Effectiveness of the defence mechanism of the turnip sawfly, *Athalia rosae* (Hymenoptera: Tenthredinidae), against predation by lizards

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Abstract

The turnip sawfly, *Athalia rosae* Linnaeus, is a pest on cruciferous crops. Larvae sequester secondary plant compounds, namely glucosinolates, in their haemolymph. When attacked, their integument is easily disrupted and a droplet of haemolymph is exuded (‘easy bleeding’). This has been shown to be an effective, chemical-based, defence against invertebrate predators. The efficiency of this proposed defence was tested against a vertebrate predator, using groups of the iguanid lizard *Anolis carolinensis* Voigt as a model predator. Caterpillars of *Pieris rapae* Linnaeus and *Pieris brassicae* Linnaeus served as control prey species that do not sequester glucosinolates. Lizards attacked far fewer sawfly larvae than pierid caterpillars. Several of the sawfly larvae were rejected after an initial attack, demonstrating unpalatability to the lizards, while the *Pieris* larvae were not rejected. However, *P. rapae* larvae topically treated with extracts of haemolymph of *A. rosae* had no deterrent effect on the lizards and no avoidance learning occurred over a period of two weeks. Adult sawflies do not easy bleed but have glucosinolates carried over from the larval stage. Lizards attacked them at a higher rate than larvae and they were never rejected. The results suggest that for the defensive effectiveness of the pest sawfly species against vertebrates the chemical cue is not necessarily sufficient. Movement and colour may be important additional factors triggering the behaviour of vertebrate predators.

Introduction

Many herbivorous insects are specialized on plants with deterrent secondary compounds, and several of these specialists are able to sequester the plant compounds for their own defence against predators (e.g. Nishida & Fukami, 1990; Bowers, 1992; Trigo, 2000). Often, unpalatability is signalled by a conspicuous warning coloration, called aposmatism (Guilford, 1990). This study focuses on the defence of the herbivorous turnip sawfly, *Athalia rosae* Linnaeus (Hymenoptera: Tenthredinidae), against a vertebrate predator. The black larvae are very effective in storing glucosinolates from various host plant species of the Brassicaceae, and concentrate them in their haemolymph more than ten-fold in comparison to their host plant (Müller et al., 2001). *Athalia rosae* can be destructive to cultivated vegetables within the Brassicaceae (Benson, 1950).

Glucosinolates are characteristic secondary compounds of Brassicaceae and form an effective defence system against generalist aquatic and terrestrial herbivores by reducing leaf palatability (Louda & Mole, 1991; Newman et al., 1992; Giamoustaris & Mithen, 1995). Glucosinolates and their hydrolysis products also show fungicidal, bactericidal, nematocidal and allelopathic properties (Chew, 1988; Fahey et al., 2001 and references therein). Several insect species,
however, are specialized on glucosinolate-containing plant species and even use these compounds as feeding and/or oviposition stimulants (Nielsen, 1988; Chew, 1995; Schoonhoven et al., 1998; Renwick, 2002), or for sequestration (Weber et al., 1986; Müller et al., 2001; Aliabadi et al., 2002). Upon harassment, larvae of *A. rosae* exude glucosinolate-containing haemolymph in a droplet through a readily rupturing integument. This phenomenon has been described as ‘easy bleeding’, and occurs in larvae of several thenthredinid sawfly species (Heads & Lawton, 1985; Heads, 1986; Boevé & Schaffner, 2003). The haemolymph of *A. rosae* has been shown to be deterrent against ants, *Myrmica rubra* Linnaeus (Hymenoptera: Formicidae), even when diluted ten-fold or more (Schaffner et al., 1994; Müller et al., 2002). Predatory wasps, *Vespula vulgaris* Linnaeus (Hymenoptera: Vespidae), rejected sawfly larvae after an initial attack and also avoided palatable prey, such as caterpillars of *Pieris rapae* Linnaeus (Lepidoptera: Pieridae), when these were treated with haemolymph of *A. rosae* (Müller & Brakefield, 2003). Thus, the defensive mechanism of the easy bleeding against invertebrates is clearly based on the chemistry. The black sawfly larvae were also avoided by chicks, *Gallus gallus* Linnaeus (Aves: Phasianidae), mainly due to warning coloration (Ohara et al., 1993). The acceptance of *A. rosae* to another vertebrate was tested to gain more insight into the defence mechanisms of the sawfly against this predator group. Both larvae and adults were used since the orange and black adults of *A. rosae* do not exhibit ‘easy bleeding’ but still contain the glucosinolates sequestered by the larvae in their haemolymph albeit in a five-fold lower concentration (Müller et al., 2001).

