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Roessingh, P.; Hora, K.H.

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Host acceptance behaviour of the small ermine moth *Yponomeuta cagnagellus*: larvae and adults use different stimuli

Peter Roessingh¹, Katja H. Hora¹, S. Ying Fung^{2,3}, Anja Peltenburg³ and Steph B. J. Menken¹

¹Institute for Systematics and Population Biology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands, e-mail: roessingh@bio.uva.nl

²Institute of Evolutionary and Ecological Sciences, Leiden University, P.O. Box 9516, 2300 RA Leiden, The Netherlands

³Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Einsteinweg 55, 2300 RA Leiden, The Netherlands

Summary. The sugar alcohol dulcitol is a strong feeding stimulant for larvae of the small ermine moth *Yponomeuta cagnagellus*. In this paper we tested the hypothesis that dulcitol also acts as an oviposition stimulant for this species. We found that the sugar-alcohol dulcitol was present on the surface of the host *Euonymus europaeus*. We also showed that (as yet unidentified compounds) can be systemically transferred (i.e. by uptake and transport via the vascular system) from *E. europaeus*, to the non-host *Crataegus monogyna* and stimulate oviposition. However, no evidence was found that this stimulatory activity was due to dulcitol. Systemic enrichment of *C. monogyna* with dulcitol did not induce oviposition on this plant. Neither was the application of pure dulcitol on artificial twigs effective. In addition it was shown that when dulcitol was removed from host plant extracts, oviposition stimulatory activity was retained in the fraction without dulcitol. Synergism between dulcitol and other stimulants could not be demonstrated, however, high concentrations of dulcitol in combination with the main stimulant(s) showed a trend towards enhanced oviposition. It is concluded that the sugar alcohol dulcitol can only play a limited role in adult host acceptance behaviour. The hypothesis that a host shift in the genus *Yponomeuta* from Celastraceae to Rosaceae might have been facilitated by the presence of small amounts of dulcitol in Rosaceae therefore needs to be restricted to larval feeding behaviour.

Key words. *Yponomeuta cagnagellus* – host-acceptance – oviposition stimulants – behaviour – dulcitol – chemoreception – phytochemistry – evolution – speciation

Introduction

A majority of insect herbivores are specialised feeders, restricted to one, or a few related species of plants. It

has been suggested that this high level of specialisation has important consequences for speciation processes (Bush 1969, 1994). Host shifts and host race formation are viewed as steps leading to speciation, even under sympatric conditions. While the likelihood of this mode of speciation is still debated (see Barton *et al.* 1988; Bush 1994), it is evident that changes in host acceptance behaviour are key events in the process, and studying the chemoeecological basis underlying host acceptance is therefore a necessary step when attempting to test sympatric speciation theory.

The genus *Yponomeuta* (Lepidoptera, Yponomeutidae) is used as a model system for the evolution of insect-plant relationships and the involvement of host shifts in speciation (Menken *et al.* 1992; Menken 1996; Menken & Roessingh 1998). Mapping host plant use on a phylogenetic tree of the Yponomeutinae and the genus *Yponomeuta* (Menken 1996; Ulenberg & Gershenson, in prep), suggests an ancestral association with Celastraceae and a unique shift to Rosaceae. Recent molecular evidence (Sperling *et al.* 1995; Lieshout *et al.*, unpublished results) indicates that one of the species, viz., *Y. cagnagellus* (Hübner), probably shifted back from Rosaceae to Celastraceae. Celastraceae are among other things characterised by the presence of the sugar alcohol dulcitol, while Rosaceae contain sorbitol, a stereo isomer of dulcitol (Fig. 1). Dulcitol is known to be a potent larval feeding stimulant for Celastraceae feeders like *Y. cagnagellus* (van Drongelen 1980; Kooi 1988a). Sorbitol similarly acts as a stimulant for species feeding on Rosaceae (Kooi 1988b). It has been proposed that the shift from Celastraceae to the relatively unrelated Rosaceae has been facilitated by a phytochemical 'bridge' provided by the presence of low amounts of dulcitol in the Prunoidea, a suborder of the Rosaceae (Herrebout *et al.* 1987; Menken & Roessingh 1998). The presence of this token stimulus is expected to increase the likelihood of larval feeding on the new host. Prunoidea contain much more sorbitol than dulcitol. Acquiring sensitivity for sorbitol after a host shift would therefore further stimulate feeding and subsequently allow utilisation of Rosaceae containing only sorbitol (Menken & Roessingh 1998). Dulcitol and

sorbitol are perceived in homologous sensory cells in *Yponomeuta* species (Roessingh *et al.* 1999), suggesting that modifications in the periphery alone (for instance, expression of another set of receptor proteins) would be sufficient to alter host selection behaviour.

