

Leaf and Floral Parts Feeding by Orange Tip Butterfly Larvae Depends on Larval Position but Not on Glucosinolate Profile or Nitrogen Level

Niels Agerbirk · Frances S. Chew · Carl Erik Olsen · Kirsten Jørgensen

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Abstract In an attempt to identify chemical signals governing the general flower and silique feeding behavior of larvae of the orange tip butterfly, *Anthocharis cardamines* (L.), we investigated feeding behavior and chemistry of two major host plants: *Cardamine pratensis* L. and *Alliaria petiolata* (Bieb.) Cavara & Grande (garlic mustard). Larvae reportedly feed mainly on flowers and siliques rather than leaves in nature, and did so when observed on the original host plants. Behavioral experiments, using detached *A. petiolata* branches, however, showed that larvae readily accepted leaves and only the final instar showed a tendency for directed movement towards floral parts. To search for semiochemicals that control plant part preference and to assess possible nutritional consequences of floral parts feeding, we determined glucosinolate profiles and total nitrogen levels of floral parts and leaves. There was only moderate difference between glucosinolate profiles of leaves and floral parts within each of two host plant species. In contrast, the profiles of floral parts differed significantly between them. *A. petiolata* was dominated by 2-propenyl glucosinolate, while *C. pratensis* was dominated by aromatic glucosinolates and branched aliphatic glucosinolates, with considerable variation among populations. Nitrogen levels tended to be higher in floral parts than in leaves in *A. petiolata*, but not in *C. pratensis*, so

floral feeding could not generally be attributed to higher N content. With the exception of a tendency of last instar larvae (L5) to move to the apex and ingest flowers and upper stem, we did not find either a plant chemistry basis or larval acceptance/rejection behavior that could explain the usual feeding of floral parts by orange tip larvae of all instars. However, by artificial manipulation of vertical larval position on host plants, we found that the frequency of leaf vs. flower feeding during 24 hr depended significantly on the initial larval position. Hence, we suggest that the placement of eggs on floral parts by ovipositing female butterflies is a major explanation of orange tip feeding habits previously known from field observations.

Key Words Larval feeding behavior and preference · Glucosinolate profile · Total nitrogen · Leaves · Flowers · Siliques

Introduction

The orange tip butterfly, *Anthocharis cardamines* (L.), accepts floral parts of a large number of glucosinolate containing plant species for oviposition, and two plant species appear to be frequent hosts: *Cardamine pratensis* L. (cuckoo flower) and *Alliaria petiolata* (Bieb.) Cavara & Grande (garlic mustard) (Wiklund and Åhrberg, 1978; Courtney, 1981; Dempster, 1997; Wiklund and Friberg, 2009). The larvae usually are described as floral parts feeders. Indeed, some field observations suggest that leaves are rejected by the larvae even when all siliques (the fruits of cruciferous plants) are consumed, thus resulting in death from starvation (Wiklund and Åhrberg, 1978). The apparent combination of female and larval preference for floral parts suggested to us that a chemical signal, perhaps a stimulant,

N. Agerbirk (✉) · C. E. Olsen · K. Jørgensen
Faculty of Life Sciences, University of Copenhagen,
Thorvaldsensvej 40,
1871, Frederiksberg, Denmark
e-mail: nia@life.ku.dk

F. S. Chew
Department of Biology, Tufts University,
Medford, MA 02155, USA

a deterrent, or an anti-nutritional factor, could be involved in plant part discrimination.

Glucosinolates, amino acid-derived secondary metabolites primarily found in the mustard order Brassicales, are crucial host plant recognition cues for pierid butterflies such as the cabbage whites (*Pieris* sp.) and related species (Renwick and Chew, 1994; Hopkins et al., 2009). Glucosinolate containing plants often contain a mixture of different structures, metaphorically known as the glucosinolate ‘profile’, which often differ between plant organs (e.g., Agerbirk et al., 2008).

Differential insect behavioral responses and their correlations with variation in individual glucosinolates have been observed (e.g., under field conditions, Rodman and Chew, 1980; Griffiths et al., 2001; Bidart-Bouzat and Kliebenstein, 2008; under laboratory conditions, Huang and Renwick, 1994; Giamoustaris and Mithen, 1995; Li et al., 2000; Gols et al., 2008; de Vos et al., 2008; Sun et al., 2009, but see also Reifenrath et al., 2005; Reifenrath and Städler, 2009; Badenes-Pérez et al., 2010). In one case that involves a monophagous insect and a glucosinolate of unusual proposed structure, host plant preference was linked to presence of a distinct glucosinolate (Larsen et al., 1992). Thus, there may be a chemosensory basis for evolution of host plant or plant part discrimination based on differential sensitivity to individual glucosinolates (Hopkins et al., 2009). Phytophagous butterfly larvae also have sensory organs for various primary metabolites (Schoonhoven et al., 2005), some of which could be correlated to total nitrogen contents of plant parts.

The purpose of this work was to search for evidence for a chemical basis of the assumed plant part preferences of orange tip larvae, and to develop a bioassay for a hypothetical semiochemical responsible for floral parts feeding. We pursued this purpose in two parallel ways: (1) by observation of caterpillar feeding choice on original host plants and after transfer to various positions on *A. petiolata*, and (2) by chemical analysis of plants collected during the period of orange tip larval occurrence.

