OIKOS 109: 503–512, 2005

The evolution of larval foraging behaviour in response to host plant variation in a leaf beetle

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Gotthard, K., Margraf, N., Rasmann, S. and Rahier, M. 2005. The evolution of larval foraging behaviour in response to host plant variation in a leaf beetle. – Oikos 109: 503–512.

The evolutionary causes of variation in host specialization among phytophagous insects are still not well understood and identifying them is a central task in insect–host plant biology. Here we examine host utilization of the chrysomelid beetle *Oreina elongata* that shows interpopulation variation in the degree of specialization. We focus on larval behaviour and on what selection pressures may favour the use of two different larval host plants (*Adenostyles alliariae* and *Cirsium spinosissimum*) in one population as opposed to specialization onto one of them as is seen in other populations. The results suggest that the degree of exploratory foraging behaviour is higher in larvae from the two-host population than in single host populations, and a field survey of the two-host population also indicated that larvae do move between host species. A field experiment indicated that predation rates on *O. elongata* larvae in the two-host population are higher on one of the host species, *A. alliariae*, than on the alternative *C. spinosissimum*. In combination with earlier results this finding suggest that larvae move between hosts to obtain better food on one host, and to get better protection from predators on the other. It appears that in this two-host situation a single plant species does not provide the most beneficial conditions in all parts of *O. elongata* life cycle and individuals may obtain different plant-specific benefits by moving between host species. This heterogeneous host situation appears to have selected for the explorative larval foraging strategy seen in the in the two-host population. In general, the results support the notion that to understand patterns of host plant use in insects it is often vital to consider a range of host related selection pressures whose relative importance may vary between life stages of the insect.

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A high degree of host specialization is perhaps the most general ecological pattern identified in studies of insect–host plant interactions and it is thought to be one of the major explanations for the remarkable diversity of herbivorous insects (Jaenike 1990, Futuyma 1991, Bernays and Chapman 1994, Thompson 1994). The general drive towards host specialization may perhaps be understood from the notion that, for pure probabilistic reasons, one particular plant species will be better than any alternative in evolutionary time. Nevertheless, there are enough generalist species present to show that the drive towards specialization is not irreversible, leaving us with the problem of explaining when polyphagy is likely to be beneficial (Bernays and Minkenberg 1997, Janz and Nylin 1998, Janz et al. 2001).

It has been suggested repeatedly that for an insect with several alternative host plants a single plant species may not provide the most beneficial conditions during all parts of the life cycle (Reavey and Lawton 1991, Scheirs et al. 2000, Janz 2002, Scheirs and De Bruyn 2002a). For

Accepted 18 November 2004

ISSN 0030-1299

example, the best host plant for the larva may not be the best site for the egg or for adult feeding. Such situations are interesting since they can lead to variable and elaborate host utilization strategies that may allow studies of general aspects of host range evolution. For example, selection may favour a dynamic use of different host plants in different life stages, which would necessitate that individuals move between host plant species. In this study we investigate several aspects of the larval foraging strategy of a population of the chrysomelid beetle, *Oreina elongata* Suffrian, in relation to the two different host plants that are used in the field (Ballabeni et al. 2001a). The aim was to explore both how and why this population use both hosts during the larval stage, rather than specializing on one of the plant species. Partly we do this by a comparison with other populations that only have one host species available in their natural habitat. This relatively simple insect–plant system presents an opportunity to study a situation where selection on the actual host range varies geographically.

Both adults and larvae of the two-host population of *O. elongata* studied here use the two asteracean species *Adenostyles alliariae* (Gouan) (Senecioneae) and *Cirsium spinosissimum* (L.) (Cardueae) and at this location the beetles are exclusively found where both plants grow close to each other. Although the adult beetles are more commonly found on *A. alliariae* (Rasmann 2002) practically all eggs in the field are found on *C. spinosissimum* (Ballabeni et al. 2001a), and this is due to a strong oviposition preference for *C. spinosissimum* (Gotthard et al. 2004). This behaviour appears to be adaptive since egg survival is significantly higher on *C. spinosissimum* than on *A. alliariae* in the field (Ballabeni et al. 2001). Intriguingly, laboratory studies show that larval growth rates are higher and development times are shorter on *A. alliariae* than on the principal oviposition host *C. spinosissimum*, and that a diet containing a mix of these plants improves larval survival in the laboratory (Ballabeni and Rahier 2000). In addition to supporting a faster larval development, *A. alliariae* contains pyrrolizidine alkaloids (PAs in the following) that are sequestered both by larvae and adults of *O. elongata* and may serve as a chemical defence against natural enemies (Dobler and Rowell-Rahier 1994, Rowell-Rahier et al. 1995, Pasteels et al. 1996). Consequently, the correlation between female oviposition preference (highest on *C. spinosissimum*) and larval growth performance (highest on *A. alliariae*) in this population is low with respect to these two hosts. In the light of these results it is interesting that after the eggs hatch on *C. spinosissimum* in the field, the number of larvae that are found feeding on *A. alliariae* increases with time and the number of final (4th) instar larvae on the two hosts plants is not significantly different (Ballabeni et al. 2001a). This change in larval distribution appears mainly to be due to larval migration rather than differential larval mortality (Ballabeni et al. 2001a). Indeed, these results have led to the adaptive hypothesis that *O. elongata* females oviposit on the plant that gives the eggs best protection but that many larvae continuously move to the plant that support the highest growth and contains PAs that can be sequestered and used as a chemical defence. In the present study we test this hypothesis by focusing the larval foraging strategy of the focal two-host population.

