

Coping with an antagonist: the impact of a phytopathogenic fungus on the development and behaviour of two species of alpine leaf beetle

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Herbivorous insects and phytopathogenic fungi often share their host plants. This creates a network of direct and indirect interactions, with far-reaching consequences for the ecology and evolution of all three parties. In the Alps, the leaf beetles *Oreina elongata* and *Oreina cacaliae* (Coleoptera: Chrysomelidae), and the rust fungus *Uromyces cacaliae* (Uredinales: Pucciniaceae) are found on the same host plant, *Adenostyles alliariae* (Asterales: Asteraceae). We compare the impact of rust infection on these two closely-related beetle species, one of which, *O. cacaliae*, is a specialist on *A. alliariae*, while the other, *O. elongata*, moves repeatedly between *Adenostyles* and an alternative host, *Cirsium spinosissimum*. Larval performance, feeding preference, oviposition choice and dispersal behaviour were studied in field and laboratory experiments. When reared on rust-infected leaves, larvae of both beetle species had lower growth rates, lower maximum weights and longer development times. Larvae and adults discriminated among diets in feeding trials, showing a preference for discs cut from healthy leaves over those bearing a patch of sporulating rust, those from elsewhere on an infected leaf, and those from an upper leaf on an infected plant. Females of the two species differed in behaviour: in *O. cacaliae* they favoured healthy leaves for larviposition, while in *O. elongata* they showed no significant preference during oviposition. In the field, larvae and adults of both species dispersed more rapidly when placed on infected host plants. The results demonstrate that rust infection reduces the quality of the plant as a host for both *Oreina* species, and they combine the ability to detect systemic infection with the evolution of evasive behaviours. For these beetles, competition with a rust clearly increases the difficulty of survival in the harsh conditions of alpine environments, and may have a profound impact on the evolution of their life history traits and host plant use.

In nature, insect–fungus relationships can take many forms, from mutualisms like those between leaf-cutter ants, termites or ambrosia beetles and their fungal “crops” (Chapela et al. 1994, Farrell et al. 2001, Aanen et al. 2002), or the transfer of fungal infectious stages or gametes by insects (Batra and Batra 1985, Roy 1993), through to predator–prey interactions with either the insect or the fungus in the role of predator (Blanford et al. 2005, Takahashi et al. 2005, Roy et al. 2006). When herbivorous insects and phytopathogenic fungi share a host plant the relationship would seem to be competitive. Yet even in this situation, a great variety of interactions are possible. For the fungus, feeding by the insect may destroy spores or mycelia (Hatcher et al. 1994a, Hatcher and Paul 2000), but it can also be beneficial if it

contributes to dispersal or aids establishment by creating wounds (Kluth et al. 2002). For the insect, fungal infection of its host plant will often act indirectly by altering host plant chemistry, with either positive or negative effects on growth rate (Hatcher et al. 1994b, Laine 2004, Mondy and Corio-Costet 2004). The participation of induced plant defence makes this a truly three-way interaction. Attack by one natural enemy may be detrimental to another if there is cross-talk between the systems of induced defence against them, or have positive effects if induction draws investment away and impedes the ability of the plant to respond to attack by a second enemy (Hatcher 1995, Stout et al. 2006). These interactions are likely to be pervasive given the enormous diversity of herbivorous insects and phytopathogenic

fungi, and have consequences at all levels of the ecology of all parties, from individual behaviour and reproductive success through to population dynamics and the evolution of specialization. There are also practical applications, for antagonistic and synergistic interactions will affect the impact of insects and fungi when they are pests on crops (Hatcher 1995), or when they are intended as biocontrol agents against invasive weeds (Friedli and Bacher 2001, Kluth et al. 2001).