Methods and Materials

Identification of Plants and Animals Flowering *C. pratensis* and *A. petiolata* were identified in the field from their characteristic morphologies. Eggs of the orange tip butterfly were tentatively identified in the field, and the identity confirmed from comparison of the morphology of the resulting larvae with the same illustrated descriptions as used previously (Agerbirk et al., 2006). Selected larvae were reared to pupation to confirm the identification based on pupal morphology. Photographs of the studied material including egg and the transition from larva to pupa have

been published elsewhere (Agerbirk and Jørgensen, 2008). Four pupae produced four female butterflies the following spring, all of which were confirmed to be *A. cardamines* by morphology.

Collection of Plants Plant shoots of flowering plants (*C. pratensis* cut above the rosette and *A. petiolata* cut below a number of fresh leaves) were collected in plastic bags at natural growth sites in May 2008 and transported to the laboratory within a few hours. Unless otherwise noted, all plants and animals were from Lake Utterslev Mose (= locality DK1), Copenhagen, Denmark. *A. petiolata* was collected within 20 m of the lake rim (e.g. 55 42' 57" N, 12 30' 22" E), while *C. pratensis* was collected at the public lawns (“Gyngemosen”) NE of the lake and NW of Highway 16 (e.g. 55 43' 19" N, 12 29' 43" E). We also sampled *C. pratensis* floral parts from two other, previously described localities (DK2, DK3) (Agerbirk et al., 2010a). The individual plants DK3a and DK3b were the same as described in that paper and were dried without lyophilization as described in that paper.

Observation of Egg Position and Caterpillar Feeding Behavior Flowering shoots of *C. pratensis* and *A. petiolata* with orange tip eggs or neonate larvae were collected as above and kept in individual, numbered beakers with tap water in the laboratory, and exposed to sunlight through an east facing glass window. If the orange tip egg could be located, the position was registered. Most L1/L2 larvae collected ($N=26$) were subject to detailed observations on the original host plants. (However, L1/L2 larvae collected on days with experimental manipulation of larval position were used directly in the behavioral experiments, and hence not observed on the original host plants). The position and behavior of each larva, as well as signs of feeding, were observed and noted at least daily between Monday and Friday, and representative specimens were photographed in order to illustrate characteristic feeding patterns. The observations on original host plants typically were carried out for 2–3 d for an individual larva, after which they were moved to fresh plants to be used for experiments. It was not meaningful to standardize the observation period because the material was collected at different stages, including eggs, newly hatched larvae, and slightly older larvae.

One intact *C. pratensis* plant (on the flower of which oviposition by an orange tip was observed the day before), with its root system and approximately 1 l of attached soil+grass, was removed from the locality, and was cultivated in the laboratory during the entire larval development including hatching and pupation (May 7–24). The spontaneous feeding behavior of the resulting larva, which was not manipulated in any way, was observed 5 d a week. When the remains of the host plant had been left, *A. petiolata* was offered for

completion of the 5th instar and pupation (pupal wt. 112 mg), to confirm identification.

Rearing of Larvae for Behavioral Experiments As soon as or before original plants started to deteriorate visibly, larvae were gently transferred (with a moist paint brush) to fresh bolting *A. petiolata* shoots, collected at the locality 0–2 d before, and kept in the laboratory immersed in beakers with tap water. They were exposed to reduced daylight through an east facing window until use. This stock of larvae, supplemented with newly collected larvae of various instars, served as the material for additional behavioral experiments.

Behavioral Experiments For behavioral experiments, *A. petiolata* side branches with a specific morphology and growth stage were used, with leaves immediately next to the lower 1–2 siliques (Fig. 1) and with flowers with fresh petals. As the natural availability of branches with this morphology (including fresh flowers) ceased late in the experimental period, we ended this type of experiment even though additional late instars were available.

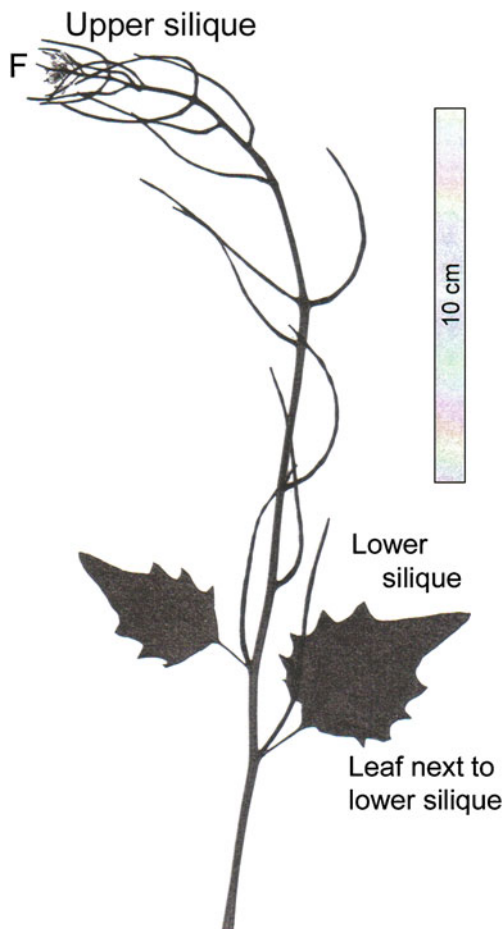


Fig. 1 *Alliaria petiolata* branch with siliques next to cauline leaves, as used for behavioral experiments with orange tip larvae. F=flowers