In this study, *A. rosae* were reared on white mustard, *Sinapis alba* L. (Brassicaceae), from which they sequester the glucosinolate sinalbin (p-hydroxybenzylglucosinolate) (Müller et al., 2001). Caterpillars of *Pieris brassicae* Linnaeus (Lepidoptera: Pieridae) and *P. rapae* were reared on the same host plant species and used for comparison of the effectiveness and mechanism of defence. According to Aplin et al. (1975), pupae of *P. brassicae* contain glucosinolates and their hydrolysis products, isothiocyanates, the latter also found in *P. rapae*. However, no glucosinolates could be found in larvae of either species in a more recent study (Müller et al., 2003). While larvae of *P. brassicae* are conspicuous colour-morphs (yellowish-green with black stripes), those of *P. rapae* are green.

To investigate the palatability of *A. rosae*, larvae of this sawfly species as well as control caterpillars of *P. rapae* and *P. brassicae* were offered for consecutive weeks to groups of the lizard *Anolis carolinensis* Voigt (Reptilia: Iguanidae), a generalist insectivore (Sexton, 1964). Lizards have been recorded as predators of sawflies (Benson, 1950) and Lepidoptera (Sexton, 1964), and their role as selective agents on evolution of unpalatability discussed controversially (Odendaal et al., 1987). *Anolis carolinensis* is a small, diurnal lizard from the southeastern part of the USA and the Caribbean area (Roughgarden, 1995; Lyytinen et al., 2003). It was captive reared in the laboratory, and used here as a model vertebrate predator. To investigate whether the haemolymph of *A. rosae* larvae, after predator attack often emitted as ‘easy bleeding’ is deterrent to lizards, palatable prey was treated with extracts of haemolymph and offered for a period of two weeks. Finally, to evaluate the defensive effectiveness of different developmental stages of *A. rosae* that do or do not evoke ‘easy bleeding’ adult turnip sawflies were offered to lizards.

### Materials and methods

#### Lizard predators

Eighteen specimens of *A. carolinensis* were obtained a year before the experiments started. They were kept in six cages (dimensions: 46 × 32 × 57.5 or 51 cm, length × breadth × height) in a climate cell (31/25°C day/night, 70% relative humidity, 12/12 h light/dark). Lizards were painted with numbers to distinguish individuals. Every cage contained one male and two female lizards, a substrate of bark chips (premium reptibark, Zoomed Laboratories Inc., Canada), a maize plant, *Zea mays* L. (Poaceae), several bamboo perches, and a shallow Petri dish with water (diameter 90 mm), renewed daily. The cage was misted twice daily. Lizards were fed buffalo worms, *Alphitobius laevigatus* Fabricius (Coleoptera: Tenebrionidae) dusted with calcium or vitamin supplement, sometimes complemented with live crickets, *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae).

#### Prey species

*Athalia rosae,* *P. rapae* and *P. brassicae* were kept in culture using a stock of *A. rosae* established from a field collection in Germany, and pierid eggs obtained from the University of Wageningen. Insects were reared on *S. alba* plants at 21°C, 70% relative humidity and 16/8 h light/dark. Larvae of comparable size were used (*A. rosae* fifth instar, *Pieris* spp. second to third instar). The two pierid species served as functional controls that do not show ‘easy bleeding’ and do not sequester glucosinolates, in contrast to *A. rosae*.

#### Feeding assays with larvae of different prey species

Groups of lizards were offered one of the prey species daily in their cages for one week. The species of prey was changed on a weekly basis for two consecutive periods of three weeks each (table 1). Each group (1–3) received prey species in a different order. A group consisted of two cages and thus six lizards. Prey larvae were starved for 1 h prior to introduction into the cages to induce more mobility. They were offered in a small Petri dish (diameter 25 mm) that was placed within a larger Petri dish (diameter 90 mm). The outer dish was filled with water to prevent prey from escaping. Five prey items were presented in each feeding trial.

