Nonvolatile Chemical Cues Affect Host-Plant Ranking by Gravid *Polygonia c-album* Females

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In a multiple-choice test, the preference of egg-laying *Polygonia c-album* (comma butterfly) females was studied for oviposition on plants bearing surrogate leaves treated with crude methanol extracts obtained from leaves of seven host-plant species: *Humulus lupulus*, *Urtica dioica*, *Ulmus glabra*, *Salix caprea*, *Ribes nigrum*, *Corylus avellana*, and *Betula pubescens*. The ranking order of surrogate leaves treated with host-plant extracts corresponded well to that reported on natural foliage, except *R. nigrum*. Thus, host-plant choice in *P. c-album* seems to be highly dependent on chemical cues. Moreover, after two subsequent fractionations using reversed-phase chromatography the nonvolatile chemical cues residing in the most polar water-soluble fractions evidently provided sufficient information for egg-laying females to discriminate and rank between the samples of more and less preferred plants, since the ranking in these assays was similar to that for natural foliage or whole methanol extracts, while the physical traits of the surrogate leaves remained uniform.

Key words: Host Plant, Preference Hierarchy, Oviposition, Stimulant

Introduction

Host-plant choice for oviposition plays a crucial role in the survival of offspring of butterflies and many other phytophagous insects, due to the low mobility and energy limitations of the larvae at a time when the host plant typically strongly influences the growth rate of larvae (Wiklund, 1975; Hough and Pimentel, 1978; Thompson, 1988; Schoonhoven *et al.*, 2005). Despite the ability of oligo- and polyphagous insects to utilize a wide range of plants, a certain preference hierarchy within this range is typically observed, which can be viewed as a facet of specialization (Thompson and Pellmyr, 1991; Nylin and Janz, 1999; Schoonhoven *et al.*, 2005; Mercader and Scriber, 2008). The ranking of acceptable plants is often variable and may be genetically determined. It may be influenced by previous experience as well as variation in environmental factors including seasonal changes in host-plant quality, induction of plant defences, and the presence of natural enemies. As a result, females are under pressure to find the most suitable environment for larvae, to maximize offspring performance (Thompson, 1988; Nylin and Janz, 1993; Janz, 2002; Schoonhoven *et al.*, 2005; Bergström *et al.*, 2006; Wennstrom, 2010).

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 (Bernays and Chapman, 1994; Renwick and Chew, 1994; Schoonhoven *et al.*, 2005). Available data suggests that ratios of stimulatory and inhibitory inputs perceived through the sensory systems and recognized as acceptable patterns by the central nervous system may be the basis for the discriminatory ability of plant-feeding insects (Städler,

2002; Haribal and Feeny, 2003; Nishida, 2005; Chachin *et al.*, 2007).

Considering that stimulants and deterrents play key roles in the acceptance of plants for oviposition, the chemical structures of those phytochemicals have been identified for a surprisingly small number of Lepidoptera species. From 1962, when David and Gardiner presented the first example of egg-laying behaviour in butterflies elicited by identified phytochemicals, until now, chemical cues which stimulate oviposition have been defined for about 30 species of butterflies or moths. Most of those species belong to a few families of Lepidoptera including Papilionidae, Pieridae, Danaidae (now considered part of Nymphalidae), and Noctuidae. The chemodiversity of egg-laying stimuli represents a wide array of compounds ranging from *n*-alkanes and terpenoids to highly polar compounds such as polyhydroxycarboxylic acids with short carbon chains (4–7 carbon atoms) and glucosinolates. The last group of phytochemicals evidently is the most often used chemical type of stimuli (Nishida, 1995; Mewis *et al.*, 2002; Städler, 2002; Macel and Vrieling, 2003; Honda *et al.*, 2004; Lee *et al.*, 2006; Li and Ishikawa, 2006; Morris *et al.*, 2009; Sun *et al.*, 2009). Females of Lepidoptera species for whom egg-laying stimulants have been identified are most often stimulated to oviposit by a few compounds, but in some cases oligophagous butterflies can show extreme synergism between multiple chemical cues (Nishida, 1995).

