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Chemosensory and behavioural responses of the turnip sawfly, *Athalia rosae*, to glucosinolates and isothiocyanates

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Summary. The turnip sawfly *Athalia rosae* sequesters glucosinolates from its cruciferous host plants in the larval stage. Investigation of the chemosensory and behavioural responses of adult *A. rosae* to glucosinolates and their volatile hydrolysis products, isothiocyanates, revealed that females detect glucosinolates by contact chemoreception and isothiocyanates by antennal olfaction. In electroantennogram recordings, four isothiocyanates (allyl [2-propenyl] isothiocyanate, benzyl isothiocyanate, butyl isothiocyanate and iberverin [3-methylthiopropyl isothiocyanate]) were active at all doses presented, including the lowest (0.1 µg), whilst the threshold for detection of three others, iberin [3-methylsulphinylpropyl isothiocyanate], methyl isothiocyanate, and sulforaphane [4-methylsulphinylbutyl isothiocyanate], was higher, at between 1 and 10 µg (source concentration of volatiles). Allyl isothiocyanate attracted experienced females in a four-chambered olfactometer, whilst naïve females showed no response. Allyl isothiocyanate also attracted mature females to baited yellow water traps in field trials, although immature females were repelled at high isothiocyanate concentrations. In laboratory behavioural bioassays the glucosinolates sinigrin (allyl [2-propenyl] glucosinolate) and sinalbin (p-hydroxybenzyl glucosinolate), stimulated ovipositor probing in mature female *A. rosae* to an extent comparable to hot-water extracts of their host plants. These responses show that glucosinolates and isothiocyanates play an important role in host finding and host recognition in *A. rosae*.

Key words. glucosinolate – isothiocyanate – host-finding – host-recognition – sawfly – *Athalia rosae* – *Delia radicum* – Hymenoptera – Tenthredinidae – Diptera – Brassicaceae

Introduction

Glucosinolates are a sulphur-based group of plant secondary chemicals characteristic of plants of the family Brassicaceae and of other Brassicales. The plants also produce the enzyme myrosinase, which is released when their tissues are disrupted, and which hydrolyses glucosinolates into volatile breakdown products, principally isothiocyanates (Fahey *et al*. 2001). These compounds are repellent or toxic to many generalist herbivores (Chew, 1988, Louda & Mole 1991, Bones & Rossiter 1996). Specialist herbivores, whilst still subject to negative effects of isothiocyanates on growth and survival (Agrawal & Kurashige 2003), are better adapted to tolerate or circumvent the glucosinolate-myrosinase-isothiocyanate system, for example by preventing the formation of isothiocyanates in the gut (Ratzka *et al*. 2002, Wittstock *et al*. 2004, Agerbirk *et al*. 2006). Furthermore, crucifer specialists commonly use glucosinolates and isothiocyanates as chemical markers for finding host-plants (e.g. Hawkes & Coaker 1976, Bartlet *et al*. 1992), stimulation of oviposition behaviour (e.g. Roessingh *et al*. 1992, Städler *et al*. 2002), and triggering of feeding behaviour (e.g. Larsen *et al*. 1992, Renwick & Lopez 1999, Renwick 2002).

The turnip sawfly, *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) is oligophagous on cruciferous plants. Its preferred hosts are cultivated crucifers such as white mustard (*Sinapis alba* L.), turnip (*Brassica rapa* L.), Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) and radish (*Raphanus sativus* L.), and it can reach pest proportions on these crops and also on young oilseed rape crops (*Brassica napus* (L.)) (Benson 1962, Liston 1995, Lamb 1989, Nagasaka & Ohsaki 2002). This sawfly species sequesters glucosinolates from its host plants (Müller *et al*. 2001, Müller & Wittstock 2005), a rare strategy for crucifer specialists (the only other known examples are two aphids (Weber *et al*. 1986) and a pentatomid bug (Aliabadi *et al*. 2002)). *A. rosae* is an 'easy bleeding' sawfly (Boevé and Schaffner 2003) in which the larval cuticle is easily disrupted on attack without long-term harm to the larva and a droplet of haemolymph containing glucosinolates is exposed to predators. The strategy appears to be effective in protecting larvae against attacks from ants (Müller *et al*. 2002), wasps (Müller & Brakefield 2003), and birds (Ohara *et al*. 1993). Comparisons between the deterrent effects of larval haemolymph containing glucosinolates and the appropriate concentrations of the pure glucosinolates suggest that

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these chemicals are involved in deterrence of invertebrate predators (Müller *et al*. 2002, Müller & Brakefield 2003).

It would seem axiomatic that insect species reliant on sequestration of specific host-plant chemicals for their own defence should detect and be attracted by those chemicals, but this has rarely been studied. For *A. rosae* itself comparatively little is known about host-plant derived chemical cues for host-finding and host-recognition and acceptance. Bogawat & Srivastava (1968) reported without giving details that sinigrin (allyl glucosinolate) was a phagostimulant for larvae of the congeneric sawfly *Athalia proxima*, and Nishida & Johki (1989) observed female *A. rosae* curling the abdomen to probe sinigrin-treated paper with their ovipositors following antennal contact, implying a role for these glucosinolates in oviposition. We set out to investigate in more detail the involvement of glucosinolates and isothiocyanates in the host use of *A. rosae* adult females, following a sequence of steps in host-plant finding and host acceptance behaviour: detection at distance through olfaction, behavioural response to isothiocyanate odours in laboratory and field tests, and responses to glucosinolates during the contact-chemoreception phase of oviposition.

Methods

Rearing of insect material

To start a culture of *A. rosae*, adults were collected in oilseed rape and mustard fields in southern Germany. Adult males and females were pooled and supplied with concentrated honey water for nutrition and Chinese cabbage leaves for oviposition. Larvae were reared on this host at 20 ºC, 16:8 L:D and supplied with sieved potting compost for pupation. The next generation of adults emerged about 10 days later and were collected every one or two days.

A. rosae is synovigenic i.e. its eggs develop fully only after eclosion from the pupa. We defined a female with no fully developed eggs as immature and a mature female as one that had one or more fully developed eggs, and considered the state of female maturity as an important factor in our behavioural experiments.