Here we examine the three-way interaction between two alpine leaf beetles, *Oreina elongata* and *O. cacaliae*, the rust fungus, *Uromyces cacaliae*, and their host plant, *Adenostyles alliariae*. In their high altitude habitats the relationships are likely to be particularly intense, for all participants have a very limited time to complete their reproductive cycle during the favourable season. Typically, only two to three months are available before the snow returns and even the summer can be interrupted by cold spells. In this paper we consider the relationship from the point of view of the insects, while future publications will complete the triangle of interactions. For the beetles, the quality and quantity of food available is critical to larval development, and is likely to have longer term effects on overwinter survival and adult performance. We use laboratory and field experiments to investigate the ecological and behavioural consequences of coexistence with a rust fungus and answer three questions:

- to what extent does rust infection of the host plant affect larval development of the two beetle species?
- If infection of the host by the rust is disadvantageous for the beetles, have they evolved behavioural mechanisms in their feeding preference, oviposition choice and dispersal that would enable them to avoid infected plants or leaves?
- Is the response the same in a specialist beetle and a closely related species that has an alternative host plant available? *O. cacaliae* is a specialist in these populations that spends the whole reproductive season on *A. alliariae*, while in *O. elongata* adults and larvae move repeatedly between *A. alliariae* and *Cirsium spinosissimum*, a host that is not attacked by the rust. Access to this alternative host may have weakened any selection for discrimination among *Adenostyles* plants or for adaptation of performance to an infected diet.

Materials and methods

Leaf beetles, host plant and rust

The leaf beetles *Oreina elongata* and *Oreina cacaliae* (Coleoptera: Chrysomelidae) are found exclusively in

mountain environments in Europe (Freude et al. 1994). *Oreina elongata* is a small (body length 6.5 to 9.5 mm), metallic-blue beetle, found at altitudes between 1600 m and 2400 m a. s. l. Its distribution is patchy, with isolated populations throughout the Alps and further south into the Apennines (Freude et al. 1994, Margraf et al. 2007). The life cycle of *O. elongata* is adapted to the high alpine environment and consists of a first summer as egg and larva, hibernation in the larval stage, pupation early in spring, then a season as a non-reproductive adult before several consecutive reproductive seasons once the adult stage is reached. In the populations studied here, host plant use is restricted to two plant species in the Asteraceae: *Adenostyles alliariae* and the thistle *Cirsium spinosissimum* (in some other sites they use *A. glabra* and *A. leucophylla*). Eggs are laid on the leaves of host plants from the beginning of July to mid-August, mainly on *C. spinosissimum*, which offers protection against predators due to its spiny leaves (Ballabeni et al. 2001a). Both adults and larvae have two modes of chemical defence: pyrrolizidine alkaloids (PAs) sequestered from hosts in the genus *Adenostyles*, and autogenously synthesised cardenolides (Dobler et al. 1996).

Oreina cacaliae resembles *O. elongata*, often with a similar metallic-blue colouration but a slightly larger body size (7.5 to 11.5 mm). It is more widespread, with a patchy distribution at altitudes between 800 and 2300 m across the mountains of Europe, from the Pyrenees in the west to the Carpathians in the east, and from the Ore Mountains in the north to the Apennines in the south (Freude et al. 1994). The life cycle is similar to that of *O. elongata*, although females may mate, but not produce offspring, in their first summer as an adult. *O. cacaliae* does not lay eggs, but deposits larvae directly on the host plant. The beetles feed and reproduce on *A. alliariae*, although in some populations they use *Senecio*, and others briefly begin the season on *Petasites paradoxus* (Asteraceae), because this is one of the first plants available as the snow melts at sun-exposed sites (Kalberer et al. 2005). *Oreina cacaliae* has lost the ability to produce cardenolides and depends exclusively on PA sequestration for defence (Dobler et al. 1996). Both *Oreina* species pass the winter buried in the soil and are only active during a very short summer season, from late May to late August, depending on the altitude and exposure of the sites.