The comma butterfly, *Polygonia c-album*, is a highly polyphagous species (Higgins and Hargreaves, 1983), even though it is not an extreme generalist like some moths. Larvae feed on herbs, vines, bushes, and trees belonging to four plant orders. Despite the diverse growth forms and the taxonomically wide range of host plants the number of plant species is limited to less than fifteen in total (Seppänen, 1970; Ebert, 1993). *P. c-album* exhibits an hierarchy of host-plant preference with the most favoured plants belonging to the family Cannabaceae followed by species of Urticaceae, Ulmaceae, Salicaceae, Grossulariaceae, and Betulaceae in roughly descending order (Nylin, 1988). These families are important hosts also for other species of *Polygonia* and related genera such as *Nymphalis* (Nylin, 1988; Weingartner *et al.*, 2006). The first three families of host plants are relatively closely related, but the remainder are only distantly related to these "urticalean rosid" plants, and also to each other. Moreover, within each family only a few species are used as hosts.

As phytochemicals are important cues for acceptance of plants for oviposition in Lepidoptera, the aim of this study was to determine the role of chemical cues in oviposition and host-plant ranking by the polyphagous *P. c-album*. This was done by comparing the ranking hierarchy observed on natural foliage with that obtained on surrogate leaves treated with foliage extracts, while keeping physical traits uniform. This study is also the first step in illuminating the mechanistic background to the enigmatic host-plant range of this species and its relatives, which have been extensively used to address questions regarding specialization, host-plant shifts, geographical range shifts, and ecologically driven speciation (*e.g.* Nylin, 1988; Nylin *et al.*, 2009; Janz *et al.*, 1994, 2001, 2009; Weingartner *et al.*, 2006; Braschler and Hill, 2007).

Material and Methods

Insects

Adults of the comma butterfly, *Polygonia c-album* L. (Lepidoptera: Nymphalidae), occur in two seasonal morphs. The "spring morph" – with dark wing undersides – hibernates before reproduction in the spring, while the "summer morph" – which has a lighter colour on the wing undersides – develops directly to reproductive adults. The offspring of comma butterflies can develop into either of the two morphs, depending mainly on photoperiod and temperature (Nylin, 1989). Under artificial breeding, a desired morph thus can be obtained by manipulation of rearing conditions. For establishment of a laboratory culture adults of the "spring morph" were collected in Åkersberga, 20 km north of Stockholm, and Järva, 10 km north-west of Stockholm, as well as outside the Stockholm University Campus, Sweden during spring 2009. Butterflies were reared at the Royal Institute of Technology, Stockholm, Sweden. Eggs were obtained on stinging nettle, *Urtica dioica* L. (Rosales: Urticaceae), and then moved to a rearing room with a photoperiod of 12 h light and 12 h dark (12L:12D) and about 18 °C. After hatching, caterpillars were maintained at these conditions until they reached the third instar. Afterwards, the light phase of the photoperiod was prolonged to 20L:4D and the temperature was increased to around 22 °C. In this way, adults of

the "summer morph", which develop directly to sexual maturation without adult diapause, were produced. After emergence, adults of both sexes were moved to the mating cages and provided with 20% honey solution in water. Matings took place in the second half of the photophase, corresponding to afternoons in the wild. The mating pairs stayed in copula until light off, thus mating pairs observed could be isolated and transferred to experimental cages. 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

Leaves of seven plant species, namely hop [*Humulus lupulus* L. (Rosales: Cannabaceae)], stinging nettle *Urtica dioica*, wych elm [*Ulmus glabra* Huds. (Rosales: Ulmaceae)], goat willow [*Salix caprea* L. (Malpighiales: Salicaceae)], black currant [*Ribes nigrum* L. (Saxifragales: Grossulariaceae)], common hazel [*Corylus avellana* L. (Fagales: Betulaceae)], and downy birch [*Betula pubescens* Ehrh. (Fagales: Betulaceae)] were collected in the area of Stockholm University and Royal Institute of Technology Campuses, Stockholm, Sweden during the last week in May 2009. Collected leaves were weighed and number of leaf equivalents calculated as follows: in the behavioural experiments to test the egg-laying activity of extracts artificial leaves of 10 cm^2 were used, and this square was counted as the size of one leaf equivalent. Then about 400 cm^2 of leaves were weighed and the weight of one leaf equivalent (10 cm^2 of leaf) for each plant species was calculated. This procedure was repeated four times and the average weight of one leaf equivalent was determined. The leaves collected for extractions were weighed and, based on the data for weight of one leaf equivalent, the number of leaf equivalents was calculated.

It was known from previous unpublished work, that only methanol extracts and fractions with the most polar phytochemicals stimulate egg laying in comma butterflies. Based on that knowledge, extraction and fractionation protocols were developed to produce samples of the polar compounds of host plants.