We hypothesized that if larval migration between plants species is adaptive in a heterogeneous host environment, selection should favour behaviours that promote this migration more strongly in a two-host population than in single host populations. If so, it is likely that larvae of the two-host population should show a more explorative foraging strategy, with higher movement rates and less specialized feeding preferences, compared to single-host populations. This was tested in a laboratory study where we compared the larval foraging strategy of this two-host population with other single-host populations.

In relation to this question we also wanted to examine to what degree the movement of larvae in the two-host population is truly directional from *C. spinosissimum* to *A. alliariae* in the field. An alternative explanation for the pattern of increasing numbers of larvae on *A. alliariae* over the season is that there is frequent migration between suitable hosts. This type of movement pattern will also tend to even out the strong distributional bias towards the principal oviposition host (*C. spinosissimum*) that is present early in the season. Therefore, we investigated to what degree larvae in the field stay on *A. alliariae* once they get to this plant, or if they continue to move between hosts and occasionally also move back to *C. spinosissimum*.

Finally, if the sequestration of alkaloids from *A. alliariae* provides protection from natural enemies it may constitute an important selection pressure that favours larval movement to this plant. This selection pressure would come in addition to the higher larval performance on *A. alliariae* and could be an important part of the explanation for why larvae in the two-host population use both hosts rather than specializing on one of them. We investigated this in a field experiment in the two-host population with the expectation that predation rate should be lower when larvae feed on *A. alliariae*.

Material and methods

Study organism and populations

Oreina elongata is a high-alpine species (altitude 1600–2300 m) with a patchy distribution throughout the Alps and further south in the Apennines. Adults and larvae

have been found feeding on four species of alpine plants in the Asteraceae: *Adenostyles alliariae, A. glabra* (Mill.), *A. leucophylla* (Willd.) (Senecioneae), and the thistle *Cirsium spinosissimum* (L.) (Cardueae). The three *Adenostyles* species are chemically and morphologically similar and contain pyrrolizidine alkaloids (PAs in the following) that are sequestered by both adults and larvae and may serve as a chemical defence. The fourth host species, *C. spinosissimum*, is distinctly different from the other species and individuals that feed only on this plant contain virtually no PAs (Dobler and Rowell-Rahier 1994, Pasteels et al. 1996).

The availability of host plants varies geographically in the Alps and in the behavioural experiment we compared three populations that represent three categories of host plant availability: the focal two-host population at Col du Petit Saint Bernard (France, altitude 2188 m) that uses *A. alliariae* and *C. spinosissimum*, the single-host population at Col du Lautaret (France, altitude 2058 m) that feeds exclusively on *A. glabra* (and has no *C. spinosissimum* in its habitat), and finally the single-host population at the Mattmark dam (Switzerland, altitude 2200 m) has only *C. spinosissimum* available and is exclusively found on this plant. The shortest distance between any of these populations is at least 200 km and they are separated by high altitude mountain ranges (alt. 3000–4000 m), making dispersal between them very unlikely (especially since adults have never been observed to fly). These populations will be referred to as the *Adenostyles* population (acronym-A), the *Cirsium* population (C), and the two-host population (TH), respectively.

Laboratory experiments on larval foraging behaviour

During two field seasons (2000, 2001) we conducted experiments to test for effects of population and host experience on larval foraging behaviour in the second and third instars, using the three described populations and two host species (*A. alliariae* and *C. spinosissimum*). Fifty females per population were brought to the field station and were kept in population cages ($30 \times 30 \times 30$ cm) with constant access to both host plants on which they oviposited freely. Eggs were collected daily and at hatching larvae were transferred to plastic boxes and were randomly assigned to either a *C. spinosissimum*-only or an *A. alliariae*-only diet on which they were reared before the behavioural experiments were started. Hence, all larvae included in the experiments had been laboratory reared from the egg and all three populations had experienced identical conditions prior to the behavioural experiments. The behavioural experiments were started when the bulk of larvae had reached the desired instars (second or third). At the start of the experiments all larvae were weighed before being individually placed in round plastic boxes ($\varnothing = 100$ mm, $h = 50$ mm) where one leaf of each *C. spinosissimum* and *A. alliariae* were available. In both experiments all larvae were started simultaneously and were put on the plant species that they had been reared on previously, which henceforth is referred to as the start plant ($N_{2000} = 20$ and $N_{2001} = 30$ larvae per population and start plant treatment leading to a total $N_{2000} = 120$ larvae, and $N_{2001} = 180$ larvae).