The lizard cages were visually isolated from one another. Two cages (one group) were observed simultaneously for 1 h after introduction of prey from behind a one-way mirror. Attacks and the fate of the larvae (consumption or rejection) were noted. Rejected larvae were recovered at the end of the observations and kept with food for 24 h to observe whether they survived the attack. If lizards refused to feed during the experiment they were offered some larvae of *A. laevigatus* afterwards to test for their feeding activity. Statistical analyses used the Chi square-test for homogeneity except with expected frequencies below five when the Fisher’s exact test was performed.

#### Feeding assays with haemolymph-coated *P. rapae* larvae

Sawfly larvae often show ‘easy bleeding’ when touched by predators. To test whether the sawfly haemolymph was deterrent, the lizards were offered *P. rapae* larvae coated with an extract of either *A. rosae* haemolymph (*Athalia*-treated) or *P. rapae* haemolymph (*Pieris*-treated). Two groups of lizards
were tested. Group A daily received *Athalia*-treated *P. rapae* larvae in the first week and *Pieris*-treated *P. rapae* larvae in the second week, group B received the prey items in the reverse order. Prior to the experiment, haemolymph had been collected subsequently from larvae reared on *S. alba* by piercing the integument with a needle without damaging the gut. Haemolymph was collected in glass capillaries, transferred into vials (2.5 ml), and immediately frozen at −80°C. Each larva was only milked once. After lyophilization, supernatant transferred to a new vial. Extraction was done with a spatula, vortexed for 60 s, centrifuged for 2 min, and the supernatant transferred to a new vial. Extraction was done three times. Ethanol was evaporated under a low air stream and freshly solvent equivalents of 6 µl fresh haemolymph were re-dissolved in 3 µl ethanol, which is sufficient to cover a *P. rapae* larva and corresponds to the amount that could typically be collected from a single *A. rosae* larva (fifth instar, similar size). Solvent was allowed to evaporate under a low air stream and freshly treated larvae offered to the lizards.

For this experiment, 21 lizards in eight cages (containing one male and one or two females) were used, including some newly acquired individuals. All lizards were familiar with *P. rapae* as prey at that time. Observations were done as described above. A group consisted of four cages with lizards. One cage from each group (A and B) was observed simultaneously in order to spread offering of the two treatments equally over a day.

**Feeding assays with adult *A. rosae***

Six lizards in two cages were offered adult *A. rosae* daily for a period of two weeks. A Petri dish with lid (diameter 50 mm) containing six sawflies was introduced to each cage, the lid removed and the lizard behaviour observed for 30 min as described above.

**Results**

**Attacks on larvae of different prey species**

The three groups of lizards responded differently to the offered prey (table 1). Group 3 did not attack any prey until the fourth week, and only during the sixth week was the number of attacks comparable to the other two groups. During the third week no *P. rapae* could be offered to this group due to shortage of larvae. Therefore, it was excluded from statistical analyses.

The prey species were attacked in different frequencies (table 1). The attack rate on *P. rapae* larvae was significantly higher than on *P. brassicae* (*P* < 0.05), and both pierid species were attacked significantly more than *A. rosae* (*P* < 0.001 for both) (fig. 1). As the lizard groups received the prey species in different orders, order had no influence on the attack rate.

One female lizard from group 2 was responsible for most of the consumptions and rejections of *A. rosae* during week 6, first rejecting (see below) two larvae in two days, then eating 12 in the following three days. In attacks of all other trials, at least two lizards were involved.

Prey offered during the second period were in most cases attacked more than in the first period. This was significant for group 1 attacking *P. brassicae* and for group 2 attacking *A. rosae* (*P* < 0.01 in both cases). However, when excluding the data from the female lizard mentioned above, the number of attacks on *A. rosae* was not significantly different between the treatments equally over a day.

![Fig. 1. Percentages of larvae attacked (= eaten + rejected) by *Anolis carolinensis* lizards belonging to groups 1 and 2. The number of each larval species offered is given in brackets below the columns. Bars with different lower case letters are significantly different at *P* < 0.001 (ab and ac) or *P* < 0.05 (bc) (Chi square-test for homogeneity).](image-url)

Table 1. The number of larvae of *Athalia rosae*, *Pieris rapae* and *P. brassicae* offered, eaten and rejected by groups 1–3 of the lizard *Anolis carolinensis*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Larvae</th>
<th>First period (weeks 1–3)</th>
<th>Second period (weeks 4–6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Offered</td>
<td>Attacked</td>
<td>Eaten</td>
</tr>
<tr>
<td>1</td>
<td><em>P. rapae</em></td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td><em>P. brassicae</em></td>
<td>45</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td><em>A. rosae</em></td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

Group 3 was not offered any *P. rapae* larvae during the first period. Values in brackets are the results excluding data of one individual female lizard.
the two periods for group 2. Compared to group 1, group 2 attacked a higher frequency of *P. rapae* and *A. rosae* larvae throughout the whole experimental period, but this was only significant for the attack on *P. rapae* during the second period (*P < 0.01*). Compared to group 2, group 1 attacked significantly more *P. brassicae* larvae during the second period (*P < 0.01*).