For this study, beetles were collected from four populations across the Alps: *O. elongata* from Emosson (Swiss Alps, Valais, 1949 m) and Piccolo San Bernardo (Italian Alps, Valle d'Aosta, 2053 m), and *O. cacaliae* from Kandersteg (Swiss Alps, Ueschinental, Bern, 1481 m) and La Fouly (Swiss Alps, Val Ferret, Valais, 1587 m). Adults of both sexes were collected in early June and kept in plastic boxes with holes for aeration, a wet filter paper lining for humidity, and fresh leaves of

A. alliariae. The food was frequently replaced, and the eggs (*O. elongata*) or first instar larvae (*O. cacaliae*) taken to begin experiments or moved to stock boxes.

Adenostyles alliariae is a common, perennial, sub-alpine and alpine plant, found at a maximum altitude of 2800 m growing on damp soils. It produces secondary compounds, pyrrolizidine alkaloids (PAs), as a defence against herbivory (Hartmann et al. 1999). Fresh leaves from uninfected plants for the lab experiments were collected every three days from Emosson, Piccolo San Bernardo, or La Fouly, transported in a cooled box, and kept in a refrigerator at 5°C for a maximum of two days until used.

Uromyces rusts occur over a wide geographical range and are parasitic upon many families of hosts (Bisby 1920). *Uromyces cacaliae* (Uredinales: Pucciniaceae) is an obligatory pathogen of *Adenostyles alliariae*. It produces teliospores from patches on the underside of leaves and can reach this stage around two weeks after infection (Bisby 1920). Obvious rust infections appear mainly at the end of June in the lower altitude sites where *O. cacaliae* occurs, and in mid July in the higher altitude habitats of *O. elongata*. Leaves bearing *U. cacaliae* for the experiments were collected from the same sites as the healthy leaves, from plants of the same age and condition.

Larval performance on healthy and infected leaves

Larval growth rate, maximum weight and development time were compared on healthy and rust-infected plants for two populations each of *O. elongata* and *O. cacaliae*. The experiment was begun with newly hatched (*O. elongata*) or laid (*O. cacaliae*) larvae. They were reared individually in petri dishes (5.5 cm Ø, 1.2 cm depth), with the base lined with plaster of Paris and a filter paper to maintain humidity. The larvae were assigned at random to one of two diets: healthy, or infected with sporulated rust. Every three days they were weighed to the nearest 0.1 mg on an electronic balance and given a fresh piece of leaf. A few days after reaching the fourth instar, the larvae were moved into larger plastic pots (9.5 cm Ø, 4.5 cm depth) with a layer of damp soil, where they were fed and weighed daily until they buried into the soil to hibernate. Experiments involving *O. elongata* were conducted in July 2003 in an unheated building at the Piccolo San Bernardo Pass, on the border between France and Italy to the south of Mount Blanc. Experiments with *O. cacaliae* were carried out in 2004 at the Univ. of Neuchâtel in a cooled incubator maintained with 15:30 h daylength and temperatures varying gradually from 6.5°C (night) to 20.0°C (day). Trials began with 15 larvae of each population and species on each diet, except

for one population of *O. cacaliae* fed on healthy leaves, where 17 larvae were used.

Growth rate was calculated as a daily growth multiplier. For each larva, the successive weights until the maximum were log transformed and then regressed against the time in days (these linear regressions gave a very close approximation to the growth process, with r^2 values of between 0.905 and 0.999). Exponential back-transformation of the slope of the line gave the daily growth multiplier, representing the coefficient by which larvae multiplied their weight each day. Larvae showed a slight decrease in body mass before they buried themselves for the winter diapause, so the maximum weight reached was used for analysis. Development time was taken as the number of days needed to reach this maximum weight. Growth rate, maximum weight, and development time were analysed in separate ANOVAs with terms for species, diet, population nested within species, the species by diet interaction, and the population by diet interaction. In these analyses, the term "species" reflects differences in species, year, and environmental conditions. A square root transformation was used to make the maximum weight data conform to assumptions of normality and homogeneity of variance, while no transformation was needed for growth rate and development time. Analyses were carried out using JMP 6.0 (SAS Inst.).