Detection of isothiocyanates by A. rosae – electroantennograms

The ability of *A. rosae* to detect seven different isothiocyanates was tested by producing dose-response curves of electroantennogram (EAG) responses across a range of four concentrations (1×10^{-3}, 1×10^{-2}, 0.1, 1 mg/ml). Isothiocyanates tested included two with straight-chain aliphatic alkane side chains (methyl and butyl isothiocyanates [supplied by Fluka, analytical quality]), one straight-chain alkene (allyl [2-propenyl] isothiocyanate [Fluka]), one aromatic side-chain (benzyl isothiocyanate [Fluka]), and three with added sulphur-groups in the side chain, iberverin (3-methylthiopropyl), iberin (3-methylsulphinylpropyl) and sulforaphane (4-methylsulphinylbutyl) (LKT Laboratory). All isothiocyanates were dissolved in paraffin oil (Fluka, flamepoint 215 ºC, for IR Spectroscopy). Fresh test solutions were prepared for each batch of females tested (three times during the recordings). 100 µl of each test solution at each concentration was applied to a separate folded filter paper (15 × 50 mm, 3 folds in length, paper from Schleicher & Schuell) each of which was placed into a standard Pasteur pipette which was sealed with a stopper until required.

Female *A. rosae* sawflies were tested 1 – 3 days after emergence from pupation. After cooling in a refrigerator (5.5 ºC) for 1 – 4 h to temporarily slow its reactions, each insect had its wings and legs amputated and was mounted ventral side up in the groove of a Plexiglas® holder. The head and the scape of the antennae were fixed to the support with histology paraffin (Merck, mp 43-44 ºC) melted locally with a temperature-controlled soldering iron (50 ºC) and the antennae were further held in place with strips of adhesive tape across the basal segments. The preparation was mounted in a water-saturated air stream (1 ms^{-1}, 22± 3 ºC) under a stereomicroscope. We were able to obtain strong responses from these preparations for up to 2 h, giving sufficient recording time to allow all compounds to be tested on each individual. To apply stimuli we used a method based on that of Guerin and Visser (1980). The airflow was split into continuous and stimulatory airstreams in a 9:1 ratio, which then reconverged prior to reaching the preparation. For each test, a Pasteur pipette containing the selected compound in paraffin oil was unsealed and inserted into the tube carrying the stimulatory airstream. On activation of a valve the stimulus was injected into the continuous airstream and passed over the preparation's antenna. The indifferent electrode, filled with a saline solution (Kaissling 1995), was inserted into the base of the antenna distal to the scape, and the recording electrode, containing saline plus 0.1 % polyvinylpyrrolidon K90 in 100mM KCl, was brought into contact with the antennal tip. We recorded the EAG signal using a lab-built amplifier with high input impedance (10^{13} Ω) and low bias current (<10 pA). Signals were filtered (electronic high-pass with cornering frequency of 0.001 Hz), amplified 100 times and digitised using Superscope II 3.0 Software (GW Instruments, Somerville, Massachusetts) on a Macintosh computer. EAG amplitudes were measured using PowerChrom v2.2.4 software (AD Instruments, Springs, Colorado).

Seven *A. rosae* females were tested. Different isothiocyanates were presented in a randomised order, working through the concentration series from the lowest to the highest concentration for each in turn. We recorded the responses of each individual to a series of five stimuli presented at approximately 5 s intervals for each compound at each concentration and also to pure paraffin oil as a control to demonstrate that it did not elicit a strong olfactory response. Before presenting each new isothiocyanate we measured the individual's response to 100 µg trans-2-hexenal, a common volatile of green leafy material to which most herbivorous insects respond, dissolved in 100 µg of paraffin and presented as a stimulus in the same manner as the isothiocyanates.

The data were analysed by calculating the mean amplitude of the five responses to each tested combination of compound and concentration, then dividing this value by the average amplitude of the associated response to trans-2-hexenal. This standardised the data both within preparations over time and between preparations. For compounds at concentrations for which there was no measurable response, an average peak height was calculated from five 0.2 sec samples of the trace recorded at the time of stimulus, to give a value for the background noise. Mean relative responses to each compound, with standard errors, were calculated for the seven females and the data were plotted as a series of dose-response curves.

Behavioural responses to allyl isothiocyanate odour

A four-chambered olfactometer was used to test whether *A. rosae* females responded to the odour of allyl isothiocyanate and how their responses were influenced by prior experience with host-plant material. The olfactometer consisted of a cylinder (4 cm high, 19 cm diameter) made of acrylic glass divided by vertical plates into four chambers. A removable walking arena (1 cm high, 19 cm diameter) made of gauze mesh (0.5 mm) with a rim of acrylic glass (0.9 cm high) was placed on top of the cylinder and covered with a glass plate (see Steidle and Schöller 1997 for full description).

For each experimental run, 10 µl of allyl isothiocyanate solution (1 µl of allyl isothiocyanate [Aldrich] in 1 ml MeOH) was dropped onto a quarter of filter paper (55 mm diameter), and the solvent allowed to evaporate for 2 min. This test filter was placed in one of the four olfactometer chambers and the other three received quarters of filter paper treated with 10 µl MeOH as controls. The olfactometer position was rotated after each trial and the filter papers renewed after every second trial. Contamination of the walking arena with sample odours or by possible pheromones from the females was avoided by cleaning the walking arena and the

covering glass plate with ethanol and demineralised water between trials. A female was introduced into the centre of the walking arena. As soon as the female started to move, its behaviour and changes in location were recorded for a period of five minutes using the recording software The Observer 5.0 (Noldus, Wageningen, Netherlands), which was then used to calculate the duration and frequency of each activity in each chamber for each individual.

Using this method, we tested both females that were naïve (i.e. had no exposure to host plants) and host-experienced females. Naïve females emerged 2 to 4 days before testing, and were kept with honeywater at 8 ºC until a few hours before use, when they were moved to room temperature. Experienced females were kept similarly for 1 to 3 days after emergence and then offered leaves of Chinese cabbage at room temperature for a day in individual Petri-dishes. The leaves were removed in the evening and the females tested on the following day. All females used were unmated in order to be certain of standardising their condition, which can be difficult when attempting to obtain successfully-mated females in a culture. Note that, like many tenthredinid sawflies, *A. rosae* exhibits arrhenotokous parthenogenesis (fertilised eggs develop into females, unfertilised eggs into males) and shows normal oviposition behaviour even when not mated.

At the end of each olfactometer assay, all females (whether initially naïve or experienced) were offered a Chinese cabbage leaf. Only females that oviposited within 5 minutes of contact with this host were assumed to be reproductively mature. Females that did not oviposit were omitted from the analysis as their states of reproductive development were unknown.