The tendency to attack prey more during the second period was similar for all groups. There was no overall tendency for one group of lizards to attack prey more than the other group, independent of larval species. Therefore it was considered justified to pool the data for the two periods and for the two groups. All lizards were, in general, hungry as they always accepted *A. laevis* larvae offered after trials in which no prey were eaten.

Attacked prey could either be eaten or rejected. When rejecting prey, lizards typically dropped the larva immediately after grasping it, without further mastication, and rubbed the sides of their beak against the substrate on the spot (beak-wiping). Of the offered pierid larvae, only one *P. brassicae* was rejected. This larva was quite large (fourth instar) and after 6 min of trying to swallow it, the lizard spat it out. Although the number of sawfly larvae attacked was lower, a larger proportion of *A. rosae* was rejected.

The two groups (1 and 2) of lizards did not differ significantly in the proportion of prey consumed and rejected per period for each of the three larval species (Fisher’s exact test). Thus, the data are pooled for the groups. But, in contrast to the data on attacks, there was a significant difference between periods. During the first period, *A. rosae* was rejected significantly more than either pierid species (*P < 0.001* for each, Fisher’s exact test; with no significant difference between the pierids). During the second period there was no difference in the lizard rejection behaviour towards *A. rosae* and *P. rapae* even when data from the female lizard noted above were excluded (Fisher’s exact test).

In total, nine *A. rosae* larvae were rejected by six different lizards. Three larvae could not be found after the observational period so their fate remained unknown. Of the remaining larvae two died and four survived.

**Attacks on haemolymph-coated P. rapae**

Both *A. rosae*-treated and *P. rapae*-treated larvae were attacked by the lizards throughout the experimental period in comparable fashion. During both weeks group A consumed significantly more larvae than group B, irrespective of treatment (*P < 0.01* for the first week, *P < 0.001* for the second week) (fig. 2). None of the *A. rosae*-treated larvae were rejected, although beak-wipe behaviour was observed on three occasions near the end of the second week following consumption. One of the *P. rapae*-treated larvae was rejected. This larva had crawled out of the dish and was picked up together with some wood chips. The lizard took it to the back wall of the cage, where it beak-wiped several times, thereafter dropping the larva which survived the attack.

**A. rosae adults**

Two-thirds of the adult sawflies were attacked and eaten within 5 min after introduction into the cages. No rejections were observed (table 2). The data did not differ significantly between the two weeks (Fisher’s exact test), and so were pooled. In comparison to the larvae (feeding assay with larvae), the adults were attacked significantly more (*P < 0.001*), and eaten or rejected in significantly different proportions (*P < 0.001*; after Bonferroni correction) (table 2).

**Discussion**

Lizards attacked larvae of the pest species *A. rosae* significantly less often than the pierid larvae (fig. 1). Several different prey traits could be responsible for the lizard behaviour. *Athalia rosae* larvae move less than those of either *Pieris* species, and thus might present a less strong visual stimulus to *A. carolinensis*. Lizards are known to be highly visual predators, depending on movement to detect prey (Burghardt, 1964; Curio, 1970; Goodman, 1971; Fleishman, 1986, 1992). The rate of attack was highest on *P. rapae*. Different attack rates on the two pierid species had been observed before (Aplin et al., 1975) and could be due to the hairiness of the *P. brassicae* larvae. Several authors have described avoidance of hairy food by vertebrate predators, such as birds (e.g. Whelan et al., 1989; Hilker & Köpf, 1994). Warning coloration might also have played a role; the dark colour of the *A. rosae* larvae could have been a more aposematic signal than the yellow and black of *P. brassicae*, both being more warningly than the uniform green larvae of...
However, the lizards had had no experience with either prey before the experiments. At the beginning of the experiments they immediately attacked only rarely sawfly larvae, making the aposematism hypothesis less likely.