It is a common knowledge that the leaf surface is covered with a protective wax layer (Samuels *et al.*, 2008). In order to destroy that nonpolar hydrophobic layer, leaves firstly were immersed for 10 s in dichloromethane (99.9%; Carlo Erba Reactifs-SDS, Val de Reuil, France). After the solvent had evaporated from the leaves, they were extracted in methanol (99.9%) for 5 min to obtain a polar extract of leaves. The methanol extract was concentrated *in vacuo* below 50 °C to 8–14 ml and was subsequently stored at -14 °C.

One third of the extract in methanol was used for the bioassay test. The rest was concentrated *in vacuo* to 5–6 ml and afterwards fractionated using medium pressure liquid chromatography (MPLC) (Fig. 1). In most liquid chromatography methods, separation occurs by different distribution of analyte molecules between a moving liquid phase and a solid stationary phase. Choice of mobile and stationary phases depends on the polarity and solubility of the analytes (Millar, 2000). Taking into consideration our unpublished data that egg-laying stimulants are dissolved in polar solvents, the reversed-phase method was selected. In reversed-phase liquid chromatography, the nonpolar stationary phase holds nonpolar analytes more strongly, thus polar molecules will elute faster. At the beginning of the fractionation process, a more polar mobile phase – *i.e.* solvent – characterized by weaker eluting strength is used to move polar compounds out of the column. Afterwards, less polar solvents with stronger eluting power are applied to elute less polar analyte molecules. Consequently, the first fraction will be comprised of the most polar compounds, and subsequent fractions will contain less and less polar molecules.

An MPLC column (500 mm length x 25 mm I.D.) was packed with reversed-phase C_{18} ZEOprep chromatography gel with particle size 40–63 μm, pore diameter 6 Å (Zeochem, Uetikon, Switzerland). The column was loaded with 178.5 ml of silica gel. For rough fractionation and to decrease back-pressure on the column, methanol and dichloromethane were used as mobile phase at a flow rate of 30 ml/min produced by a high-flow pump FMI, model QD (Fluid Metering Inc., Syosset, USA). Pre-chlorophyll and chlorophyll fractions were eluted with methanol and the post-chlorophyll fraction was obtained using dichloromethane as well as methanol when the column was conditioned for the next run. Only the pre-chlorophyll fraction was concentrated *in*

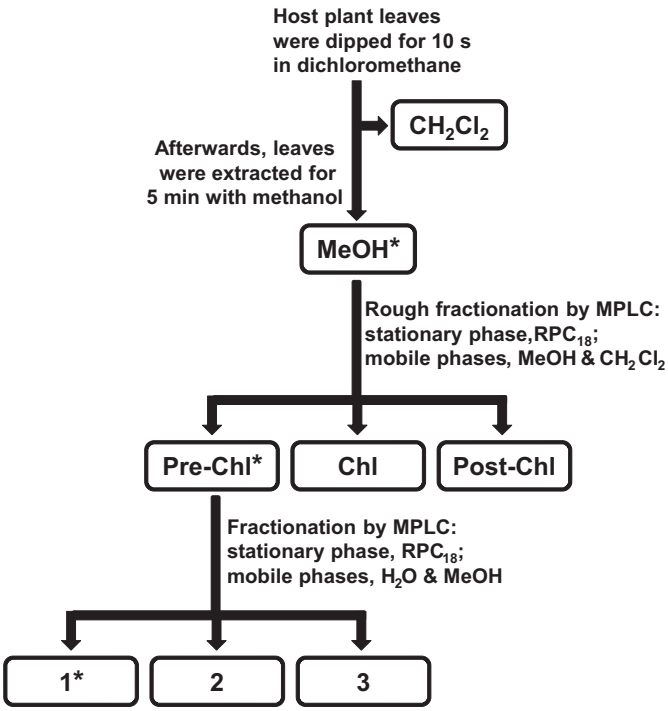

Fig. 1. Preparation of samples used for preference tests. Asterisks indicate samples which stimulated egg-laying behaviour and were used for preference tests. Pre-Chl indicates pre-chlorophyll fraction, Chl and Post-Chl represent chlorophyll and post-chlorophyll fractions, respectively. The extraction and fractionation procedures were similar for leaves of all seven host-plant species.

vacuo and used for bioassay experiments as well as for further fractionation (Fig. 1).