Two plant part preference experiments were carried out. The purpose of the ‘leaf/silique choice experiment’ was to test whether an absolute preference for silique feeding could be demonstrated in a choice situation, where both a silique and a leaf were within close proximity to the larva. Larvae of known instar were gently transferred to lower parts of *A. petiolata* branches (one larva per branch in separate beakers), with about half of the larvae of each instar placed on lower siliques and the other half placed on leaves immediately next to lower siliques (Fig. 1). The feeding during the 24 hr experimental period was registered by careful inspection of the branch for feeding traces at the end of 24 hr. Occasional observation of some larvae during the experiment also was carried out in order to qualify the subsequent inspection of the branch for feeding traces. Selected branches with larva and feeding traces were photographed with a background 5×5 mm grid after the experiment, and ingested amounts were estimated by comparison with un-touched leaves.

The purpose of the ‘vertical position experiment’ was to test whether the vertical position of the larva, either near the plant apex with flowers or at lower levels near the leaves, would influence the subsequent plant part feeding choice of the larva (as suggested by the outcome of the leaf/silique choice experiment). Larvae were placed either on the uppermost silique, near the flowers, or on the lower-most silique, next to a leaf (Fig. 1). Evaluation of the feeding choices was as described for the leaf/silique choice experiment.

Whether or not occurrence of leaf feeding depended on the initial position of the larva was tested by Rice’s conditional binomial exact test (Rice, 1988) of 2×2 matrixes with leaf feeding vs. no leaf feeding as a function of larval position. (Fisher’s exact test gave the same pattern of significance vs. non-significance). The significance level was set at 5% (two-tailed test), and results for L1-L4 were pooled because there was no indication of differences among these instars. Whether or not flower feeding depended on initial larval position was tested in the same way.

Plant Sampling and Dissection for Chemical Analysis - Plants for chemical analysis were sampled (in parallel to larvae and plants for behavioral experiments) at the main study locality (DK1) in May (2008), which was the oviposition and larval feeding period of orange tip butterflies at the locality. Plants for chemical analysis were dissected and lyophilized immediately after arriving in the laboratory; the time from collection in the field to dissection and lyophilization was 3 hr or less. In dissection for chemical analysis, upper stem was defined as the upper inflorescence (with flowers) and included flower stalks; middle stem was defined as the lower inflorescence (with

siliques) and included silique stalks; and lower stem was defined as the basal part of the stem, from the lowermost silique downwards. Leaves were cauline (stem) leaves, not rosette leaves. Siliques and flowers included only these organs, as the stalks attaching them to the stem were included with stems. For nitrogen analysis of specific plant organs, we aimed at pooling only from the same individual, but in the case of flowers, siliques, and upper stems, the samples in most cases had to be pooled from several individual plants to provide sufficient amounts for the analysis.

Determination and Identification of Glucosinolates Glucosinolates were determined by extraction of lyophilized plant parts in boiling 70% MeOH, binding to anion exchange columns, enzymatic desulfation, elution (in 5×1 ml H₂O) and subsequent HPLC with diode array detection of desulfo derivatives relative to an external standard of sinigrin (**15**) treated similarly in parallel (Agerbirk et al., 2007). The exact HPLC conditions were different for the two plant species: HPLC conditions for *C. pratensis* samples were optimized specifically to achieve separation of all glucosinolates known from this species (Agerbirk et al., 2010a). For *A. petiolata*, a Supelcosil LC-ABZ column, 25 cm×4.6 mm, 5 μm, was used with flow rate 1 ml/min, and elution by 2 min of H₂O followed by a 48 min linear gradient from 0 to 60% MeOH, a brief wash with MeOH, and equilibration with H₂O. Peak identification by comparison with authentic reference compounds supplemented by LC-MS of selected samples was as previously described (Agerbirk et al., 2010a). Glucosinolate levels in *A. petiolata* were log₁₀-transformed to remedy non-normal distribution of the original data, and subjected to statistical analysis. Due to unexpected occurrence of several chemotypes of *C. pratensis* with qualitative differences among them, the various chemotypes were reported separately, and quantitative statistical tests of glucosinolate levels in this plant were not considered meaningful.

Nitrogen Determination Total nitrogen (and carbon) contents of lyophilized samples from locality DK1 were determined on an elemental analyzer (Carlo Erba model NA 1500, Carlo Erba, Milan, Italy) in the laboratory of

Jeffrey Dukes at University of Massachusetts, Boston, MA, USA. Two samples (*A. petiolata* leaves, %N 3.27, and upper stems, %N 5.99) deemed unreliable by the analysis-lab due to abnormal values for associated standards were excluded from the data and calculations. Numbers for %N were converted to proportions (0.00–1.00), which were arcsin transformed to remedy non-normal distribution of the original data.

Statistical Analysis of Chemical Data Levene's test was used to confirm that transformed glucosinolate and nitrogen levels met the variance homogeneity assumptions of ANOVA. Differences between nitrogen and glucosinolate levels among plant parts within each species were examined by ANOVA. Where the ANOVA was significant, unplanned multiple comparison among plant parts was made using Scheffé *post-hoc* test with significance level set at 0.05. In one case with *N*=1 for one plant part, this plant part was excluded before ANOVA with *post-hoc* test.