Statistical analysis

Data were recorded on three behaviours, walking, grooming and immobility. Most females showed little grooming behaviour – about one third showed none at all in the assay period – so we have concentrated on the duration of the walking behaviour in our analysis.

As the data on walking duration were not normally distributed, they have been analysed using a non-parametric Friedman 2-way Analysis of Variance with chamber (test with allyl isothiocyanate solution, 3 solvent controls) as the experimental treatment and individual females treated as randomised blocks without replication. Data from naïve and experienced females were analysed separately. Where the overall ANOVA result was significant, a multiple comparisons test (following Sokal and Rohlf 2000) was used to identify the significant pairwise differences. Data are presented as medians with standard errors of the median.

Field responses of A. rosae to allyl isothiocyanate

We set up two experiments to investigate field responses of *A. rosae* to allyl isothiocyanate using yellow water traps, known to be effective for trapping sawflies in the genus *Athalia* (Barker *et al*. 1997). These traps were 'baited' with allyl isothiocyanate to investigate how this chemical influenced the trap catch rate of *A. rosae*.

Water traps were constructed using light aluminium trays (25 cm long, 12 cm wide, 10 cm deep, 1.5 litres in volume). Traps were sprayed with standard yellow car paint ('Daffodil yellow', Migros A.G., Switzerland). PVC tubes (7 mm diameter, 50 mm long) were filled with test solutions and sealed with PVC caps at each end. In our first experiment we tested pure allyl isothiocyanate, with empty tubes as controls, and the second used different concentrations of allyl isothiocyanate dissolved in paraffin oil, with pure paraffin-oil filled tubes as controls. These tubes allowed the slow release of the isothiocyanate through the tube walls, so that the chemical 'bait' remained active over a period of time. This design has previously been used to attract cabbage root fly (*Delia radicum* [L.]) to water traps (Finch *et al*. 1980).

In use, each trap was filled with a weak detergent solution (one drop of household liquid detergent per trap) and a tube containing the selected test solution or control was added. Traps were emptied at 3 - 4 day intervals. All insects were collected and the traps cleaned and refilled; the 'bait' tubes were also replaced. The samples were sorted under the microscope in the laboratory and all *A. rosae* recorded and sexed. All female sawflies were dissected to assess whether they were immature, with no eggs fully developed, or mature, with one or more fully developed eggs.

The following experiments were carried out:

1) Testing the effect of undiluted allyl isothiocyanate

In August 2002 two pairs of transects were laid out in a field at Delémont, Switzerland, containing young re-growth oilseed rape (B. napus) on which mature *A. rosae* larvae had been observed feeding about a week prior to the experiment. Transect pairs were placed 10 m apart starting 7 m into the field. The five traps in each transect were spaced at 10 m intervals; this should be sufficient distance to minimise interference between treatments as the traps have a maximum effective range of about 5 m (Finch *et al*. 1980). At each position from the field edge one trap was randomly assigned as the control, receiving an empty tube, and the other received a tube containing pure undiluted allyl isothiocyanate (Fluka). These constituted paired blocks, controlling for any gradient in insect catch from the field edge to the centre. The two transect sets of 5 trap pairs were set up from different field edges and were spaced over 100 m apart.

Catches were collected over two three-day intervals. For the first period, we identified and counted not only the *A. rosae* in the traps but also the cabbage root flies (*D. radicum*) as a check that the traps were effective in emitting allyl isothiocyanate.

2) Testing a range of concentrations of allyl isothiocyanate

From June to July 2003 we set up a more extensive experiment in fields in the Rhine Valley, Germany, to the south of Freiburg-im-Breisgau. In this area white mustard is widely grown as a green manure; it is not treated with pesticides and *A. rosae* infestation levels can be high (A. Barker pers. obs.). We selected four mustard fields where the crop was in-between the stem elongation and early-flowering stages and in each set out a randomised block design with four 'bait' treatments:

1) 50 % solution allyl isothiocyanate (50:50 allyl isothiocyanate and pure paraffin oil (Fluka, flamepoint 215 ºC)
2) 5 % allyl isothiocyanate in paraffin oil
3) 0.5 % allyl isothiocyanate in paraffin oil
4) Paraffin oil control

In the central 40 m of each field, we set out four transects of traps spaced 10 m apart, starting with the first trap at 10 m from the field edge and continuing at 10 m intervals across the field. To control for changes in insect density patterns from the edge to the middle, the four traps at each distance were treated as a block and the treatments randomised between them.

Statistical analyses of field trials

1) Testing the effect of pure allyl isothiocyanate

Catches of *A. rosae* in each trap over the two time periods were summed (for *D. radicum*, which was more common, we only assessed the numbers caught after the first three day period). As the data approximated to a normal distribution, a paired *t*-test was conducted on the pairs of control/treatment traps in matched positions. Catches of *A. rosae* males and females and of *D. radicum* were analysed separately. Accidental damage to a trap in one position reduced the sample size to nine.

2) Testing a range of concentrations of allyl isothiocyanate

Although four fields were used in this experiment, catches in two of them were negligible. We analysed the results from the other two fields using an analysis of variance with field and

blocks-within-fields treated as blocking factors and treatment as the main experimental factor using GENSTAT 5.0 v. 3.2 (Genstat 5 Committee 1993). Data were summed over all catch dates and log (x+1) - transformed to normalise the residuals. Catches of males and females and of mature and immature females were analysed separately. In addition we carried out a planned comparison test (Sokal and Rohlf 2000) to find out whether traps baited with allyl isothiocyanate, irrespective of concentration, attracted mature females.

The influence of plant extracts and glucosinolates on oviposition behaviour

Our first oviposition behaviour experiment was conducted in midsummer when a large pool of females was available. Females were removed from the pool and used once only in testing on one of three treatments:

1. 200 µg/ml of pure sinigrin (allyl glucosinolate as monohydrate, Carl Roth, Karlsruhe) dissolved in distilled water.
2. Hot water extract of mustard leaves (*S. alba*) made by placing 5 mg of chopped fresh leaf into 1ml of freshly boiled distilled water which was left to cool. This method extracts glucosinolates (among other water-soluble compounds) from the leaf whilst inactivating the myrosinase enzyme responsible for their breakdown to isothiocyanates (N. Agerbirk, pers. comm.).
3. Distilled water control

One or two replicates of each treatment were tested on each day of the experiment in a random order. In total 124 females were tested, 41 on the hot water extract, 41 on the control and 42 on sinigrin.