The amount of pre-chlorophyll fraction which was left after the oviposition bioassays was further fractionated by MPLC (Fig. 1). The methanol from the sample was evaporated and the aliquot was dissolved in 5 ml water (99.9%; Carlo Erba Reactifs-SDS) to be able to start fractionation with the weakest eluting solvent in the reversed-phase method in order to achieve highest separation efficiency. A column (190 mm length x 5 mm I.D.) was loaded with the same stationary phase as in the previous fractionation. Water/methanol with decreasing polarity was used as a mobile phase at a flow rate of 10 ml/min. Fraction 1 was eluted with water, fraction 2 with methanol, and finally fraction 3 was obtained with water when the column was conditioned for the next run. Fraction 1 contained the most polar compounds followed by fraction 2 then 3 which contained constituents with decreasing polarity. Based on our previous bioactivity test, the most polar fraction 1 elicited egg-laying behaviour (unpublished data), thus only that sample was used in multiple-choice bioassays.

The extraction and fractionation procedure was the same for leaves of all species of plants.

Bioassay of oviposition response

Green sponge cloth Wettex (Huhtamaki Sweden AB, Stockholm, Sweden) was used to make 10-cm^2 surrogate leaves. Each artificial plant had two such surrogate leaves. In total five leaf equivalents of extract (two and half leaf equivalents on each leaf side) were applied, and when methanol was used as a solvent it was allowed to evaporate before the beginning of the bioassays. After evaporation of methanol, surrogate leaves were moistened by gently spraying deionized water on the surface of each 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 five leaf equivalents of a methanol extract of *U. dioica* 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 the tests. Our previous pilot study revealed that pure deionized water, methanol, and dichloromethane did not affect oviposition of females.

Extracts of the seven plants studied were divided into two groups, based on earlier studies with real plants (*e.g.* Nylin, 1988): the group of more preferred plants consisted of *H. lupulus*, *U. dioica*, *U. glabra*, and *S. caprea*, while the second group was comprised of less preferred plants including *R. nigrum*, *S. caprea*, *C. avellana*, and *B. pubescens*. Extracts of goat willow (*S. caprea*) were used in both groups, to facilitate reconciliation of results from the two set-ups. Simultaneous presentation of more than four types of extracts was not deemed feasible in the relatively small cages used in the bioassays. A solvent control was not included in the multiple-choice test as the responsiveness of every female to sample versus control was checked every day before the experiments. Groups of two to three one-week-old mated females were used for oviposition experiments.

Four artificial plants, each treated with an extract of a certain plant species, were placed in the corners of a cage (70 cm x 70 cm x 70 cm) equipped with a transparent roof. 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, Amsterdam, Holland). Location of a model plant in a cage was changed by clockwise moving the plant to the location of the neighbouring one every 15 min to eliminate corner biases. In total, a bioassay lasted for 1 h. The sets of artificial plants bearing the extracts of the less preferred plants were tested first, followed by the ones treated with extracts of the more preferred plants. Less and more preferred groups of the plants were tested once with the same group of females. Oviposition tests were conducted in the second half of a photoperiod, during the period when the egg-laying activity of females was highest. The number of eggs on each "plant" was counted at the end of each experiment. Only eggs oviposited on the artificial plants were included in the data analysis. Twenty one, 9, and 15 groups (comprised of 49, 22, and 36 females in total) were tested using methanol extracts, pre-chlorophyll fractions, and fractions 1, respectively.

Statistical analysis

The groups of four more and less preferable plants were analysed separately. The nonparametric Quade test for dependent samples (Conover, 1999) was used to determine significant differences between the percentages of eggs laid on artificial plants within the test group. The differences found to be significant at the $P < 0.05$ level are indicated in Fig. 2. Nonparametric Kruskal-Wallis ANOVA and Wilcoxon matched pair test were used for evaluation of data regarding the absolute number of eggs. All statistical methods were run using the computer programme package StatXat version 9.