During the three days that the behavioural experiments lasted we regularly observed where in the box each larvae positioned itself (two time times per day in 2000, three times per day in 2001). In the 2000 experiment there were three potential positions a larvae could take: on the box or on either of the host leaves (i.e. the plastic box was treated as one compartment). In the 2001 we wanted to estimate larval movements in the box at a slightly finer scale and divided the plastic boxes into five equally sized compartments (marked with a permanent marker). This produced seven potential positions that a larva could take: any of the five box compartments or either of the host leaves. This data allowed us to calculate the proportion of observations when larvae had changed position, and the proportion of observations where larvae were found on their start plant. We also calculated the number of individuals that were never observed to leave the start plant.

To estimate larval feeding preferences we counted the number of feeding marks made on each plant species during the three-day period. In the 2001-experiment we also used image analysis to calculate the leaf areas that had been consumed (NIH Image, version 1.62) and estimated the relationship between leaf area and dry weight of both plant species (equation for *C. spinosissimum*: dry weight (mg) $= 0.029 \times$ leaf area (mm^2), $N = 80$, $r^2 = 0.85$; for *A. alliariae*: dry weight (mg) $= 0.028 \times$ leaf area (mm^2), $N = 79$, $r^2 = 0.89$). There was no significant difference between plant species and none of the intercepts were significantly different from 0. These equations allowed us to estimate actual larval consumption of each host plant. Further analysis showed a strong positive correlation between the proportion of feeding marks that a larva made on the start plant (the only measure of feeding preferences noted in 2000) and the proportion of plant material actually consumed of that plant (linear regression: $F_{1,170} = 805.5$, $P < 0.0001$, $r = 0.91$). Since both measures estimated the same underlying feeding preference we used the proportion of feeding marks on the start plant as our estimate of feeding, as it simplified comparison between years. For all data (movement estimates and feeding) we investigated the effect of population origin, start plant and year of experiment.

Directionality of larval movement in the two-host population

The rationale for this field survey was to use the presence of PAs in larvae to detect larval movement from *A. alliariae* to *C. spinosissimum*. A positive result would indicate that larval movement is not unidirectional from *C. spinosissimum* to *A. alliariae* and that larvae in this population move between hosts repeatedly during development. If larvae collected on *C. spinosissimum* in the field contain more PAs than larvae reared on a *C. spinosissimum*-only diet in the laboratory we may deduce that the field-collected larvae, at some point, have fed on *A. alliariae*. On the other hand, no difference between these categories of larvae in the level of PAs would indicate low rates of movement from *A. alliariae* once this plant is reached. Obviously, we cannot distinguish movement from *C. spinosissimum* to *A. alliariae* with this method but the presence of movement in this direction was already documented in earlier field studies (Ballabeni et al. 2001a).

Late in the season (August 30, 2001) we collected 10 *O. elongata* larvae from *C. spinosissimum* plants in the two-host population. These larvae were starved for 24 h (to allow plant material in the gut to be excreted) and then weighed and frozen at −80°C. The equivalent treatment was given to 12 larvae from the same population that had been reared their whole life in the laboratory on a *C. spinosissimum*-only diet. All larvae were either 3rd or 4th instar when they were killed. We extracted the total fraction of PAs of all 22 larvae individually using a standard protocol (Pasteels et al. 1996). We analyzed the presence and quantity of PAs in each larva by gas chromatography–mass spectrometry (GC-MS, Agilent 5973) using split/splitless injection (injector temperature 250°C), aliquots of 2 µl were injected onto a HP1-MS 30 m × 0.25 mm × 0.25 µm column. Initial oven temperature was set at 40°C for 3 min and thereafter increased 15°C min^{-1} to 300°C, where it was held for 10 min. The MSD was run under the following conditions: transfer line 230°C, source 230°C, quadrupole150°C, ionization potential 70 eV, and a scan range 0–400 amu. Heliotrine was used as internal standard. The identity of PAs was confirmed by comparing the spectra from the samples with the NIST reference library.

Field experiment on larval disappearance rates in the two-host population

The aim of this field experiment was to estimate potential differences in host-specific mortality due to natural enemies if larvae are manipulated to use a single host plant. Early in the season of 2001 we collected approximately 30 mating pairs of *O. elongata* from the two-host population. The beetles were handled in the same way as described in the behavioural experiment. Eggs were collected daily and at hatching larvae were transferred to plastic boxes where either *C. spinosissimum* or *A. alliaria*e were provided as larval food until they were released in the field. Hence, all larvae used had been reared from the egg in the laboratory and had no prior experience with the natural situation.