The second series was carried out in autumn when fewer females were available. For these we tested 16 females using a paired design, to maximise use of individuals and reduce variation due to between-female differences. Two treatments were tested:

1. 200 µg/ml pure sinalbin (p-hydroxybenzyl glucosinolate, the principal glucosinolate of *S. alba*, Carl Roth, Karlsruhe) in distilled water
2. Distilled water control

Each female was observed on both treatments in a random order.

Adults for this experiment were given a 24 h initial exposure to Chinese cabbage at ambient temperature to stimulate egg development, which is faster in females exposed to hosts (Barker & Molotsane, unpubl. data). After 24 h the females were moved to a container with males to allow them to mate but with no host leaves (to prevent them ovipositing once eggs developed), supplied with honey water, and returned to the rearing conditions. Under these conditions eggs mature in about 5 days, so females were used in experiments only when they were at least 6 days old.

Immediately before the start of the experiment, female *A. rosae* were placed in small groups in a container with a leaf of Chinese cabbage. Individuals that laid at least one egg were immediately removed to a pool for inclusion in the experiment, as they were evidently mature. Females that failed to exhibit oviposition behaviour within 30 minutes were assumed to be immature or sterile and were excluded.

For the experimental observations, filter paper was cut to the shape of a mustard leaf-shaped template (a simple but deeply lobed leaf approximately 9 cm by 5 cm). Paper leaf models were coated with 0.5ml of a test solution and then left to dry. Fresh solutions and new leaf models were made up on each experimental day and each model leaf was used with a maximum of two females. The stem of each leaf model was inserted vertically into a separate 6 cm-sided cube of dry florist's green block and supported with a wooden cocktail stick. The blocks were placed individually onto the base of a transparent cylindrical pot (1.3 l capacity, 10 cm diameter) with a 3 cm diameter hole cut into one side sealed with a foam bung. A small piece of cotton wool dipped into a 10 % honey water solution was also placed on the base to provide energy and moisture to sawflies during observations. Cylinders were set up in a windowless room with strong overhead lighting at a temperature of a minimum of 22 ºC to encourage insect activity. Weather conditions were noted and experiments were not carried out on rainy days, when females seemed to be much less active.

At the start of each observation a single female was removed from the pool and added to one of the pots under observation. Two or four females were watched simultaneously. Behavioural observations started as soon as all females were in place. We recorded position (on pot, on the leaf model, or on cotton wool) and activity (still, walking, grooming, probing with ovipositor, feeding/drinking with mouthparts, flying). Durations of all activities in each position were timed with a stopwatch and noted by hand for 30 minutes. Ovipositor probing was defined as a distinct bending of the abdomen into a full 90º-angle, bringing the whole distal end of the ovipositor sheath and the tip of the ovipositor into contact with the leaf model.

Statistical analysis for behavioural assays

The sawflies spent the majority of their time grooming, sitting still and walking. We analysed the effect of treatments on the time spent on leaf models and on the durations of these three behaviours performed whilst on the treated leaf models. In the sinigrin/hot water leaf extract/control trial, the data were not normally distributed (data checked using the Shapiro-Wilk test and residuals plotted and examined after one-way ANOVA). They were therefore analysed using log-linear modelling assuming quasipoisson errors with a log-link function and estimating the dispersion parameter in the model using GENSTAT 5.0 (Genstat 5 Committee 1993). The PREDICT function of GENSTAT was used to supply the predicted means and standard errors from the model. For the 'time on leaf model' analysis all females were analysed including those which had never contacted leaves ($n = 124$). For the analyses of behaviour on leaf models, however, only those females which had spent some time on the leaf model were included (93 females). In the sinalbin/control trial the females spent on average much longer on the experimental leaf models (of both treatments) and the data were normally distributed. They were therefore analysed in GENSTAT 5.0 by ANOVA with females as a block factor (data were paired for each female on the two treatments). Again, all females ($n = 16$) were analysed for the time spent on leaf models but only the 15 that spent time on both treatments were analysed for their behaviour when in contact with the models.

Three other behaviour patterns were also observed: oviposition probing and pre-flight and flight behaviour. These were too brief to be timed accurately so their durations were not analysed. However we were able to analyse the number of oviposition probes on leaf models per female. These data were discrete with many zeroes, so we used a non-parametric Kruskal-Wallis test (Sokal & Rohlf 2000) for the data from the sinigrin/hot water extract/control trial and a Wilcoxon's signed-ranks test designed to analyse paired ranked data (Sokal & Rohlf 2000) for the data from the sinalbin/control trial.

Results

Electroantennogram responses

The antennae of the *A. rosae* females responded to all the isothiocyanates tested (Figure 1). The compounds tested fell into two groups in terms of their activity. One group, allyl, benzyl and butyl isothiocyanate and iberverin, were active even at the lowest dose offered; extrapolating back on the graph suggests that the threshold value for these compounds would lie at about 0.01 µg or lower. The response to allyl isothiocyanate and possibly to butyl isothiocyanate and iberverin appeared to be nearing saturation at 100 µg, but the response to benzyl isothiocyanate was clearly still

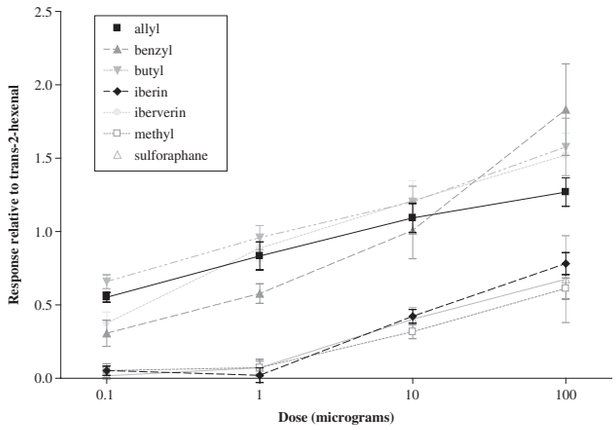

Fig. 1 Dose-response curves of EAG recorded from antennae of *A. rosae* stimulated by different isothiocyanates, expressed relative to their response to 100 µl of trans-2-hexenal (i.e. a response of 1.0 indicates a mean amplitude of response to the test compound equal to that of trans-2-hexenal). Data show means and standard errors for seven females

increasing. For the second group, consisting of iberin, methyl isothiocyanate, and sulforaphane, the antennae were much less sensitive and the threshold value was several orders of magnitude higher, at 1 µg. There was no measurable response to the paraffin oil solvent.