Results

Oviposition Site Almost all eggs or egg shells located on *C. pratensis* or *A. petiolata* were on siliques or flowers, including their basal parts, while a single egg was observed on a leaf close to a silique (Table 1A). Usually, only a single egg or larva was observed per plant, but in some cases two (Fig. 2) or even three orange tip eggs or larvae were present naturally.

Plant Part Choice on Original Host Plants Feeding habits of L1/L2 instars on the original host plants nearly always (88%) included silique feeding. Flower feeding also was frequent (46%), while only few larvae ate leaves at all (Table 1B) (but one fed exclusively on a leaf during the first and the initial part of the 2nd instar). The flower feeding behavior by early instars on *C. pratensis* was quite stereotypic, with the feeding starting from the basal part of the flower (Fig. 2) whether or not the flower was open. Floral parts egg position and feeding by orange tips was obviously dominant for both host plant species, and it was

Table 1 Egg position and feeding choice by orange tip L1/L2 larvae hatched on original host plant shoots

A. Egg position		N	Egg on:	Leaf	Silique	Flower	Not located
Host plant							
<i>Cardamine pratensis</i>		11		0	2	3	6
<i>Alliaria petiolata</i>		15		1	6	6	2
B. Feeding choice ^a		N	Feeding from:	Leaf	Silique	Flower/flower bud	
Host plant							
<i>Cardamine pratensis</i>		11		0	9	6	
<i>Alliaria petiolata</i>		15		2	14	6	

^a The observation period was restricted to the L1 and L2 instars and typically 2–3 days

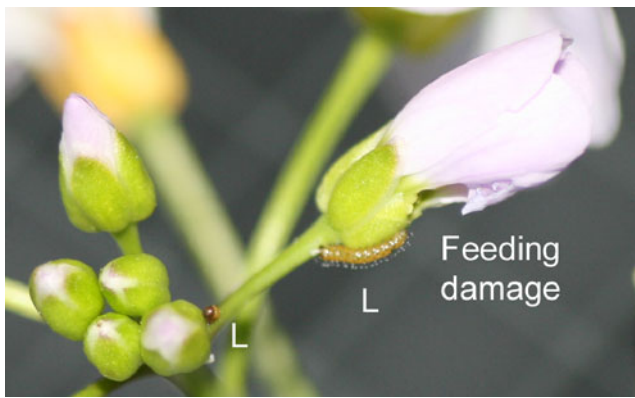


Fig. 2 Second instar orange tip larvae (“L”) feeding on flowers of *Cardamine pratensis*. The entire flower was usually eaten, starting from the outside of basal parts as illustrated (“Feeding damage”)

not considered relevant to test statistically whether slight differences between patterns on the two plant species existed. For comparison of plant part distribution with published field data, see “Discussion”.

The feeding behaviour of a single larva through all five instars on an intact *C. pratensis* plant complemented the observations. In the first three instars (L1-L3), the larva ate flowers and siliques. The 4th instar (L4) also ate upper stem, and 5th (final) instar (L5) ate the remaining stem except the lower 9 cm (ca. one third) as well as all cauline leaves and part of the rosette leaves.

Leaf/Silique Choice Experiment The probability of leaf feeding was not significantly different for larvae placed initially on a leaf vs. larvae placed initially on a nearby silique ($P=0.21$). Irrespective of their initial position, larvae frequently ingested leaves as well as siliques (Table 2A). The amounts of leaf ingested were typically substantial i.e., more than the minute test bites seen when another species

of insect larvae probed unacceptable *Barbarea vulgaris* plants (Agerbirk et al., 2003). Flower feeding was infrequent for L2-L4 instars in this experiment. Apparently, the frequency of feeding on each plant part was a consequence of a limited mobility within 24 hr, and was sufficient to reach the leaf even when they were placed at the silique and vice-versa, but was generally insufficient to reach the more distant flowers.

A single L5 larva was included in the experiment, but was not included in the pooled results for statistical testing due to its atypical behavior: The individual was placed on the leaf next to the lower silique, from which approximately 3 cm² (ca. 1/5 of the entire leaf) was eaten. Rather than eating the remaining leaf or neighboring silique, it moved overnight to the flowers and upper siliques, which were ingested entirely. This observation led us to include as many L5 larvae as available in the vertical position experiment.

Vertical Position Experiment This experiment, in which larvae were placed on either lower or upper siliques, confirmed the importance of larval position inferred above. Young to intermediate instars (L1-L4) placed near leaves exhibited frequent leaf feeding (50%) and no flower feeding, while larvae placed near flowers exhibited frequent flower feeding (45%) and no leaf feeding (Table 2B). The probability of leaf feeding was significantly higher when larvae were placed on lower siliques than when placed on upper siliques ($P=0.01$). Likewise, the probability of flower feeding was significantly higher when larvae were placed on upper siliques compared to lower siliques ($P=0.015$). Based on the combined experiments, we concluded that any preference of L1-L4 larvae for floral parts (siliques and flowers) would be too weak to be of practical use in a bioassay for a hypothetical stimulant or deterrent. Indeed,

Table 2 Feeding choice by orange tip larvae during 24 hr after experimental manipulation of larval position

Instar	Placed at	N	Feeding from:	Leaf	Silique	Flower/flower bud
A. Leaf/silique choice experiment: larvae placed on either lower <i>Alliaria petiolata</i> silique or the leaf immediately next to it						
L2-L4	Lower silique	10		5	7	0
	Leaf at silique	6		5	4	1
L5	Leaf at silique	1		1	1	1 ^a
B. Vertical position experiment: larvae placed on either lower <i>Alliaria petiolata</i> silique (at leaf) or upper silique (at flowers)						
L1-L4	Silique at leaf	10		5	8	0
	Silique at flowers	11		0	9	5
L5	Silique at leaf	4		1	4	3 ^a
	Silique at flowers	3		0	3	3 ^a

^a Ate all flowers as well as flower-supporting part of stem

For statistical evaluation: See text

feeding choice by young and intermediate instars (L1–L4) seemed to be governed mainly by proximity, at least under our laboratory conditions.