At the start of the field experiment second and third instar larvae were randomly assigned to one of three treatments: Enclosed, Glue or Non-enclosed, which were crossed with the two host plants *C. spinosissimum* and *A. alliariae*. For all three treatments we choose host plant pairs consisting of one *C. spinosissimum* and one *A. alliariae* plant growing without physical contact but within two meters of each other. We placed ten larvae on each individual plant and all larvae were put out on the same plant species that they had been reared on in the laboratory. In order to minimize emigration from the experimental plants we made sure that there was no leaf contact with any other plants and larvae could only leave the plants by moving down to the ground. The Glue treatment was set up to estimate to what degree this route of emigration could influence plant-specific disappearance rates and it was created by applying a 5 cm broad band of odourless insect glue (Stickem-special, Seabright Laboratories, CA, USA) on the bottom of the plant stalks. In the Enclosed treatment we removed all visible arthropods from the experimental plants, which were then enclosed with a fine meshed bag that inhibited all emigration and protected experimental larvae from predators while still allowing repeated counting of them (Insect Rearing Sleeve, 30 × 70 cm, MegaView Science Education Services Co., Taiwan). Lastly, in the Non-enclosed treatment we left the plants unmanipulated so that natural sources of larval disappearance (predation, emigration down the stem, falling off) apart from leaf to leaf emigration between plants were affecting the larvae. A comparison between the Enclosed and Non-enclosed treatments would indicate the effect of all external sources of disappearance (excluding the known leaf-to-leaf migration between plants) while a comparison between the Glue and the Non-enclosed treatments would indicate any difference in host-specific emigration down the stalk of the plant.

We estimated disappearance rates from the plants in the Non-enclosed and Glue treatments by counting all remaining larvae twice a day during seven days. Larvae in the Enclosed treatment were only counted once a day since disappearance rates were very low. In the Glue treatment we also counted the number of larvae that got stuck in the glue. The experiment was performed within the host plant patch where the adult mothers had originally been collected and

larvae were put out in the field at the rate at which they reached the desired instars (second or third). Consequently, the experiment stretched over approximately three weeks covering a significant part of the larval growth season. All treatments were started in parallel to minimize uncontrolled effects of weather variation and seasonal progression. The numbers of plant pairs (one plant of each host species) in the treatments were: 15 in the Non-enclosed treatment, 10 in the Enclosed treatment and 5 in the Glue treatment (leading to a total number of larvae in each treatment of 300, 200, and 100, respectively). Logistic problems led to a smaller sample size in the Glue treatment than planned. The larger sample in the Non-enclosed treatment was chosen because we expected the measurement error to be larger in this unmanipulated treatment.

Statistical treatment

The experiments on larval feeding behaviour were first analyzed by a MANOVA where the effects of population, start plant and year of experiment (all fixed factors) and their interactions on the three response variables were analyzed (proportion of observations when larvae had moved, proportion of observations on start plant, proportion of feeding on start plant). We then performed the corresponding univariate ANOVAs on each response variable to investigate how they were affected by the three factors. To better meet model assumptions all proportions were arcsine-square root transformed ($X' = \text{arcsine}(\sqrt{X})$) prior to analysis (Sokal and Rohlf 1995). Finally, we used a generalized linear model (GLM) to the analyze differences between populations, start plants and years in the number of larvae that were never observed to leave their start plant (binomial data with a logit link function).

To analyze larval disappearance rates from plants in the field we used Cox proportional hazards regression (Cox 1972), which is the one of the standard methods for analyzing time to event data such as disappearance rates (Fox 2001). Larvae that remained on the plants after the seven days of experiment were coded as censored observations. To control for differences among plant pairs we stratified all analyses by this variable. Since we wanted to investigate potential differences in disappearance rates between the plant species within each treatment we performed one analysis per treatment. The proportional hazards assumption was checked for each model.

The untransformed data are displayed in all graphs and all statistical analyses were performed with Statistica 6.1 and/or Stata 8.0 for Macintosh.

Results

Larval foraging behaviour

All three main effects (population, start plant and year) and their two-way interactions were significant in the multivariate analysis (the three-way interaction was non-significant at $P = 0.58$ and was dropped from the analysis). This indicated that, when we controlled for the effects of year, there was variation among populations in the general foraging strategy and the populations seemed to forage differently on the two plant species (Table 1a).