Behavioural responses to allyl isothiocyanate odour

Eight of the 14 naïve female *A. rosae* and 14 of the 23 experienced females laid eggs when offered hosts after the test so were included in the data analyses as being reproductively mature. Naïve female *A. rosae* showed no measurable response to allyl isothiocyanate (Figure 2a), spending similar amounts of time walking over the treated chamber as the controls (Friedman ANOVA with 3 d.f.: χ^2 = 0.450, P = 0.930). In contrast, females that had previously experienced host plant material showed a significant difference between treatments (Friedman ANOVA with 3 d.f.: χ^2 = 16.114, P = 0.001, n = 14). They spent over twice as long walking above the test chamber with the allyl isothiocyanate than above all controls (Figure 2b) and a multiple-comparisons test showed that there was a significant pairwise difference (at $P \leq 0.01$) between the test and all three control chambers. Ten of the 14 experienced females were observed fanning their wings with high frequency over the test chamber and making attempts to fly for very short distances (behaviour included in "walking" as it appeared only for short periods). This frequently carried them out of the test chamber which they rapidly re-entered to continue circling the test chamber. This 'excitement' behaviour was not observed in naive females.

Field responses to traps baited with allyl isothiocyanate

1) Testing the effect of undiluted allyl isothiocyanate

Density of *A. rosae* and of the cabbage root fly, *D. radicum* was very high during the trapping period leading to high

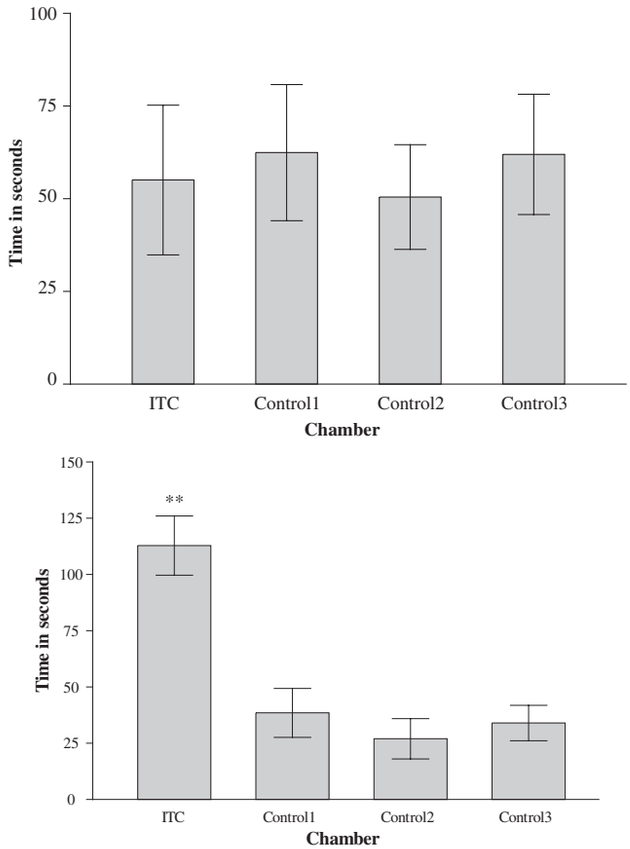

Fig. 2 Time spent by (a) naïve (n = 8) and (b) experienced (n = 14) females in walking above the allyl isothiocyanate-treated (= ITC) chamber and controls in four-chambered olfactometer. Data represent medians and standard errors of the medians; naïve females showed no differences between treatments but experienced females spent more time above the allyl isothiocyanate chamber than over the controls (** represents P<0.01, see text for analyses)

catches. The allyl isothiocyanate was attractive to *D. radicum*, with twice as many flies caught in baited traps (mean catch = 29.67, S.E. = 1.24) as in control traps (mean = 14.33, S.E. = 4.98, Paired t-test: t_8 = 3.108, P = 0.017). However, both male and female sawflies appeared to be repelled by the allyl isothiocyanate; this was significant for females (control mean = 19.33, S.E. = 3.36, baited mean = 13.67, S.E. = 2.45, t_8 = 2.422, P = 0.042), and marginally significant for males (control mean = 24.56, S.E. = 4.34, isothiocyanate baited mean = 17.78, S.E. = 2.91, t_8 = 2.126, P = 0.066).

Dissection of female sawflies showed that most of them were immature, without fully formed eggs. Mean catches of immature females from control traps were significantly higher with 17.8 females per trap (S.E. = 3.31) compared to an average of 12.4 per baited trap (S.E. = 2.20; paired t-test t_8 = 2.523, P = 0.036). Catches of mature females were comparatively low; there were no detectable differences in catches between trap types (mean in control = 1.33, S.E. = 0.33, mean in baited trap = 1.11, S.E. = 0.48; paired t-test t_8 = 373, P = 0.719).

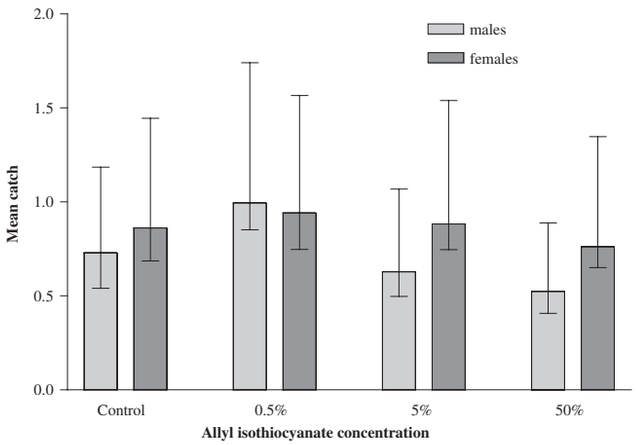

Fig. 3a Mean catch per trap of male and female *A. rosae* in water traps baited with allyl isothiocyanate at different concentrations. Means and standard errors are back-transformed from log (x+1)-transformed data, n = 80. There were no significant difference between treatments (Treatment factor in ANOVA; see text)