Seven available L5 larvae also were included in the vertical position experiment, and all except one (from lower silique) moved to flowers and ingested the entire upper part of the inflorescence within 24 hr, while only a single had eaten from leaves (Table 2B). For this instar, the probability of flower feeding did not depend on the initial position of the larvae ($P=0.94$). Despite the low number of replicates, we interpret this result as a tendency for L5 instars to prefer floral parts.

Glucosinolate Profiles of Leaves and Floral Parts The two main hosts of orange tip butterflies at the DK1 locality, an urban lake habitat in the greater Copenhagen area, appeared to be *A. petiolata* and *C. pratensis*. There was no difference in the kinds of glucosinolates in vegetative or floral parts of *A. petiolata*, but when the levels were compared, there was a significant effect of the plant parts in statistical analysis by ANOVA. Flowers and upper stems contained higher levels of the major glucosinolate sinigrin (**15**) and the phenolic indole glucosinolate **12** (with proportional traces of **10**) relative to leaves (Table 3, Fig. 3).

In the other common host plant at the locality, *C. pratensis*, the mixture of glucosinolates was more complex. Plants from the main locality (DK1) contained three dominant glucosinolates – the phenolic glucosinolate sinalbin (**8**), the *O*-methyl derivative **9**, and the hydroxylated aliphatic glucosinolate **4** – and a number of minor glucosinolates including the indole glucosinolate **10** (Table 4, Fig. 3). The glucosinolate profiles of floral and vegetative parts were similar, with no obvious indications of a particular glucosinolate profile or higher glucosinolate level of floral parts.

As a hypothetical floral parts signature was expected to be of general nature, we included floral parts of the same plant

species from other localities (DK2, DK3). Floral parts of individual *C. pratensis* plants from these localities showed qualitative differences from both floral parts and leaves at the main locality in terms of glucosinolate profiles. In a plant from locality DK2, *sec*-butylglucosinolate (**3**) rather than the hydroxy derivative **4** was the dominating aliphatic glucosinolate in flowers. At locality DK3, flowers of two individual plants had glucosinolate profiles that deviated even more from those at locality DK1, as the methylated aromatic glucosinolate **9** was nearly absent (Table 4). Plant DK3a accumulated benzylglucosinolate (**7**) and a short chain hydroxylated aliphatic glucosinolate (**2**) in flowers while both **3** and **4** were absent. In contrast, plant DK3b accumulated the non-hydroxylated **3** but not **7**. The distinctive profiles of floral parts from localities DK2 and DK3 were similar to leaf glucosinolate profiles of the same plants reported elsewhere (Agerbirk et al., 2010a).

Nitrogen Levels in Different Plant Parts In *A. petiolata*, total N content was higher in upper stems than in leaves, and there was also a tendency for high N levels in flowers. In the case of *C. pratensis*, however, there was no statistically significant difference in N content among floral parts and leaves (Table 5).

Discussion

An initial purpose of the behavioral experiments was to establish a bioassay for a hypothetical semiochemical (Agerbirk et al., 2003; Miles et al., 2005; Nielsen et al., 2010) responsible for floral parts feeding. Branches of *A. petiolata* (Fig. 1) with adjoining lower siliques and upper leaves (in contrast to the distance between these organs on the main stem), as well as a controlled laboratory environment, were used in an attempt to maximize the sensitivity for any larval preference. However, the larval feeding behavior seemed to be governed mainly by the position of the larvae in laboratory experiments with young and intermediate instar larvae (L1–L4). We believe that the laboratory test situation was relatively similar to the natural situation in terms of physical conditions and plant chemistry because relatively fresh *A. petiolata* branches were used. It can be argued that biochemical changes in the detached *A. petiolata* branches kept in the laboratory may have compromised a semiochemical responsible for plant part preference. Obvious consequences of the laboratory rearing at lower light intensity and significantly less UV light than in the field could be decreases in levels of photosynthetic products or UV induced metabolites with a significant turnover rate. Indeed, a recent experiment used for demonstrating specific movement to flowers of third

Table 3 Glucosinolate profiles ($\mu\text{mol/g}$ dry wt., mean (s.d.)) of floral and vegetative parts of *Alliaria petiolata* in May

Plant part	10	12	15	<i>N</i>
Flowers	0.29 (0.05)	4.6 (0.6) a	73.8 (11.1) ab	4
Siliques	0.03 (0.03)	0.8 (0.8) b	31.6 (20.7) bc	3
Leaves	0.02 (0.02)	0.1 (0.1) b	35.0 (2.6) c	3
Upper stem	0.17 (0.02)	4.3 (0.9) a	124.9 (11.5) a	2
Middle stem	n.d.	0.3 (0.1) b	14.4 (4.4) c	3
Lower stem	n.d.	0.1 (0.0) b	0.9 (0.5) d	3
Significance	–	***	***	

n.d. not detected

Statistical significance of differences in major glucosinolates between plant parts were tested by ANOVA ($P<0.001$: ***, $P<0.01$: **, $P<0.05$: *, $P>0.05$: ns, not tested: -). Significant differences in a *post-hoc* Scheffé test ($P<0.05$) are indicated with different letters