The three-way interactions in the univariate analyses were all non-significant ($P > 0.15$) and were excluded from the final models (Table 1b). In all three cases there was an effect of year, either due to a significant main effect or to an interaction with one of the other factors (Table 1b). Nevertheless, the results for the two movement estimates (prop. obs. with position changes, prop. obs. on start plant) show strong effects of both population and start plant as well as their interaction. It seems clear that all populations were more likely to move and leave their start plant when it was *A. alliariae* (Fig. 1a–d). The population effect on these variables seems mainly to be due to the two-host population being more likely to move in general as well as being more likely to leave the start plant. Post-hoc tests of the difference between populations for both movement variables showed that the two-host population differed significantly from the other populations (Tukey HSD, $P < 0.001$ in all four cases), whereas the two single host populations did not differ from each other significantly in any of the movement variables ($P > 0.19$ in both cases). Finally, the interactions between population and start plant on the two movement estimates appears mainly to be due to a difference between the two-host population and the other two populations in how they behave when they are started on *C. spinosissimum*. Post-hoc tests of the population by start plant effects indicate that the differences between the two-host population and the other two were always significant when they were started on *C. spinosissimum* (Tukey HSD, $P \leq 0.001$ in all four cases), whereas there was no significant difference between the two single host populations ($P > 0.27$ in both cases). On the other hand, when larvae were started on *A. alliariae* only one of the comparisons between the two-host and the single host populations that showed a significant difference ($P = 0.037$ for TH vs A in the proportion of obs. with position changes, $P > 0.17$ in the three other comparisons), and none of the comparisons between single host populations were significant ($P > 0.99$ in both cases).

The relative amount of feeding on the two plant species was significantly affected by the start plant and the interaction between population and start plant (Table 1b). No difference in preference between hosts

Table 1. Results of (a) MANOVA and (b) univariate ANOVAs for the analysis of larval foraging behaviour and feeding preference from both years of experiments. All proportions were arcsine-square root transformed. All three-way interactions were non-significant and were dropped from the models.

a) MANOVA	Wilk's λ	df	F	P
Year	0.79	1	24.17	<0.0001
Population	0.83	2	9.16	<0.0001
Start plant	0.71	1	36.75	<0.0001
Population × start plant	0.95	2	2.24	0.038
Population × year	0.91	2	4.37	0.0003
Year × start plant	0.97	1	2.81	0.040
Residual		277		
b) Univariate ANOVAs	**df**	**MS**	**F**	**P**
Proportion obs. when larvae had moved:				
Year	1	0.18	2.63	0.11
Population	2	1.28	18.51	<0.0001
Start plant	1	3.13	45.45	<0.0001
Population × start plant	2	0.23	3.38	0.035
Population × year	2	0.58	8.46	0.0003
Year × start plant	1	0.03	0.47	0.49
Residual	281	0.07		
Proportion obs. on start plant:				
Year	1	2.93	20.94	<0.0001
Population	2	1.94	13.82	<0.0001
Start plant	1	14.90	106.20	<0.0001
Population × start plant	2	0.64	4.54	0.011
Population × year	2	0.12	0.84	0.43
Year × start plant	1	0.13	0.93	0.34
Residual	281	0.14		
Proportion feeding marks on star plant:				
Year	1	0.21	0.91	0.34
Population	2	0.14	0.62	0.54
Start plant	1	7.96	34.16	<0.0001
Population × start plant	2	1.02	4.39	0.013
Population × year	2	0.12	0.50	0.61
Year × start plant	1	1.64	7.03	0.0085
Residual	277	0.23		

would predict this measure to be independent of start plant. However, this was clearly not the case for the two single host populations, which fed more on the start plant when it was *C. spinosissimum* (Fig. 1e, f). The feeding preference of the two-host population was less clear since *A. alliariae* was used the most in the first year whereas there was more feeding *C. spinosissimum* in the second year (Fig. 1e, f). The post-hoc tests showed that there was no significant difference between start plants in the two-host population (Tukey HSD, $P = 0.73$) while this difference was highly significant in both single host populations ($P < 0.001$ in both cases).

Larvae that were never observed to leave their start plant represent the most extreme form of non-explorative foraging behaviour in these experiments and we investigated how this type of behaviour varied between populations and host species (Table 2). The number of individuals that was found on the start plant at all observations differed significantly between plant species and populations but there was no significant effect of year (GLM, year: $Z = 0.39$, $P = 0.70$, population: $Z = -4.71$, $P < 0.001$, plant species: $Z = 4.14$, $P < 0.001$, none of the interactions were significant $P > 0.34$ in all cases). Hardly any larvae stayed on *A. alliariae* throughout the experiments while relatively many larvae from the two single-host populations stayed on *C. spinosissimum* when they were started there (Table 2). It appeared that the difference between populations was entirely due to the larval behaviour on *C. spinosissimum*, and to further investigate the effect of population we performed an additional analysis where we only included individuals that were started on *C. spinosissimum*. Since the *Cirsium* population was intermediate in the frequency of individuals that stayed (Table 2), we used it as a baseline in a comparison with the two other populations. Larvae of the two-host population left *C. spinosissimum* in significantly greater numbers compared to the *Cirsium* population, whereas the same comparison for the *Adenostyles* population was just above significance at the 0.05-level (GLM. $Z_{A\text{-}C} = 1.92$, $P = 0.055$, $Z_{TH\text{-}C} = -2{,}75$, $P = 0.003$).