Additionally, A. rosae larvae were rejected significantly more often than the pierid caterpillars. This rejection was immediate; lizards dropped the larvae without further mastication and beak-wiped. Beak-wiping behaviour has been noted for A. carolinensis (Sexton, 1960, 1964; Stanger-Hall et al., 2001; Sword, 2001) and other lizard species (Boyden, 1976) as a clear indicator of prey unpalatability. Thus, it appears that the sawfly larvae were also chemically defended. Unpalatable prey can be recognized by lizards using taste buds on their tongue (Schwenk, 1995), a perception which can be reinforced by the prey colour, leading to association between the two stimuli (Sexton, 1960, 1964; Sexton et al., 1966; Shafir & Roughgarden, 1994; Sword, 1999, 2001; Sword et al., 2000; Stanger-Hall et al., 2001).

However, the rate of attacks on A. rosae increased during the second period and fewer rejections occurred. Most noteworthy was the single female lizard in group 2 that first rejected two A. rosae larvae but then ate twelve on the subsequent three days without any sign of rejection behaviour. Thus, at least some individual lizards may be able to overcome their initial taste aversion.

No comparable rejection to that of A. rosae was observed for the pierid larvae. The one P. brassicae larva that was eventually rejected was only dropped after a 6 min struggle, and was clearly too large to be swallowed whole. Our finding of higher acceptance of both Pieris species than A. rosae could be correlated to the fact that the larvae of P. brassicae and P. rapae do not sequester bitter-tasting glucosinolates (Müller et al., 2003) unlike the sawfly larvae (Müller et al., 2001).

However, although A. rosae larvae were rejected, it was not possible to demonstrate with the coating experiments that the active chemically deterrent principle of the sawfly haemolymph is sufficient on its own. Both A. rosae-treated and P. rapae-treated P. rapae larvae were eaten, and consumption rates tended to increase during the second week, regardless of treatment (fig. 2). Lizards might not have yet re-associated the appearance of P. rapae with unpalatability. The lizards had eaten P. rapae before in the first experiment and found them palatable. Since the visual system is of paramount importance in A. carolinensis (Burghardt, 1964; Fleishman, 1986), contradictory chemical information might need more time to overrule established preferences. Indeed, near the end of the second week some lizards in group B started wiping their beaks after consuming A. rosae-treated pierid larvae but there was no learning of avoidance. Any unpalatable compounds of A. rosae haemolymph might also not have been extracted by the procedure or were diluted too much in the solutions. Chemical analyses of some A. rosae-treated Pieris larvae showed glucosinolates to be present in a slightly lower concentration compared to that in A. rosae larvae. Direct application of A. rosae haemolymph on P. rapae would have been a more preferable method but has the disadvantage of being very time-consuming and requiring the simultaneous availability of large stocks of two larval species over a long period.

The behavioural response of the vertebrate predator is in clear contrast to invertebrate predators such as ants and wasps. Ants were deterred by the haemolymph of sawfly larvae even in a manifold dilution (Schaffner et al., 1994; Müller et al., 2001) and wasps avoided P. rapae caterpillars (which are usually accepted as food) when these were coated with sawfly haemolymph (Müller & Brakefield, 2003). However, in the latter experiment, fresh, not further processed haemolymph was applied.

Adult A. rosae were attacked more rapidly by the lizards and in higher numbers emphasizing the crucial importance of movement for prey detection by the lizards. Movement seems to overrule colour as well as chemistry. Thus the bright orange and black pattern of the adults, which could be viewed as warning coloration, together with glucosinolates carried over from the larval feeding, was apparently not sufficiently deterrent (table 2). This contrast with the larvae could be due to the fact that adults do not easy bleed, and perhaps the glucosinolate concentrations in adults fall below the detection threshold of the lizards. It was not observed whether the lizards would reject adult A. rosae after some (long) time lag from commencing to take them, but this seems unlikely since the consumption rate remained unaltered and no rejections occurred over the course of two weeks.

In summary, these observations demonstrate that the defence mechanism of the larvae of the pest species A. rosae known to be effective against invertebrate predators can also be effective against vertebrates. The larvae were frequently rejected and some survived lizard attacks. However, while against invertebrate predators the deterrent principle of the sawfly haemolymph overrules other cues, this is not the case for lizards. The results suggest that only the combination of several strategies such as low mobility, warning coloration, easy bleeding, and distastefulness offers an effective protection against this vertebrate predator.

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