Results

Our previous work (unpublished data) has indicated that from all fractions and extracts including those obtained with dichloromethane, only crude methanol extracts originaing from leaves of seven host-plant species, as well as pre-chlorophyll samples and the fractions 1 obtained from the first and the second fractionations (Fig. 1), respectively, stimulated the egg-laying behaviour of comma butterflies; thus those samples were used for preference experiments. In the groups comprised of samples originating from more preferred host plants, 28, 19, and 20 eggs on average per female were recorded on artificial plants treated with crude methanol extracts, pre-chlorophyll fractions, and fractions 1, respectively, and these differences were not significant (Kruskal-Wallis ANOVA, $P = 0.063$, $H = 5.870301$). In the less preferred sample groups, artificial plants treated with crude methanol extracts, pre-chlorophyll fractions, and fractions 1 obtained 8, 6, and 8 eggs on average per female, respectively, and the differences observed were not significant (Kruskal-Wallis ANOVA, $P = 0.47$, $H = 1.497522$). Significant differences in the numbers of eggs were however recorded when comparing more and less preferred sample groups within the same sample preparation type, *i.e.* methanol extracts, pre-chlorophyll fractions, and the fractions 1 (Wilcoxon matched pair test, $P > 0.001$ for all 3 types).

Taking into consideration the large variation in egg numbers laid by individual females, portions

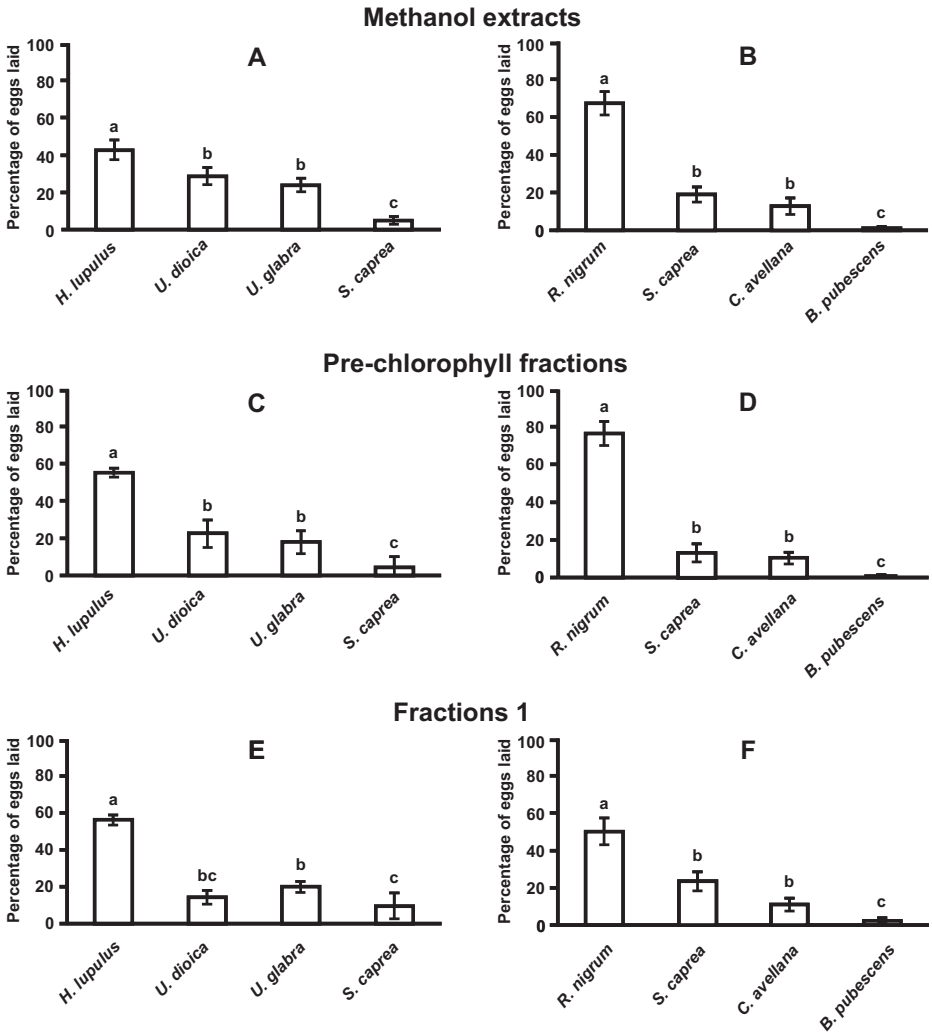

Fig. 2. Mean percentages of eggs laid by *Polygonia c-album* females on surrogate leaves treated with extracts and fractions of 7 host-plant species tested in multiple-choice bioassays. Values indicated by the same letters are not significantly different ($P \leq 0.05$) by the Quade test; vertical bars represent standard errors. Groups of 2 to 3 one-week-old mated females were used in oviposition experiments and 21, 9, and 15 groups comprised of 49, 22, and 36 females in total were tested using methanol extracts, pre-chlorophyll, and fractions 1, respectively.

of eggs in percent rather than their absolute numbers were used for determination of the preference of the host plants.