Directionality of larval movement in the two-host population

We could identify three different PAs in our samples: Seneciphylline, Senecionine and Platyphylline. The two latter compounds were always present in very small quantities and we therefore only included Seneciphylline

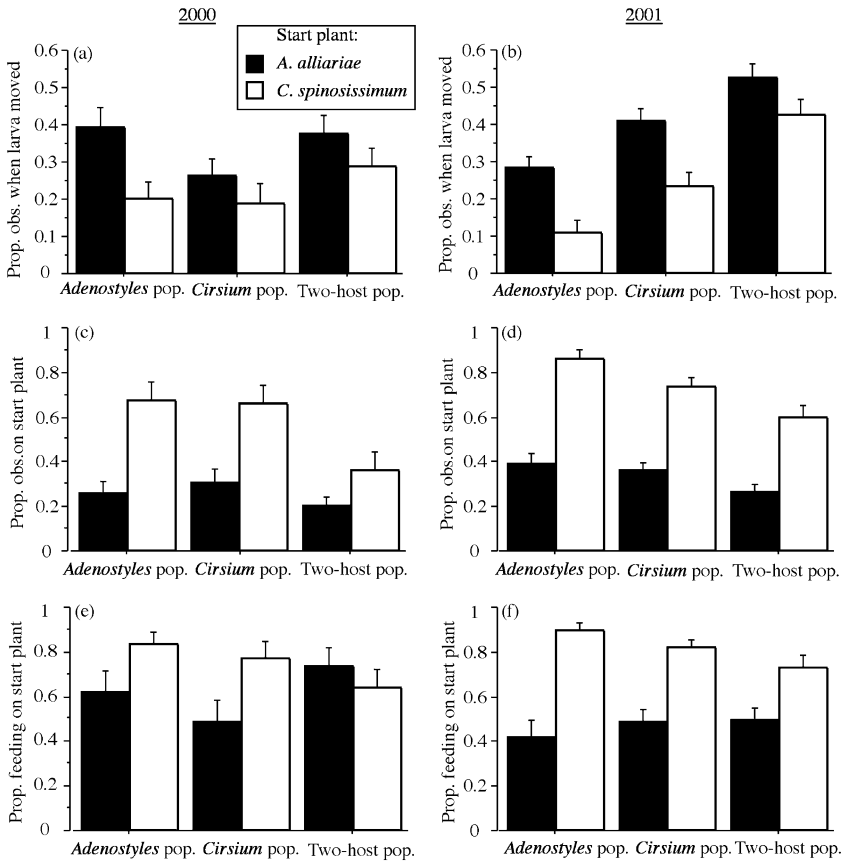

Fig. 1. Results from the two experiments on larval behaviour and feeding preferences. The left column show results from the 2000 experiment (a, c, e) while the 2001-experiment is displayed in the right column (b, d, f). Means ± 1 SE is given for the proportion of observations when larvae had changed position (a, b), proportion of observations on the start plant (c, d), and proportion feeding performed on the start plant (e, f). The data is split by population and start plant (the plant species that each larva was put on at the start of the experiment). N_{2000} was between 17 and 20 individuals per treatment, whereas N_{2001} was between 27 and 30.

in the quantitative analysis. All larvae contained PAs although five of the laboratory-reared larvae contained only unquantifiable traces. Therefore, we first analyzed the results assuming that these five individuals contained no PAs and then reanalyzed the data assuming that they had the same concentration as the larva with the lowest quantifiable concentration ($=0.009$ μg mg^{-1}). The analyses gave practically identical results and only the first analysis is presented.

Despite the small sample we found that the concentration of PAs in the field-collected larvae was significantly higher than in the laboratory-reared larvae (mean $\pm$1SE for field $=0.17 \pm 0.07$, for laboratory $=0.022 \pm 0.007$, Mann–Whitney: $Z_{10,12} = -3.03$, $P = 0.0024$). There was no significant difference in average weight between groups (mean $\pm$1SE for field $=21.1 \pm 3.2$ mg, for laboratory $=26.8 \pm 2.3$ mg, ANOVA,: $F_{1,20} = 2{,}21$, $P = 0.15$) and PA-concentration did not change with larval size (field: correlation $=0.24$, $N = 10$, $P = 0.52$; laboratory: correlation $=0.042$, $N = 12$, $P = 0.90$).

Table 2. The number of larvae of each population that was never observed to leave the start plant, in relation to the total number of individuals in each treatment. Results from the two years of experiments are pooled since there was no significant difference between them.