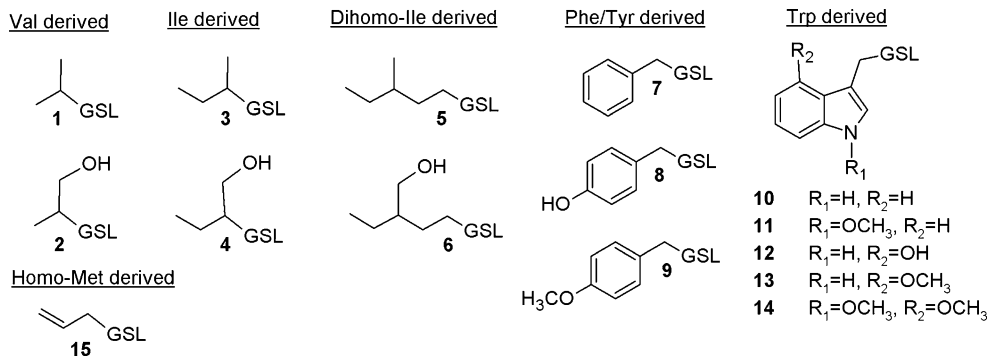


Fig. 3 Glucosinolates detected in *Cardamine pratensis* or *Alliaria petiolata* from the investigated localities, and two glucosinolates (**6** and **14**) known from other accessions of *C. pratensis* but not detected in this investigation. GSL: The constant part of the glucosinolate molecule, C (SGlc)NOSO₃⁻. Systematic (and common names, if in general use) of the glucosinolates (GSLs) are: **1**, 1-methylethylGSL (isopropylGSL); **2**, 1-(hydroxymethyl)ethylGSL; **3**, 1-methylpropylGSL (*sec*-butylGSL); **4**:

1-(hydroxymethyl)propylGSL; **5**, 3-methylpentylGSL; **6**, 3-(hydroxymethyl)pentylGSL; **7**, benzylGSL (glucotropaeolin); **8**, 4-hydroxybenzylGSL (sinalbin); **9**, 4-methoxybenzylGSL; **10**, indol-3-ylmethylGSL (glucobrassicin, GB); **11**, 1-methoxy**10** (neoGB); **12**, 4-hydroxy**10** (4-hydroxyGB); **13**, 4-methoxy**10** (4-methoxyGB); **14**, 1,4-dimethoxy**10** (1,4-dimethoxyGB), **15**, 2-propenylGSL (sinigrin)

instar *Pieris brassicae* (Smallegange et al., 2007) involved intact plants (in a greenhouse). However, the feeding on detached original host plants kept in the laboratory was almost exclusively from floral parts, suggesting that even if plant biochemistry changed due to the laboratory conditions, the positional effect was sufficient to enable the larvae to behave as in the field. Hence, biochemical changes due to the laboratory conditions are not likely to have influenced the plant part choices of the larvae. In

agreement with this argument, the spontaneous behavior of young larvae, when their position had not been manipulated, agreed well with published field observations (Wiklund and Åhrberg, 1978; Courtney, 1981; Dempster, 1997), except that Dempster (1997) described floral feeding to precede silique feeding in general on *C. pratensis*. This slight difference from our observations may depend on local conditions such as relative phenologies of insect and host plant (Wiklund and Friberg, 2009).

Table 4 Glucosinolate profiles (μmol/g dry wt.) of floral and vegetative parts of *Cardamine pratensis* plants from three localities in May

Locality:	DK1				DK2			DK3a	DK3b		
	Flo.		Sil.	Lea.	Stem			Flo.	Sil.	Flo.	Flo.
				Upper	Upper	Middle	Lower				
Glucosinolate											
<i>Aliphatics</i>											
1	tr.	0.1	0.2	0.1	0.1	0.2	0.5	0.7	0.2	0.3	
2	1.5	1.9	2.3	2.2	1.9	1.9	0.1	0.1	4.7	0.1	
3	0.2	0.1	0.2	0.6	0.5	0.3	9.4	12.1	n.d.	7.2	
4	2.9	3.0	5.1	5.9	4.6	3.8	n.d.	n.d.	n.d.	tr.	
5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	tr.	
<i>Aromatics</i>											
7	tr.	n.d.	tr.	n.d.	tr.	n.d.	tr.	n.d.	13.7	n.d.	
8	6.0	7.5	26.3	7.2	4.6	4.0	10.8	10.2	10.2	14.4	
9	6.0	6.0	4.1	13.6	9.6	8.7	15.8	16.6	0.2	0.1	
10	0.6	0.6	0.2	0.7	0.5	0.6	0.7	0.9	0.6	0.4	
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr.	n.d.	n.d.	
12	n.d.	n.d.	n.d.	n.d.	tr.	tr.	tr.	tr.	0.1	0.1	
13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr.	
Not identified	1.0	1.1	2.5	1.0	0.6	0.5	0.2	0.1	0.1	0.7	
Total	18.3	20.3	40.9	31.3	22.5	19.9	37.7	40.9	29.8	23.4	
SD of total	1.7	1.0	8.9	–	–	–	–	–	–	–	
<i>N</i>	2	3	3	1	1	1	1	1	1	1	