However, the simple evolutionary scenario outlined above is not complete. In *Yponomeuta*, like in many Lepidoptera with relatively small first instar larvae, host choice is primarily determined by the ovipositing female. Egg batches are deposited on the stems of relatively large woody plants (*Euonymus*, *Prunus*, *Malus*). The first stadium larvae are small (less than one mm long) and do not balloon. A successful host shift therefore eventually requires changes in adult oviposition behaviour as well as changes in feeding behaviour. The need for simultaneous adaptations affecting both oviposition and feeding behaviour clearly reduces the likelihood of a host shift. This constraint can partly be alleviated by assuming that the same host plant compounds stimulate both larval and adult acceptance behaviour. This situation is found in a number of phytophagous insects. *Pieris* butterflies for instance are stimulated to oviposit by mustard-oil glucosinolates (van Loon *et al.* 1992), while these glucosinolates at the same time act as feeding stimulants for the larvae. At least 10 other examples can be found in Städler (1992). When the same plant compound affects the behaviour of larvae and adults, a parsimonious scenario for the sensory modifications underlying a host shift can be envisioned. The response profiles of receptor neurones are determined by the presence of particular receptor proteins. If genes for (modified) receptor proteins are expressed *both* in adults and larvae, peripheral changes affecting the behaviour of the larvae can directly affect the behaviour of the adult, and vice-versa (Menken & Roessingh, 1998). In the present paper we test this hypothesis, using *Y. cagnagellus* and determine if the sugar alcohol dulcitol, in addition to being a larval feeding stimulant (Herrebout *et al.* 1987), also acts as an oviposition stimulant for adult moths. From this hypothesis a number of predictions can be formulated:

- 1) Adult moths have no access to the interior of their host, so dulcitol must be present in surface extracts

Fig. 1 The sugar alcohols dulcitol (1) and sorbitol (2) are stereo isomers and differ only in the position of a single hydroxyl group. *Yponomeuta cagnagellus* is only sensitive to dulcitol, while *Yponomeuta* species feeding on Rosaceae are sensitive to sorbitol

of *Euonymus europaeus* L. (Celastraceae), the host-plant of *Y. cagnagellus*.

- 2) Dulcitol is the main soluble sugar alcohol in *E. europaeus* extracts (Fung & Herrebout 1988). Since dulcitol is taken up by branches of non-hosts and transferred to the leaves (Herrebout *et al.* 1987) it is expected that oviposition on non-hosts will be induced by systemic uptake of dulcitol.

- 3) Dulcitol alone, or dulcitol acting as a synergist in a diluted surface extract should stimulate oviposition on artificial substrates.

- 4) Host-plant extracts from which dulcitol is removed should show reduced stimulatory activity – or no stimulatory activity at all – relative to the activity of the complete extract.

Testing these predictions will provide information about the relative importance of dulcitol as oviposition stimulant for *Y. cagnagellus* and the possible roles of this compound in the (historical) host shifts in the genus *Yponomeuta*.

Material and methods

Insects

In early summer (June–July) fifth stadium larvae of *Y. cagnagellus* were collected from their hosts *E. europaeus*, reared at 18°C, L:D 17:7 and fed on fresh host leaves until pupation. Pupae were individually transferred to glass vials (8 cm long, Ø 2 cm) closed with a plug of cotton wool. Emerging moths were provided with small pieces of agar containing a 1% sucrose solution and allowed to mature for three weeks. In the third week the insects were transferred to a climate chamber 20°C, L:D 16:8 with an offset L:D rhythm, shifting dawn to 16:00 h. To promote sexual maturation a section of host-plant stem was placed in the vials. About one hour before the start of the photophase groups of 20 pairs of mature moths were introduced in large perspex cylinders (70 cm high, Ø 30 cm) and observed. Pairs in copula were taken from the cylinder and females were allowed to develop mature oocytes for another week. These procedures increase the likelihood that females are fertilised and egg batches will be deposited in the bioassays.