Feeding preference

In a three-choice experiment, adults and larvae of both species were offered a choice between leaf discs from healthy plants, those carrying a sporulating patch of the rust, and those from an infected leaf but cut to avoid sporulating patches of the rust. Tests were carried out individually in petri dishes (9 cm Ø, 1.5 cm depth) lined with a moist filter paper. Leaf discs of 13 mm Ø were cut from fresh leaves using a cork borer and single discs of each type were arranged randomly and symmetrically around the edge of the dish. Adults and larvae were then allowed to feed for 24 h (individuals never ate an entire disc, so that all choices were still available at the end of the experiment). The experiment was run in a cooled incubator with conditions as described above. After 24 h, the leaf discs were dried and pasted on a transparent sheet, scanned, and the area remaining measured using Scion Imaging software.

After excluding individuals that did not feed (5, 0, 7 and 2 replicates in the four categories), data were available from 25 adults and 30 larvae of *O. elongata* and 11 adults and 16 larvae of *O. cacaliae*. Four Friedman ranks tests were used to determine if each species and life stage discriminated consistently among the diets, and then pairwise Wilcoxon signed ranks tests were used to detect where the preference lay (using

SPSS 14.0 for both tests). The sequential Bonferroni method (Rice 1989) was used to correct for the number of pairwise comparisons for the entire table of Wilcoxon tests.

The experiment was repeated the following year under identical conditions but as a four-choice trial, with the addition of a leaf disc from an infected plant taken from an upper leaf that showed no sign of rust attack. Again, some individuals did not feed (11, 5, 8 and 10 replicates in the four categories), leaving data from 16 adults and 63 larvae of *O. elongata* and 32 adults and 45 larvae of *O. cacaliae*.

Oviposition and larviposition preference

Laboratory choice experiments were used to test for a preference between healthy and rust-infected leaves during oviposition in *O. elongata* and larviposition in *O. cacaliae*. In mid June, pairs of beetles were placed in $19 \times 9 \times 8$ cm plastic boxes with holes for aeration and a wet filter paper lining for humidity. At either end of the box, fully infected and healthy leaves were offered on pieces of stem, to simulate their natural position and orientation. The boxes were arranged at random in a room at 20°C with natural light, and the females given 24 h to oviposit (*O. elongata*) or 12 h to larviposit (*O. cacaliae*). Eggs were counted at the end of this period, whereas experiments involving *O. cacaliae* were monitored throughout the day to determine the choice of larviposition. Larvae and eggs laid on the box were considered as a no-choice and excluded from the analysis. Females laid relatively few eggs or larvae (an average of 16.2 for *O. elongata* and 1.8 for *O. cacaliae*) so the data were analysed with a simple non-parametric sign test in SPSS 14.0 to test for a consistent preference in each species.

Dispersal rates from healthy and infected plants in the field

Field trials were carried out to investigate the effect of rust infection on the movement patterns of adults and larvae of the four populations. At each site, 10 adults and 10 larvae were marked with Tipp-Ex and deposited individually on *A. alliariae* plants, half on healthy plants and half on those strongly infected with *U. cacaliae*. Over a period of 6 h in the middle of the day, the 20 plants were checked every 2 h to count the number of individuals still remaining. The experiments occurred at the beginning of August under good weather conditions.

The data were analysed using parametric survival analysis, treating the act of leaving the plant as “mortality”. The `SurvReg` function was used in S-PLUS 7.0 (Anonymous 2005), coding those individuals that remained for the full six hours as right-

censored and all others as interval-censored (because their leaving times could only be estimated to within two-hour intervals). A value of 0.001 was added to data with a lower bound of zero to allow log terms to be treated. Different models were compared using likelihood ratio tests, and the independent variables were entered as factors (introducing them as strata did not significantly improve the fit). The data were first analysed for each species separately, with terms for population (two for each species), stage (adult or larva), and plant (healthy or infected). The population term was never significant, so the two datasets were combined to analyse a single model with terms for species, stage, plant, and all their interactions. The order in which terms were added had very little effect on their significance. S-PLUS offers 10 possible distribution families, but all gave very similar p values and only the results from the analysis using a Weibull distribution are presented. This distribution was suggested by the approximately linear relationship between $\ln(t)$ and $\ln(-\ln(1/S(t)))$, where $S(t)$ is the proportion of individuals that remain at time t (Gross and Clark 1975, Fox 2001).