Bold: More than 10% of total glucosinolate level in that plant part. Glucosinolates **6** and **14**, known from other accessions of *C. pratensis*, were not detected in any sample

^a Flo.: Flowers; Sil.: Siliques; Lea.: Leaves

Table 5 Nitrogen contents (% n wt./dry wt.) of floral and vegetative parts of two common host plants in May

Plant part	<i>Alliaria petiolata</i>		<i>Cardamine pratensis</i>	
	Mean (SD)	<i>N</i>	Mean (SD) ^a	<i>N</i>
Flowers	5.10 (0.17) ab	3	2.76 (0.45) a	3
Siliques	4.37 (0.22) b	3	3.34 (0.30) a	3
Leaves	3.76 (0.73) b	2	3.00 (0.45) a	4
Upper stem	6.63 (0.58) a	2	3.10 (0.17) a	2
Middle stem	2.97 (0.64) c	3	2.15 (0.51) ab	3
Lower stem	1.54 (–) –	1	1.19 (0.05) b	2
Significance	*** ^a		***	

Statistical significance of differences in major glucosinolates between plant parts were tested by ANOVA ($P < 0.001$: ***, $P < 0.01$: **, $P < 0.05$: *, $P > 0.05$: ns). Significant differences in a *post-hoc* Scheffé test ($P < 0.05$) are indicated with different letters. ^aThe level of significance was *** whether or not lower stem was excluded in ANOVA.

The feeding behavior of the single larva followed through all instars on the same plant immediately suggested a biological advantage for flexible feeding behavior. The inflorescence of the *C. pratensis* individual chosen by the female was too small to support full development of the larva if only siliques and flowers were ingested. Larval migration to other plants would be needed if only floral parts were accepted, but such movement to other, perhaps distant plants poses an obvious risk (Wiklund and Åhrberg, 1978; Dempster, 1997). Hence, utilization of vegetative parts would allow larvae to avoid or delay risky migration. Although this observation of a single larva can be dismissed due to lack of replication, we find it of interest and worthy of additional investigation as a complement to the traditional assumption of the existence of leaf avoidance (Wiklund and Åhrberg, 1978). The outcome of our experimental manipulations and the observation of occasional leaf feeding by young larvae on original host plant shoots (Table 1) confirm the observed spontaneous leaf acceptance by orange tip larvae.

The apparent preference for flowers by L5 larvae was reminiscent of a similar behavior reported for *Pieris brassicae* L3 and *Athalia rosae* L4 larvae (Smallegange et al., 2007; Bandeili and Müller, 2010), and suggests that proximity was not the only factor controlling orange tip L5 larval feeding. The most direct advantage of the tendency for feeding from the top would be to avoid accidental cutting of the main plant axis by the vigorously feeding L5 instars, which would lead to loss of the upper parts of the plant. As usual for late instar caterpillars (Theunissen et al., 1985), L5 larvae ingested a tremendous amount of material. In this case it was comparable to the remaining parts (after feeding by previous instars) of the relatively small *C. pratensis*. Thus, a strong plant part preference might have little effect on this plant species, because the majority of the plant (except tough

lower parts) would likely be ingested during the L5 developmental stage. In contrast, *A. petiolata* individuals were substantially larger plants, and floral parts preference by L5 larvae could be a means to ensure that potentially nutritious apical parts were prioritized. A similar benefit in terms of larval nutrition could be a consequence of the female's choice of floral parts for oviposition. This hypothesis is assessed below in light of our data on glucosinolate profiles and nitrogen content of plant parts. These analyses also represent an independent search for a semiochemical responsible for floral parts feeding.

A comparison of the glucosinolate profiles of vegetative and floral parts of two host plant species revealed no consistent floral parts glucosinolate profile. Based on the analyses of the chemically simple *A. petiolata*, a preliminary hypothesis for a hypothetical glucosinolate 'signature' or profile of floral parts could be a higher total level of glucosinolates, or a higher level of indole glucosinolates (10–14) or perhaps of aromatic (7–14) or phenolic (8+12) glucosinolates in general. Based on the literature, a different balance of aromatic vs. aliphatic glucosinolates (van Loon et al., 1992; Huang and Renwick, 1994) or of *O*-methylated vs. non-substituted aromatics (Sun et al., 2009) would be candidate signatures that could possibly be distinguished by insect sensory organs. Based on separately reported analytical chemistry research (Agerbirk et al., 2010a), we obtained reliable glucosinolate profiles of *C. pratensis* floral parts and leaves. However, none of the hypotheses of a floral parts 'signature' was supported when data for three populations of *C. pratensis* were considered. From the data in Table 4, a tendency for higher levels of 10 in floral parts than in leaves is suggested, but levels of 10 were higher in leaves than in floral parts in the individual plants from locality DK2 and 3 (Agerbirk et al., 2010a; unpublished results), so a hypothesis of a role of 10 was not generally supported. Indeed, the difference in glucosinolate profile between the two species and between individuals from different populations of *C. pratensis* appeared to be much greater than any systematic differences between vegetative and floral parts (Table 4). Consequently, there appears to be no basis for using glucosinolate profiles for larval distinction of floral parts from leaves.