Host-plant surface extracts

Leaf surface extract of *E. europaeus* (20 gram leaf equivalent) was prepared following Städler & Roessingh (1991). Stem surface extracts were produced as described in Hora & Roessingh (1999b). Briefly, 10 meter of leafless branches of *E. europaeus* plants were collected in the field early in September and cut into 12 cm sections. These pieces were gently washed for 5 seconds in 0.5 l CH₂Cl₂ followed by two times a 5 sec wash in 1 l portions of MeOH. The stem sections were not sealed so minor contamination of the surface extract with internal compounds cannot be excluded. Only the MeOH fraction is biologically active in oviposition assays (Hora & Roessingh, 1999b). The volume of the MeOH fraction was reduced in a rotation evaporator at 300 mm Hg and 40°C to approximately 25 ml. The activity of this stock solution (stored at 4°C) was stable for at least one year. The concentration of the extracts is expressed in twig surface equivalents (TSE) per ml, the surface (cm²) of stem extracted per ml solvent.

Bioassays

Artificial twigs (ATs) as described by Hora & Roessingh (1999b) were constructed from glass Pasteur pipettes (WU, Mainz, Germany). The glass surface was covered with masking tape (TESA, Beiersdorf

BDF). Two orthodontic elastics (6.4 mm diameter) were placed around the AT to simulate nodes on the stem. Surface extracts or surface extract fractions were sprayed on the AT at a concentration of 1 TSE per cm² of treated surface. This concentration mimics the normal concentration of the extracted compounds on the host. Pairs of ATs were placed at 25 mm distance in small perspex cylinders (150 mm long, Ø 42 mm). In each cylinder one to five mated pairs of moths were introduced and the number of egg batches for each treatment was used to evaluate the stimulatory power of the applied compounds.

The effectivity of host compounds as oviposition stimulant was also tested in choice test with real twigs, making use of systemic transfer of plant compounds (Herrebout *et al.* 1987). Mated pairs of *Y. cagnagellus* were individually placed in glass jars (20 cm high, 10 cm diameter) and given a choice between a twig of the host *E. europaeus* or a twig of the non-host *Crataegus monogyna* Jacq. (Rosaceae). Both hosts have smooth bark and are structurally similar. The experimental manipulation consisted of placing each twig in separate 20 ml glass vial or both together in a the same vial. This latter condition allows compounds produced by the host to be taken up by the non-host. In some replications 1% agar was added to the water to allow easier manipulation of the vials. Care was taken to ensure that the distance between the branches in the two experimental conditions was comparable.

Effects of pure sugar alcohols on oviposition was evaluated using twig pairs of the non-host *C. monogyna*, placed in vials containing a 1% agar solution with 2% pure dulcitol. As a control 2% mannitol was used. Both compounds (99% pure) were obtained from Sigma.

Yponomeuta cagnagellus is a batch layer. Therefore, in all bioassays the moths were allowed to oviposit for their remaining life span (three to four weeks) to maximise the number of egg batches produced in the assays.

Fractionation

Part of the methanol fraction of the stem extract (340 TSE) was further separated into two fractions using solid phase extraction on 3 ml C₁₈ disposable extraction columns (Mallinckrodt-Baker inc., Philipsburg). Columns were conditioned by washing with two column volumes of 100%, 80%, 50%, 20% and 0% MeOH in H₂O. The sample was dissolved in a 0.5 ml of MeOH, diluted with 3 ml H₂O, and applied on the column with several column volumes of H₂O. This fraction was called the "C₁₈ polar" fraction and is expected to contain the polar compounds like sugars and sugar alcohols. The column was subsequently eluted slowly with 1.5 ml (three times 0.5 ml) of increasingly apolar solvents respectively MeOH, EtOH and Acetonitrile. All these fractions were combined and are called the "C₁₈ apolar" fraction.