Population Start plant	*Adenostyles* pop.	*Cirsium* pop.	Two-host pop.
A. alliariae	1/49	0/45	0/49
C. spinosissimum	24/49	15/50	2/49

Larval disappearance rates in the field

Of the total of 600 larvae that were put out in the field 429 disappeared from the plants during the seven day experiment. There was no significant difference in disappearance rate between plant species in the Enclosed treatment (Cox proportional hazards regression, stratified by plant pair: $Z = 0.83$, $df = 1$, $P = 0.41$, Fig. 2). However, in the two other treatments larvae disappeared significantly faster from *A. alliariae* than from *C. spinosissimum* (Cox proportional hazards regression, stratified by plant pair: Non-enclosed treatment $Z = -2.81$, $df = 1$, $P = 0.005$, Glue treatment $Z = -2.45$, $df = 1$, $P = 0.014$, Fig. 2). In these treatments the average chance of staying on the plant after 60 h and onwards was almost twice as high on *C. spinosissimum* compared to *A. alliariae* (Fig. 2).

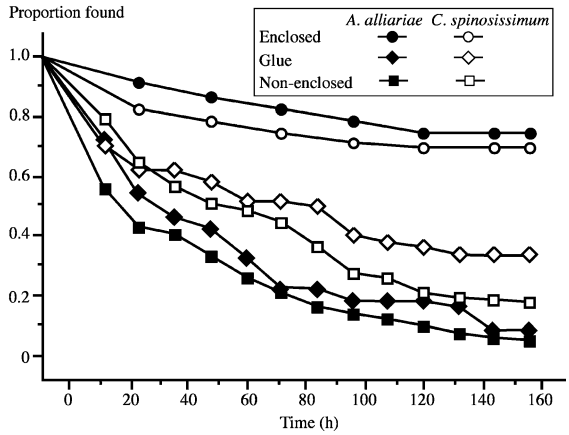

Fig. 2. Results from the field experiment on larval disappearance rates in the two-host population showing survival functions separated by three experimental treatments and plant species. $N_{enclosed} = 100$ individuals per plant species, $N_{glue} = 50$ individuals per plant species, $N_{non\text{-}enclosed} = 150$ individuals per plant species.

There was no difference between plant species in how many larvae were caught in the glue. During the seven days of experiment 10 larvae got stuck at the *C. spinosissimum* plants while 9 larvae were found in the glue on *A. alliariae* (out of 50 larvae put out on each species).

Discussion

The results of the two first studies suggest that individual larvae of the two-host population move between and utilize both their host species. In line with predictions, larvae of the two-host population displayed a significantly higher level of exploratory feeding behaviours compared with single-host populations in a laboratory setting (Fig. 1, Table 1, 2). The variation among populations suggests that this behavioural difference has a genetic basis and may be due to geographic differences in local selection pressures. Larvae from the two-host population stood out from single-host populations in being generally more active (Fig. 1a, b), more likely to leave the host they had been feeding on earlier (Fig. 1c, d, Table 2), and by showing no obvious feeding preference (Fig. 1e, f). Although the foraging behaviour of all three populations showed similar effects of host species (leave *A. alliariae* to a higher degree) it seems that this difference typically was smaller in the two-host population than in the single-host populations. Indeed, in the extreme case of staying on the start host throughout the experiment there was no effect of host plant in the two-host population as opposed to the situation in two single host populations (Table 2). All variables except the "stay-data" were affected by the year of experiment and this may be due to differences in host plant quality between years that we could not detect by visual inspection of plants. However, the main result – a significant interaction between population and host plant – was in all cases stable against this variation (Table 1). Together with the post-hoc tests this result suggest that larvae from the two-host population are more prone to leave a suitable host plant and to explore their host plant environment compared to larvae from the two single-host populations.

The similar response of the two single host populations in response to host plant was surprising, given the difference in their host plant environment. In particular it is difficult to explain why larvae of the *Adenostyles* population show a strong preference for *C. spinosissimum*; a plant species they never encountered in the natural habitat. Earlier studies of these populations suggest that both single host populations have slightly higher larval growth rates on their native hosts, but otherwise it seems as if the degree of local adaptation in larval performance on these two hosts is relatively limited (Ballabeni et al. 2003). Moreover, females from all three populations show a strong oviposition preference for *C. spinosissimum* over *A. alliariae* when the plants are presented simultaneously (Gotthard et al. 2004). Together these results suggest that a general preference for *C. spinosissimum* may be an ancestral condition in these populations of *O. elongata*. Since the *Adenostyles* population never encounters *C. spinosissimum* it is possible that selection for a change in host plant ranking is relatively weak (Gotthard et al. 2004).

In contrast to predictions for the two-host population the results of the behavioural experiment do not suggest that larvae of this size range (2nd and 3rd instar) preferentially move from *C. spinosissimum* to *A. alliariae* as was suggested by earlier field observations (Ballabeni et al. 2001a), but rather that they are adapted to use both host species during larval development. This conclusion was also supported by the comparison of the PAs in larvae feeding on *C. spinosissimum* in the field and in the laboratory. The relatively high amounts of PAs found in larvae collected on *C. spinosissimum* in the two-host population indicate that larvae do not stay on *A. alliariae* when they reach this host but continue to move between hosts. The small amounts of PAs that were found in the laboratory-reared larvae remains still to be explained but could be due to the high amounts of plant-derived alkaloids that females transfer to their eggs (Dobler and Rowell-Rahier 1994).