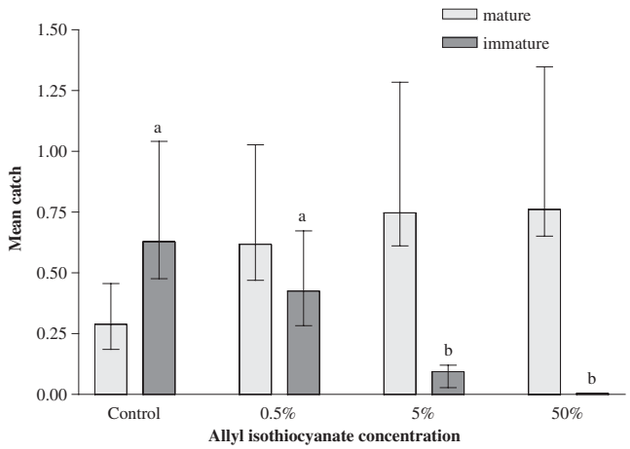

Fig. 3b Mean catch per trap of mature and immature female *A. rosae* in water traps baited with allyl isothiocyanate at different concentrations. Means and standard errors back-transformed from log (x+1)- transformed data, n = 80. There were significant differences in catches of immature females between pairs of treatments (Tukey HSD test: significant differences at P<0.05 indicated by different letters over bars). Catches of mature females did not differ between pairs of treatments but a planned comparison test showed that traps baited with allyl isothiocyanate, irrespective of concentration, attracted significantly more mature females than control traps (see text for analyses)

2) Testing a range of concentrations of allyl isothiocyanate

In this experiment overall catches were lower, reflecting a lower field density of *A. rosae* than in the previous experiment, but with a larger number of traps the data set was still informative. Overall, we found that the concentration of allyl isothiocyanate in paraffin in the 0 % – 50 % range had no significant influence on the total catches of either male (effect of allyl isothiocyanate concentration: $F_{3, 66}$ = 0.745, P = 0.529) or female ($F_{3, 66}$ = 0.080, P = 0.970) *A. rosae* (Figure 3a). However, when the catches of mature and immature females were examined separately, it could be seen that immature females showed a negative response to rising concentrations of allyl isothiocyanate (Figure 3b; Treatment effect in ANOVA: $F_{3, 66}$ = 5.376, P = 0.002). As the concentration rose, the catch reduced sharply, to the point where no immature females were caught in the 50% allyl isothiocyanate baited traps. With mature females there were no significance differences between pairs of treatments (Treatment effect in ANOVA: $F_{3, 66}$ = 1.467, P = 0.232) but there was an attraction to allyl isothiocyanate across all concentrations tested (Figure 3b) which was confirmed by the significance of the planned comparison between the three isothiocyanate treatments and the control ($F_{1, 66}$ = 4.097, P = 0.047).

Influence of glucosinolates on oviposition behaviour

None of the treatments tested had a significant effect on the amount of time females spent on the test leaf models. Females in the sinigrin/hot water leaf extract/control trial (Figure 4a) spent relatively little time on leaf models of all treatments, with an average of less than 180 seconds (3 minutes) in 30 minutes of observation. Although inspection of the data (Figure 4a) suggested that females were spending more time on leaf models with hot water leaf extract than on controls or sinigrin-treated models, the data were highly variable and there was no statistically significant difference between treatments ($F_{2, 121}$ = 1.25, P = 0.289). Once on the leaf model, females were most frequently observed walking over the surface, followed by grooming and resting. They did not differ in the time spent on these activities on different treatments (Walking $F_{2, 90}$ = 0.48, P = 0.618, Resting $F_{2, 90}$ = 1.37, P = 0.259; Grooming $F_{2, 90}$ = 2.54, P = 0.085). Females in the sinalbin/control experiment (Figure 4b) spent much longer (around 900 seconds) on the leaf models, about half the trial time, but there was no difference in residence time between controls or sinalbin-treated leaf models ($F_{1, 15}$ = 0.83, P = 0.376). Both time spent resting on leaf models and grooming time were longer than in the previous experiment, but neither activity was linked in duration to treatment (Resting $F_{1, 14}$ = 3.61, P = 0.078; Grooming $F_{1, 14}$ = 0.02, P = 0.886). The time females spent walking was similar to that observed in the previous trial, but there was a marginally significant tendency for them to walk for longer on sinalbin-treated than control leaf models ($F_{1, 14}$ = 4.62, P = 0.050).

Sinigrin, hot-water mustard leaf extract and sinalbin all increased the number of times that females probed the leaf models with their ovipositors (Figure 5). About 30% and 35 % of females on sinigrin treatment and the hot water mustard leaf extract respectively showed some probing behaviour, as opposed to only 7 % on controls, and the overall number of probes was significantly higher on these two treatments than the control (Kruskal Wallis test: χ^2 = 10.73, P = 0.005). There was no difference in the number of probes between the hot water mustard leaf extract and the sinigrin treatment (Mann-Whitney test on these two treatments: χ^2 = 0.001, P = 0.974). Sixty-seven percent of females on

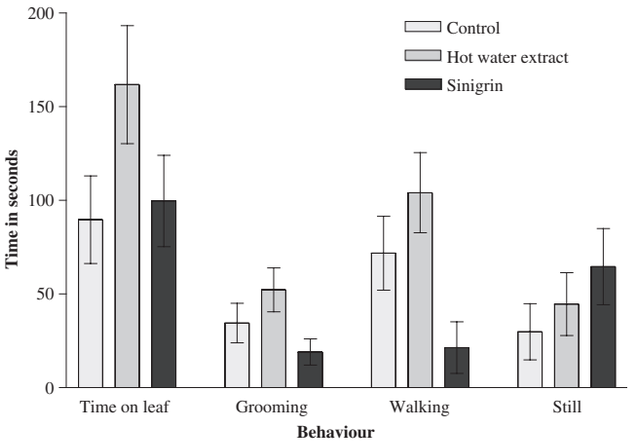

Fig. 4a Behaviour of *A. rosae* females on leaf models with three treatments: water (control), a hot water *S. alba* leaf extract, and sinigrin. Data shown are average time spent on leaf models by all females (n = 124) and time spent on the leaf in three main activities by females that contacted model leaves (n = 93). Means and standard errors predicted by a linear model assuming a Poisson distribution. There were no significant differences between treatments (see text)