Activities of crude methanol extracts

In a multiple-choice test, groups of egg-laying *P. c-album* females ranked artificial plants treated with crude methanol extracts. In the group bearing artificial plants treated with the extracts of leaves collected from the more preferred host plants, surrogate leaves impregnated with the extract of *H. lupulus* received the highest portion of eggs and differed significantly from the remaining treatments (Fig. 2A). The activities of extracts obtained from *U. dioica* and *U. glabra* did not differ significantly from each other, and these samples were medium efficient as egg-laying stimulants. Surrogate leaves treated with the extract of *S. caprea* received the smallest portion of eggs, significantly less than those bearing ex-

tracts from the three other plants. In the group of artificial plants treated with the extracts of leaves obtained from less preferred host-plant species a significantly larger portion of eggs was registered on surrogate leaves impregnated with the extract of *R. nigrum* than on leaves with the other plant extracts (Fig. 2B). The percentage of oviposition on surrogate leaves treated with either *S. caprea* or *C. avellana* extracts did not differ significantly from each other, but was significantly higher than that of surrogate leaves treated with the extract of *B. pubescens*.

Activities of samples obtained after the first fractionation

Hierarchical ranking patterns observed for the artificial plants treated with the pre-chlorophyll samples were the same compared to those determined for leaves treated with the crude methanol extracts for both more and less preferred host-plant groups (Figs. 2C and D).

Activities of samples obtained after the second fractionation

Groups of egg-laying *P. c-album* females showed a good ability to rank also artificial plants treated with fractions 1, containing only polar compounds (Figs. 2E and F). The activity pattern of artificial plants treated with fractions 1 obtained from the previous pre-chlorophyll fractions was slightly different. Among the group of more preferred host plants (Fig. 2E), the most active fraction 1 was still that originating from *H. lupulus* leaves, but the fraction obtained from *U. glabra* was more active than that of *U. dioica.* Thus, the egg-laying stimulating property of the latter species tended to decrease relative to the other species after the second fractionation. However, the activities of the last two fractions mentioned did not differ significantly from each other. In the group of less preferred host-plant species, the ranking pattern was similar to the one in the previous two experiments (Fig. 2F). There was a slight tendency towards a more similar ranking of the four plants, in that the proportion of eggs laid on leaves treated with fractions from the most preferred plant *R. nigrum* tended to decrease, and the activity of fractions from the least preferred *B. pubescens* tended to increase. The portion of eggs laid on leaves with fractions from *S. caprea* also tended to increase, compared to the earlier experiments.

Discussion

We found that *P. c-album* females readily oviposited on surrogate leaves treated with crude methanol extracts of host plants or the most polar fractions obtained after subsequent fractionation of extracts and active fractions on reversed-phase chromatographic medium. Our data suggest that at least part of the egg-laying stimulants consists of relatively polar phytochemicals. These results follow the general pattern that most oviposition stimulants identified for butterfly species are polar compounds (Nishida, 1995; Städler, 2002; Macel and Vrieling, 2003; Honda *et al.*, 2004).

Moreover, chemical cues present in host-plant leaves provided sufficient information for egg-laying females to discriminate between more and less preferred plants in a manner broadly consistent with data obtained from real plants. A ranking hierarchy of host plants by *P. c-album* was first published by Nylin (1988) where it was shown in two-choice experiments, using *U. dioica* as a standard reference, that *H. lupulus* was the most preferred plant followed by *U. dioica*, *U. glabra*, *S. caprea*, *Ribes alpinum* L. (Saxifragales, Grossulariaceae), *B. pubescens*, and *C. avellana*. Later studies have confirmed the relative rankings of in particular *U. dioica*, *U. glabra*, *S. caprea*, and *B. pubescens* (*e.g.* Janz *et al.*, 1994, 2009; Bergström *et al.*, 2006). In our experiments, the hierarchy of preference of extracts and fractions obtained from the more preferred plants corresponded well with the published results, however in the group of less preferred plants, extracts and fractions obtained from *C. avellana* were significantly more active compared to those of *B. pubescens*. In addition, samples obtained from *R. nigrum* were more active than those from of *S. caprea*; however, in this case direct comparison of our data with earlier publications is not possible as different species of currants were used (*R. nigrum* and *R. alpinum*, and also *R. uva-crispa* in Janz *et al.*, 2009).