The second question of why larvae in the two-host population move between and use both hosts was addressed in the experiment on larval disappearance rates in the field. In contrast to expectations, the experiment indicated that the disappearance rate was higher when larvae were feeding on the PA-containing *A. alliariae* than when they were feeding on *C. spinosissimum* (Fig. 2). This difference may in principle be

caused by differential emigration and/or differential predation on the two host species. The experimental setup was designed to reduce the known leaf-to-leaf migration between plants and the Glue treatment indicates that migration down the stem was not different on the two hosts. A third route of emigration could be that larvae allow themselves to fall to the ground and then search for a new host, and we could not experimentally control for this. However, there are no field observations suggesting that this is a common migration strategy. We therefore find it more likely that the main cause of the pattern is a difference in the host-specific predation risk. One additional argument in favour of the predation hypothesis is that the immobile eggs show a very similar pattern of higher disappearance rates from *A. alliariae*, which is most certainly due to a higher predation risk on this plant (Ballabeni et al. 2001b).

There is a possibility that the relative risk of predation on the two hosts varies between years and drawing definite conclusions of the results of one year is risky (see Scheirs and De Bruyn 2002b for a general discussion). Nevertheless, the similarity between this experiment and the experiment on egg mortality indicates that the pattern of higher predation rates on *A. alliariae* has some consistency between years. Despite these considerations the result was surprising since protective effects of PA:s are documented in several insects (Brown 1984, Masters 1990, Rowell-Rahier et al. 1995, González et al. 1999, Eisner et al. 2000). However, recent evidence shows that a very common predator in the two-host population, the harvestman *Mitopus morio*, is able to detoxify and feed on PA-containing *Oreina* larvae (Hartmann et al. 2003). It has been argued that the hairy and spiny leaves of *C. spinosissimum* provide the eggs with mechanical protection against predators (Ballabeni et al. 2001b) and it is possible that the same is true for small larvae. In any case, the results of this experiment give no support to the original hypothesis that larval predation risk in the two-host population typically is lower on *A. alliariae* than on *C. spinosissimum*, and that this would favour larval migration to *A. alliariae*.

The major benefits of larval feeding on *A. alliariae* in the two-host population appears to higher larval growth and developmental rates, and that a mixed diet of *A. alliariae* and *C. spinosissimum* improves survival (Ballabeni and Rahier 2000). A reformulation of the host plant utilization hypothesis for the two-host population would state that females oviposit on the host that is associated with the lowest mortality for eggs and larvae (*C. spinosissimum*), but that larvae also forage on the alternative host because it supports a higher larval performance (*A. alliariae*). However, after feeding on this alternative host they often move back to the oviposition host, possibly because larval predation risk seems to be lower on that host. This type larval foraging strategy will even out the strong distributional bias towards the principal oviposition host that is present early in the season, and is likely to cause the pattern of increasing numbers of larvae feeding on *A. alliariae* with progression of the season (Ballabeni et al. 2001a). In line with this hypothesis the behavioural experiment suggested that the two-host population have indeed been selected for a high degree of explorative larval foraging.

It has frequently been proposed that to understand patterns of host plant utilization in insects it can be vital to consider a range of host related selection pressures whose relative importance may vary between life stages of the insect (Reavey and Lawton 1991, Roitberg and Mangel 1993, Scheirs et al. 2000, Janz 2002, Scheirs and De Bruyn 2002a). Moreover, the role of larval behaviour in the evolution of insect–host plant associations has only received relatively little attention (Dethier 1988, Singer and Stireman III 2001, Oppenheim and Gould 2002). These studies suggest that the mixing of many hosts is unlikely to be maintained by improved larval growth performance alone (Singer 2001, Singer and Stireman III 2001), and that behavioural adaptations may influence the evolution of dietary specialization (Oppenheim and Gould 2002). The present results support these claims and we conclude that the host utilization strategy of *O. elongata* in the two-host situation appears to be maintained by a combination of selection pressures that favour the use of different hosts in different life stages and a larval behaviour that allows alternate use of both hosts.

Acknowledgements – We thank Ted Turlings for help and advise with the chemical analysis and Martin Tschan for providing chemical results. Sören Nylin, Russell Naisbit and Stig Larsson provided helpful comments on earlier drafts. Mathieu Rapp assisted in the field and in the laboratory. Philippe Kuepfer, Barbara Barisani Ephyse Noussan, Lucie Vaser, the Association Internationale du Jardin Alpin de la Chanousia, and the Ordine Mauriziano, Torino, Italy, provided logistic support. This study was financed by grants 3146850.96 and 31-64'864.01 from the Swiss National Science Foundation.

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