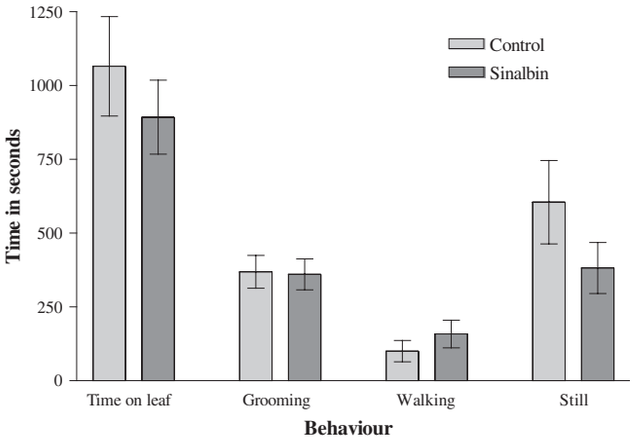

Fig. 4b Behaviour of *A. rosae* females on leaf models treated with water (control) and sinalbin, showing mean time spent on leaf models by all females (n = 16) and mean time spent on three main activities by females that contacted leaves (n = 15). Means and standard errors from raw data. There were no significant differences between treatments (see text) except for a marginally significant difference (P = 0.050) in the time spent walking on the two treatments

sinalbin-treated leaf models probed them one or more times, as opposed to 13 % on water controls, and with significantly more probes being observed on sinalbin than on controls (Wilcoxon's signed-ranks test: Z = 2.820, P = 0.005).

Discussion

Our results showed that the sawfly *A. rosae* was able to detect isothiocyanates through antennal olfaction and that experienced female sawflies oriented towards sources of allyl isothiocyanate in static olfactometer trials, although naïve females did not (Figure 1 and 2). In the field, mature females were attracted by allyl isothiocyanate, but immature females were repelled by it at high concentrations (Figure 3). In the contact phase of oviposition, the glucosinolates sinigrin and sinalbin triggered oviposition-probing behaviour to the same extent as a hot-water extract of a host leaf, which would have included the leaf's glucosinolates (Figure 5). These results show that isothiocyanates and glucosinolates are integrally involved in the host-finding and host use behaviours of *A. rosae*.

Effects of isothiocyanates and glucosinolates on physiology and behaviour have been reported for many cruciferous insects. The lepidopterans *Pieris brassicae* L., *P. rapae* L. and *Plutella xylostella* L., the coleopteran *Ceutorhynchus assimilis* Payk. and the dipteran *D. radicum* (Evans and Allen-Williams 1992, van Loon *et al*. 1992, de Jong & Städler 1999, Renwick *et al*. 2006) have all been shown in electrophysiological studies to have antennae sensitive to low levels of isothiocyanates. *C. assimilis* and the lepidopteran *Hellula undalis* Fab. (Bartlet *et al.* 1993, Mewis *et al*. 2002) were attracted to isothiocyanates offered in olfactometer experiments, and allyl isothiocyanate-baited traps have been shown to attract both *D. radicum* and

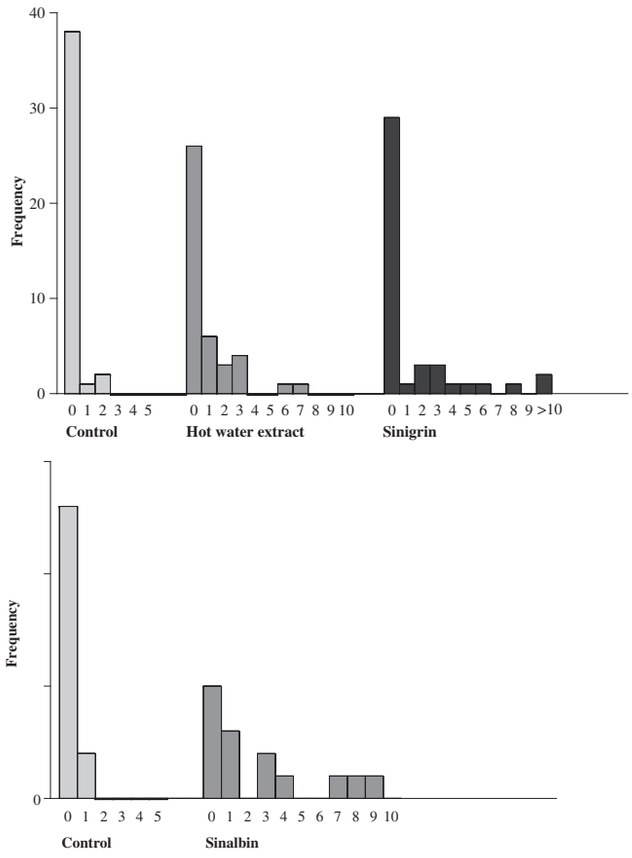

Fig. 5 Frequency distribution of numbers of probes by females on **(a)** control, hot extract-treated and sinigrin-treated model leaves, and **(b)** control and sinalbin-treated model leaves. Each record represents one female, so for example, in Fig. 5a, 38 control females never probed, one probed once and two probed twice. See text for statistical analyses

cruciferous flea beetles (Chrysomelidae) (Finch *et al*. 1980, Vincent & Stewart 1984). *D. radicum*, the closely-related turnip root fly *Delia floralis* Fallen, and *Pieris* spp. butterflies are stimulated to lay eggs on leaf models or non-host leaves treated with glucosinolates (Roessingh *et al*. 1992, Huang & Renwick 1994, Simmonds *et al*. 1994, Städler *et al*. 1995, Städler *et al*. 2002). There is a general pattern that emerges that cruciferous insects can detect and will orient towards isothiocyanates both over short distances in controlled laboratory studies and in the more complex conditions of field trials, and that contact with glucosinolates stimulates their oviposition behaviour (although only a few insect species have been tested at all these different levels). We have been able to integrate *A. rosae*'s sensory and behavioural responses to show that they fit into this pattern.

As *A. rosae* is oligophagous on a variety of crucifers with different chemical profiles, and can sequester most but not all classes of glucosinolates (Müller *et al*. 2001), the ability to respond to a range of isothiocyanates which we have demonstrated here may have a role in allowing it to find different hosts. Our EAG results suggest that there may be differential responses to different isothiocyanates, although one has to be cautious as these results will be partially confounded by the different volatility of different compounds and hence the different concentrations reaching the antenna. It is worth noting though that in equivalent experiments with *P. xylostella* using a similar range of compounds in the same laboratory (Renwick *et al*. 2006), a very different relative response pattern was observed that could be directly related to the known oviposition responses of *P. xylostella* to host compounds, suggesting that *A. rosae*'s compound-specific responses may also have an adaptive significance. It will be interesting to extend our electrophysiological and behavioural work to a greater range of host plant volatiles with the ultimate aim of investigating possible synergistic effects, such as those detected for the cabbage seed weevil *C. assimilis* (Bartlet *et al*. 1997) and the cabbage root fly *D. radicum* (Städler *et al*. 2002), which may better reflect the sawfly's response to the chemical profiles of real plants. Similarly, our assays of contact chemoreception could be broadened to other host plant chemicals; the extended time spent on leaves with whole leaf extract relative to those with just sinigrin, although not statistically significant, might be a hint that other water-soluble chemicals are involved in arresting sawflies on host leaves.