A similar glucosinolate profile of flowers and vegetative parts, with a general tendency for higher levels in flowers of undamaged plants, also had been reported from *Arabidopsis thaliana*, *Raphanus sativus*, *Brassica nigra*, and *Sinapis alba* (Brown et al., 2003; Strauss et al., 2004; Smallegange et al., 2007; Bandeili and Müller, 2010). Glucosinolates were constitutively high but generally less inducible in radish flowers compared to leaves, as expected for an organ with high fitness value (Strauss et al., 2004). Our data do not exclude the possibility that floral parts could be deficient in the enzyme myrosinase (which converts glucosinolates to defensive products such as

isothiocyanates), but a recent report demonstrated high levels of myrosinase in floral parts of the crucifer *S. alba* (Bandeili and Müller, 2010), showing that floral parts high in glucosinolates may indeed also be high in myrosinase.

It was a surprise to discover that *C. pratensis* plants from different localities had different glucosinolate profiles; a more extensive investigation of glucosinolate variation in the species is published separately (Agerbirk et al., 2010a). Two subspecies of *C. pratensis* with different chromosome numbers had different probabilities of oviposition by orange tip butterfly females in Sweden (Arvanitis et al., 2007, 2008), but the *C. pratensis* populations at three Danish localities investigated here had identical chromosome numbers (Agerbirk et al., 2010a).

In *A. petiolata*, there was a tendency for higher N contents in upper parts, although the difference from leaves was statistically significant only for upper stems (which were frequently eaten by late instar orange tip larvae). Given the low number of replicates, this result should be interpreted with caution. However, the *A. petiolata* parts high in N were also high in glucosinolates and were both from the plant apex, suggesting that the measured tendency reflects a real biological phenomenon. As nitrogen is considered to be a limiting resource for herbivorous insects (Mattson, 1980), floral parts feeding may thus be a nutritional advantage in the case of feeding on *A. petiolata*. Position-dependent levels of nitrogen and glucosinolates also have been reported by Traw and Feeny (2008) for various leaf-positions of *Brassica nigra* and *B. kaber* (syn. *Sinapis arvensis*). However, in the case of *C. pratensis*, floral parts did not have higher total N than leaves (Table 4). Hence, our data do not support the hypothesis that floral parts feeding in general provide a nutritional advantage for orange tip larvae in terms of N content.

Nutritionally available N can only be approximated by total N if the majority of plant N is in a form available for protein or nucleotide biosynthesis; N in secondary metabolites may not be available for such biosynthesis. Intake of nitrogen in glucosinolates by orange tips and related species is balanced 1:1 by excretion of nitrogen in a nitrile (Agerbirk et al., 2006), a nitrile-derived functional group (Vergara et al., 2006; Agerbirk et al., 2007), or possibly inorganic ammonia from hydrolysis of nitrile groups (Agerbirk et al., 2010b). However, even a high glucosinolate level of 100 $\mu\text{mol/g}$ dry wt. would correspond only to 0.14% N for non-indoles (and 0.28% N for the indoles 10–14), so glucosinolate N was only a low fraction of the total N content of any plant part and total N could be regarded as a proxy of nutritionally available N. This result agreed with another investigation (Traw and Feeny, 2008).

In summary, we found a tendency for floral parts preference for L5 larvae, but no evidence for any L1–L4 larval behavioral preference for floral parts. For a classical

example of a floral parts feeding butterfly, it was surprising that the feeding preferences of the larvae were so unspecific compared to the recently discovered within-plant selective foraging by intermediate to late instar *P. brassicae* and *A. rosae* (Smallegange et al., 2007; Bandeili and Müller, 2010). If the tendency of late instars to move to flowers is a real phenomenon, it still is not certain that a chemical signal is involved; we did not, for example, test the effect of positioning the inflorescences up-side down (Bandeili and Müller, 2010). Much of the natural tendency for young orange tip larvae for floral parts feeding can apparently be attributed to the choice of floral parts for oviposition by female butterflies (Wiklund and Åhrberg, 1978). Having established that floral parts are as rich and diverse in glucosinolates as vegetative parts, the wide range of host plants used by orange tip butterflies may imply that there is no basis for selection of a chemically less well defended plant part (Courtney and Chew, 1987). However, flower feeding recently has been demonstrated to lead to faster growth of two species of glucosinolate adapted larvae (Smallegange et al., 2007; Bandeili and Müller, 2010), supporting a hypothesized overall nutritional or micro-environmental benefit of feeding on floral parts.

Alternative explanations for flower oviposition in *Anthocharis* could be phylogenetic conservatism (the entire clade of anthocharines and euschloeines oviposits on floral and fruiting parts), perhaps due to mutual dependency with other behavioral patterns such as the “red egg syndrome” (Shapiro, 1981; Nomakuchi et al., 2001) or an optimized host plant search strategy: exclusive investigation of flowers may save valuable time during the oviposition period or allow the search for nectar source plants (Wiklund and Åhrberg, 1978) and plants for oviposition to be combined. The present demonstration of flexible but position-dependent larval plant part choice, glucosinolate diversity in floral parts and variable but relatively high N levels in floral parts underlines the importance of the positioning of eggs by ovipositing females, and shows that crucifer floral parts may be as diverse and well defended as crucifer foliage.

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