Dulcitol quantification

Dulcitol in dried extracts was determined as its n-butyl boronic acid ester as previously described (Fung and Herrebout 1988). GLC analyses were carried out on a Packard 436S GC equipped with a splitter, a CP-Sil 19 CB fused silica column (25 m × 0.32 mm i.d.), and a FID detector. Nitrogen was used as carrier gas (80 kPa). Temperature program: 5°C/min from 180°C to 240°C (5 min hold). The temperature of the injection port was 250°C and of the detector 260°C.

Results

Dulcitol was present on the surface of *E. europaeus*

Stem and leaf surface extracts of the host *E. europaeus* were analysed for the presence of dulcitol (Fig. 1). Both leaf surface extract (one sample) and stem surface extract (3 samples) contained dulcitol. The concentration in the leaf surface extract was 0.38 mg/gle. The concen-

Fig. 2 The percentage oviposition mistakes by *Yponomeuta cagnagellus* on the non-host *Crataegus monogyna* when twigs of the host and the non-host were offered in the same vial (N = 70 pairs) or in separate vials (N = 69 pairs). Three independent replicates of the experiment were conducted with a total of 139 pairs ($P < 0.05$, Wilcoxon matched pair signed rank test).

tration in three stem surface extracts was variable (resp. 2.3 µg/TSE, 3.8 µg/TSE and 70 µg/TSE). In both the stem and the leaf surface extract dulcitol was the major sugar alcohol present. Only traces of the stereo isomer sorbitol could be detected.

Host plant compounds induced oviposition in the non-host *C. monogyna*

The systemic uptake of dulcitol and subsequent transportation of compounds to the leaves have been demonstrated by Herrebout *et al.* (1987). These authors showed that *Y. cagnagellus* larvae feed and successfully develop on the non-host *Prunus padus* when branches were placed in a dulcitol solution. Using this approach we investigated if oviposition on a non-host (*C. monogyna*) would be stimulated by compounds produced by *E. europaeus*, the normal host plant of *Y. cagnagellus*. *Crataegus monogyna* is the host of the sister species of *Y. cagnagellus* and normally not accepted for oviposition (Bremner *et al.* 1997; Hora & Roessingh 1999a). Transfer of oviposition stimulating compounds was tested by placing branches of the two plant species either together (N = 70 pairs) or individually (N = 69 pairs) in glass vials and oviposition "mistakes" on the non-host in choice experiments were recorded (Fig. 2). When branches were in the same water, almost 20% of the batches were deposited on the non host, compared to about one percent when there was no contact via the water ($P < 0.05$, Wilcoxon matched pair signed rank test). This result is not due to a few aberrant females. When branches were in the same vial 15 out of 70 females made oviposition mistakes, while only one female out of 69 made a mistake when branches were in

separate vials ($P < 0.001$, G-test of independence). There was no significant difference between the total number of egg batches laid per female in each treatment (on average 2.7 and 2.5 egg batches/female).

Systemic dulcitol enrichment did not induce oviposition in the non-host C. monogyna

After it was established that uptake of host plant compounds influenced oviposition on *C. monogyna*, the stimulatory effect of pure dulcitol was tested in a choice test in which two branches of *C. monogyna* were provided, one in a vial containing 2% dulcitol, one with 2% mannitol. This latter compound was used to control for non-specific effects of sugar alcohols. Neither of the two treatments turned out to be acceptable for the moths and by 29 females, only one batch was laid on the mannitol control and none on the dulcitol treatment. The insects in this rearing batch were capable of oviposition since in another experiment 25 females produced 24 egg batches. The lack of oviposition on *C. monogyna* after systemic dulcitol enrichment, combined with the high level of oviposition on this plant when placed in one vial with *E. europaeus*, suggests that although certain plant compounds from *E. europaeus* induced oviposition the presence of dulcitol can not explain this effect.

Dulcitol alone did not stimulate oviposition on artificial twigs

In addition to the experiments with dulcitol systemically taken up by non-host twigs, pure dulcitol solutions were also sprayed on the surface of ATs and tested for oviposition stimulatory activity. The amount of dulcitol applied per cm^2 of AT was equivalent to that found in a 1 TSE surface extract that was shown to stimulate oviposition effectively (Hora & Roessingh 1999b). The stimulatory power of dulcitol turned out to

be minimal. Out of 47 females only 7 laid egg batches (0.15 egg batch/female) and no significant difference was found between dulcitol and the control mannitol. As a control 50 females from the same rearing batch were also tested on the original surface extract to verify their condition and establish the effectivity of the extract. In this control experiment good stimulatory activity was found and an average of 1.4 egg batches/female was produced.