One novel discovery in our study has been the extent to which females' behavioural responses to allyl isothiocyanate were dependent on their physiological maturity and oviposition experience. Fully mature females were attracted by this compound in both laboratory and field experiments, but naïve females in olfactometer tests showed no response to it, and females with no mature eggs in field experiments were repelled by allyl isothiocyanate. They were still attracted by the visual cue of the yellow water traps but avoided them if allyl isothiocyanate was present, apparently in a dose-dependent manner (Figure 3b). The lack of response by naïve females in olfactometer trials may be directly due to the lack of experience with host plants; modification of oviposition behaviour following exposure to host plants has been demonstrated in other insect herbivores (Szentesi & Jermy 1989) including the cruciferous *P. rapae* (Traynier 1979). However the active repellency of allyl isothiocyanate to immatures was a strong response; one would not expect this solely as a result of inexperience. A logical possibility is that pre-reproductive females were using plant cues to reinforce a migration strategy. *A. rosae* is known sometimes to make substantial migrations in search of new habitats (Benson 1962, Nagasaka 1992, Barker pers. obs.). Since isothiocyanates are released from plant cells mainly via myrosinase-catalysed hydrolysis of glucosinolates following cell damage (Bones and Rossiter 1996), at high concentrations they may indicate to newly-emerging females that herbivore population densities on local plants are already high and that dispersal to new sites would be advantageous. In contrast, once females have developed eggs they have less fat in reserve for migration and only live a few days, so they will gain more from finding hosts and laying their eggs regardless of the state of damage of the plants. We note that *D. radicum*, which was also positively attracted to the allyl isothiocyanate in our traps, is a root feeder that is actually attracted to damaged plants (Baur *et al*. 1996). There are other examples of migration strategies being linked to host-plant relationships and reproductive state, most notably among aphids that have differing seasonal forms and even alternate hosts in different seasons. For example the black bean aphid, *Aphis fabae* Scop., produces three forms: one that migrates in spring from the primary host, spindle tree (*Euonymus europaeus* L.), to various summer secondary hosts including broad bean (*Vicia faba* Moench), a second that flies to locate new hosts in summer in response to overcrowding, and a third form that migrates in autumn to relocate spindle. Summer migrants respond to visual plant cues after relatively short flights and are attracted strongly to odours from undamaged but not damaged bean plants, suggesting a facultative migration strategy aimed at rapid location of new hosts rather than overall dispersal; in contrast the autumn form are true migrants which show responses to plant visual cues only after long flight periods, and which do not respond to host plant odours (Nottingham and Hardie 1989, Nottingham *et al*. 1991) .

One practical difficulty in working with *A. rosae* has been a problem common to all attempts to study the chemical ecology of tenthredinid sawflies, namely that they lay eggs obligately into plant tissue, making it very difficult to create artificial model oviposition targets on which they will lay eggs. Although some studies with other insect families have had success with treating leaves from non-host plants, these will still have characteristic secondary chemicals and surface waxes which make them unlikely to be accepted by insects that lay into leaf tissue; brief trials with this technique were wholly unsuccessful (Barker, unpublished data). The assay we used, quantifying ovipositor probing behaviour on a paper leaf model, was a development of that used by Nishida and Johki (1989) and was also similar to the technique used as a measure of the ovipositional responses of a willow-feeding sawfly *Euura lasiolepis* Smith to compounds characteristic of their host plants (Roininen *et al*. 1999). The ovipositor probing behaviour we quantified is closely linked to oviposition itself, being the last step in the pre-oviposition sequence and the normal precursor to egg-laying (Lee *et al.* 1998). Nishida and Johki (1989) recorded

the number of females that showed this ovipositor probing behaviour on sinigrin-treated paper and controls; we resolved our data further by recording the number of individual probes (i.e. separate touches of the sheath tip onto the surface) made by each female and showing that this differed significantly between treatments. Like Nishida and Johki (1989), we showed a positive response to sinigrin equivalent to the response to a hot-water whole leaf extract, and we also demonstrated for the first time a positive response to sinalbin, the principal glucosinolate of *S. alba*, one of *A. rosae*'s main host plants in Europe.

These results show that glucosinolates stimulate oviposition responses. However, at present it remains unclear whether glucosinolates are available to females walking across a normal leaf surface. Although Griffiths *et al.* (2001) found glucosinolates in leaf surface solvent extracts, experiments using adhesives instead of solvents (Reifenrath *et al*. 2005) found that glucosinolates are not naturally present in detectable quantities in the outermost wax layer of two crucifers, *Brassica napus* L. var. 'Martina' and *Nasturtium officinale* R. Br. By extension, this suggests they are absent from the leaf surfaces of other crucifers, which would make them less readily available to insects in normal contact with the leaf surface than previously supposed. It is possible that scratches to the surface from tarsal claws and spines or even the ovipositor itself during probing may bring sawflies into sensory contact with glucosinolates.

A final question is the extent to which *A. rosae*'s requirement for sequestered glucosinolates for larval defence determines its responses to isothiocyanates and glucosinolates. Many cruciferous insects respond to these compounds, but *A. rosae* is the only holometabolous insect known to sequester them. The possibility that oviposition responses of adult holometabolous insects can be based on compounds sequestered during the larval stage, whilst apparently logical, has barely been investigated. The only example other than *A. rosae* of which we are aware is a swallowtail butterfly, *Battus philenor* (L.), which is stimulated to oviposit by the aristolochic acids it sequesters from its host as a caterpillar (Sachev-Gupta *et al*. 1993, Sime *et al*. 2000). Adult oviposition behaviour and larval feeding behaviour in insects appear to have different genetic origins and thus are potentially independent (Thompson *et al*. 1990; Keese, 1996; Janz *et al*. 2001); however, in insects that rely on larval sequestration as a defence strategy one would expect these traits to have evolved in close parallel to allow selective oviposition on a host with appropriate chemistry. As this would predict, *A. rosae* has host-finding and oviposition behaviours that are attuned to confirming the presence of glucosinolates in potential hosts.

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