This appears to be the first evidence that chemical cues play an essential role in the host-plant preference in Nymphalini butterflies (a tribe which besides *Polygonia* and the nominate genus *Nymphalis* also contains *Vanessa*-type butterflies). Such evidence is rare even from the entire large

family Nymphalidae (but see Honda *et al.*, 2004; Reudler Talsma *et al.*, 2008).

Host-plant utilization in butterflies is often evolutionarily conservative with respect to plant taxa (Janz and Nylin, 1998). Physical plant traits can affect host selection to an important extent, and utilization of a particular plant growth form can also be a conservative aspect of the insect-plant interaction (Janz and Nylin, 1998). However, the taxonomical conservatism of phytophagous insects is most likely best explained by similar plant chemistry in related plants (Schoonhoven *et al.*, 2005). Our data matched with this paradigm as the ranking hierarchy of natural leaves corresponded well with that of surrogate leaves treated with extracts obtained from host-plant species, when the physical traits of the surrogate leaves were uniform.

The chemical information perceived by an insect at a leaf surface consists of opposing positive and negative cues, and the final decision by a gravid female to oviposit or not depends on the balance of these inputs. In *P. c-album*, our data are consistent with a ranking that is mostly determined by positive cues in the fractions of highest polarity, possibly even the same or similar compounds in different plants. If so, this is somewhat unexpected given the peculiar host-plant range of the species: the extracts were from seven plant species representing as many families and four different orders, plus all four plant growth forms (herbs, vines, bushes, and trees).

In line with this reasoning, the low proportions of eggs deposited on surrogate leaves treated with samples obtained from *S. caprea*, compared to those of *U. dioica* and *U. glabra*, could be a result of the extraction of oviposition deterrents from the interior of goat willow leaves after destruction of the wax layer. It is known that the foliage of *S. caprea* is rich in phenolics (Sagareishvili *et al.*, 1990) and those compounds deter feeding and egg laying of generalist herbivore species (Kelly and Curry, 1991; Topp *et al.*, 2002), thus they could act as oviposition deterrents for polyphagous *P. c-album* females as well. The slightly higher relative activity of the most pure fraction 1 from *S. caprea* compared with those of crude extract and pre-chlorophyll fraction could be a result of partial removal of such deterrents. In addition, a similar pattern of increase in relative activity of the most pure fraction was observed for *B. pubescens* samples, and again this could perhaps be explained by removal of oviposition deterrents, as foliage of this species is also rich in phenolics and other inhibitory phytochemicals (Ossipov *et al.*, 2001; Riipi *et al.*, 2002). An alternative explanation for the somewhat less clear rankings using fraction 1 is the removal of less polar additional positive cues from the most preferred plants during the fractionation.

Volatile compounds also play a role in host-plant detection in butterflies (Heinz, 2008), however, our results suggest that they were not essential for ranking surrogate leaves treated with host-plant extracts and fractions. We did not count the number of landings that females made on surrogate leaves; however the preference hierarchy was very similar for crude extracts, which contained some volatiles, compared to the polar water-soluble fractions 1 which were comprised of only nonvolatile chemicals.

Since most butterflies are specialists, it can be assumed that polyphagy in butterflies is a transient state, *i.e.* polyphagous butterflies tend to re-specialize, often on ancestral plants but sometimes on novel hosts (Nylin and Janz, 1999; Janz *et al.*, 2001; Weingartner *et al.*, 2006). Such changes in host range and preference hierarchy followed by narrowing host-plant range and local specialization can aid the formation of new herbivore species, and may be an important factor in explaining the great diversity of phytophagous insects (Janz *et al.*, 2006; Janz and Nylin, 2008). Several hypotheses have been proposed to explain the role of plant chemistry during a host shift by a phytophagous insect species, and they all predict that insects are more likely to colonize new hosts containing secondary compounds that are chemically related to those of the ancestral hosts (Murphy and Feeny, 2006). As noted above, our data suggests that all seven host-plant species investigated here might share similar oviposition stimulants, as the egg-laying behaviour was elicited by the most polar water-soluble fractions, however further bio-guided fractionations are needed to confirm this speculation. In addition, structure elucidation of active phytochemicals including stimulants and deterrents is needed to characterize the host-plant recognition patterns used by comma butterflies to discriminate between plant species for egg deposition.

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