The dulcitol containing fraction of the surface extract did not stimulate oviposition

A direct test to evaluate the role of dulcitol as oviposition stimulant for *Y. cagnagellus* was performed by testing fractions of active stem surface extract on artificial twigs. A solid phase C_{18} column was used to split the surface extract in a relative polar and a relative apolar fraction. Under the hypothesis that dulcitol is a major oviposition stimulant good stimulatory activity is expected from the polar, sugar-alcohol containing fraction. The presence of the majority of the dulcitol in the polar fraction was confirmed on a GC. The polar fraction contained 70 μg of dulcitol per TSE (99.4%), the apolar fraction 0.4 μg /TSE (0.6%). A total of 16 tubes was used with five gravid females/tube. As can be seen in Fig. 3, 87.2% of the egg batches were found on the apolar fraction, which contained only a trace of dulcitol. The polar fraction with the majority of the dulcitol received only 12.7% of the batches, and 50% of these (6 batches) were produced in a single tube.

Limited synergism between dulcitol and other host plant compounds

There is a possibility that while dulcitol alone is not an effective stimulus for oviposition, it acts as a synergist in conjunction with other compounds. Three types of experiments were performed to investigate this possibil-

Fig. 3 Oviposition of *Y. cagnagellus* and dulcitol content of fractions prepared on a C_{18} solid phase column. One TSE of stem surface extract from *Euonymus europaeus* was used. The fraction without dulcitol still stimulates oviposition. Data from 15 experiments with a total of 75 female moths

Fig. 4 Effects of dulcitol suppletion (1 TSE) on oviposition of *Y. cagnagellus* on a diluted stem surface extract (0.01 TSE) from *Euonymus europaeus*. Dulcitol did not increase oviposition significantly. Data from 2 experiments with a total of 25 female moths. Error bars indicate the standard error

ity. In a first series of experiments diluted stem surface extracts (0.01 TSE) were used. This concentration was on the threshold of the behavioural response for this extract (Hora & Roessingh 1999b), but does still somewhat stimulate oviposition. Against this threshold background, the effect of 1 TSE of dulcitol was tested. If dulcitol is stimulating oviposition as a synergist, the treatment with added dulcitol is expected to receive significantly more egg batches. However, in 25 experiments no significant effect was found by adding the full amount of dulcitol (2.3 µg/cm²) to the diluted stem surface extract (Fig. 4). Overall an average of 0.96 egg batch per female was produced in these experiments. This number is not significantly different from the fecundity of control female on the natural host ($P > 0.05$), which shows that even the diluted extract is still effective.

In a second series of experiments a comparison was made between the active apolar C₁₈ fraction, lacking dulcitol, and a recombination of the apolar and the polar C₁₈ fractions. If dulcitol acts as a synergist a significant increase of oviposition on the combined fractions is expected. In 15 experiments with 5 females each, no significant increase in oviposition was found, although a trend towards more oviposition on the combined fractions was present ($P = 0.0832$, Wilcoxon matched pair signed rank test). In the third series of tests the active apolar C₁₈ fraction (lacking dulcitol) was tested against this same fraction with 1 TSE of dulcitol added (70 µg/cm²). If dulcitol plays a role as synergist this last treatment is expected to receive more egg batches. Five experiments with five females each were performed and no significant difference was found, but again a trend towards an increase on the ATs with added dulcitol was observed ($P = 0.0679$, Wilcoxon matched pair signed rank test).

Oviposition stimulants from E. europaeus are stable for up to one year

All experiments with fractions were based on surface extracts produced in the previous season and stored at 4°C until used. Since good oviposition stimulatory activity on ATs was evoked by these extracts (see Fig. 3 and 4), it can be concluded that the (unknown) stimulants present on the surface are stable when stored at 4°C.

Discussion

Dulcitol plays a limited role in the oviposition behaviour of Y. cagnagellus

In this paper the hypothesis was tested that the larval feeding stimulant dulcitol also acts as an oviposition stimulant for *Y. cagnagellus*. In situations where the same compound stimulates both larvae and adults, a simple scenario for a host shift can be envisioned in which genes that allow perception of feeding stimulatory compounds by larvae are also expressed in the adult, and stimulate oviposition on the new host.

We found that dulcitol was indeed the major sugar-alcohol present on the surface of the host-plant *E. europaeus*. We also found that (as yet unidentified compounds) can be systemically transferred from *E. europaeus* to the non-host *Crataegus monogyna* and stimulate oviposition. However, no evidence was found that this stimulatory activity was due to dulcitol. Systemic enrichment of *C. monogyna* with dulcitol did not induce oviposition on this plant. Neither was the application of pure dulcitol on artificial twigs effective. When dulcitol was separated from the behaviourally active host plant extracts, oviposition stimulatory activity was retained in the fraction with-

out dulcitol. Only a (non significant) trend was found in experiments testing for synergistic effects of dulcitol. Taken together, these results effectively falsify the hypothesis that dulcitol is a major oviposition stimulant for *Y. cagnagellus*.

Properties of the oviposition stimulants and implications of the limited role of dulcitol

Hora & Roessingh (1999b) showed that host-specific compounds in the methanolic surface extracts of *E. europaeus* stimulate oviposition by *Y. cagnagellus* on artificial twigs. The experiments described in the present paper confirm this observation, and demonstrate that the compounds involved are stable during storage at 4°C for at least one year. As yet no other information on the identity of these compounds is available. The observed systemic transfer of stimulatory compounds is expected under the hypothesis that dulcitol (a soluble carbohydrate) plays a role as oviposition stimulant. However, the data presented in this paper show that the oviposition stimulatory activity of the surface extract is not due to dulcitol. This result suggests that feeding behaviour and oviposition behaviour were modified by independent mutations, rather than by a single mutation affecting chemoreception both in larvae and adults.

Dulcitol may act as a synergist

It is well known that in Lepidoptera compounds that affect behaviour sometimes show strong synergism (Honda 1995). A striking example is given by Feeny *et al.* (1988) who showed that oviposition in the black swallowtail *Papilio polyxenes* (Lepidoptera, Papilionidae) is stimulated by a mixture of two host plant compounds, while the individual compounds evoke little or no behavioural response. We therefore tested for a synergistic interaction between the inactive polar C₁₈ fraction (containing more than 99% of the dulcitol) and the active apolar C₁₈ fraction (containing less than 1% of the dulcitol), as well as for the effect of adding pure dulcitol to fractions. Although no significant effects were found, several experiments displayed a trend towards an increase of oviposition when dulcitol was present. In case of the combination of the dulcitol containing fraction and the active C₁₈ fraction this effect can be explained by an imperfect partitioning of the unknown stimulatory compound over the fractions. The protocol for the C₁₈ column was optimised for the separation of dulcitol. While the majority of the active compound was present in the apolar fraction, it is not known how much active compound was left in the polar fraction. The trend towards an increase could simply be explained by the (unknown) increase in the concentration of the main stimulant. However, in experiments in which pure dulcitol was added the effect (when real) must be attributed to the presence of dulcitol. Although the results are not statistically significant, together the last two experiments suggest that dulcitol can not yet be excluded as a possible synergist. It is

however clear that the sugar alcohol only plays a minor role in host acceptance behaviour compared to the main stimulant present in the more apolar fraction.

Conclusion

We found no evidence that the larval feeding stimulant dulcitol plays a significant role in the host acceptance behaviour of *Y. cagnagellus*. One or more unidentified host-specific plant compounds *other* than dulcitol are involved in the control of oviposition. This means that the parsimonious host shift scenario suggested by Menken & Roessingh (1998) in which the sugar alcohol dulcitol (and a single gene for its detection) affects *both* adult and larval behaviour, has to be rejected. The implication is that host shifts might be constrained by the need for independent mutations affecting larval feeding behaviour and adult oviposition behaviour. It should be noted, however, that we have only limited knowledge of the nature of feeding and oviposition stimulants for *Y. cagnagellus*, and it can not be excluded that other compounds than dulcitol stimulate both feeding and oviposition.

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