Results

Larval performance on healthy and infected leaves

For both *O. elongata* and *O. cacaliae*, larvae reared on rust-infected leaves of *A. alliariae* showed significantly lower growth rates, lower maximum weights, and longer development times than those reared on healthy leaves (Table 1, Fig. 1). The two species differed (*O. elongata* grew more rapidly, was smaller at the maximum and developed in a shorter time), but this may in part represent an effect of the rearing conditions or variation between years. The populations did not differ within each species and all four populations showed the same effect of diet.

Twenty-seven of the 122 larvae died during the trial, but mortality was not related to diet (mortality of *O. elongata* was 4/30 on healthy leaves, 6/30 on the infected diet, G-test of independence with Williams’s correction, $G_1 = 0.459$ $p = 0.498$; for *O. cacaliae* mortality was 6/32 on healthy and 11/30 on infected, $G_1 = 2.442$ $p = 0.118$).

Feeding preference – three-choice trials

Adults and larvae of both species discriminated among the three diets (Table 2, Fig. 2). After sequential Bonferroni correction of the pairwise Wilcoxon signed ranks tests (Table 3), this was found to be a result of a significant preference for healthy leaf discs over those

Table 1. ANOVAs on the three larval performance parameters for the two populations of *O. elongata* and of *O. cacaliae* reared on healthy or infected diets. The species term reflects differences between species, experimental years, and rearing conditions. The population and population by diet terms were nested within species.

| Source | DF | SS | F | p-value |
|-------------------------|----|--------|---------|---------|
| Daily growth multiplier | | | | |
| Species | 1 | 0.1143 | 521.343 | <0.001 |
| Diet | 1 | 0.0130 | 59.495 | <0.001 |
| Pop[species] | 2 | 0.0011 | 2.503 | 0.088 |
| Diet × species | 1 | 0.0002 | 0.812 | 0.370 |
| Diet × pop[species] | 2 | 0.0006 | 1.337 | 0.268 |
| Error | 87 | 0.0191 | | |
| Maximum weight | | | | |
| Species | 1 | 24.642 | 182.567 | <0.001 |
| Diet | 1 | 1.255 | 9.297 | 0.003 |
| Pop[species] | 2 | 0.743 | 2.753 | 0.069 |
| Diet × species | 1 | 0.013 | 0.093 | 0.761 |
| Diet × pop[species] | 2 | 0.016 | 0.061 | 0.941 |
| Error | 87 | 11.743 | | |
| Development time | | | | |
| Species | 1 | 199.82 | 24.763 | <0.001 |
| Diet | 1 | 237.88 | 29.480 | <0.001 |
| Pop[species] | 2 | 8.30 | 0.514 | 0.600 |
| Diet × species | 1 | 0.60 | 0.075 | 0.785 |
| Diet × pop[species] | 2 | 3.43 | 0.213 | 0.809 |
| Error | 87 | 702.01 | | |

with sporulating rust present (for both species, but marginally non-significant for *O. cacaliae* larvae). *O. elongata* adults (and marginally non-significantly for the larvae) also discriminated between healthy leaf discs and those from infected leaves but with no visible sign of the rust, while the larvae preferred the latter type of disc over those with sporulating rust present.

Overall, 37 of the 82 individuals tested (45%) fed only on the healthy leaf, significantly more than would be expected if these were just the individuals that by chance first fed on that disc and then remained (exact binomial probability $\text{bin}[82, 1/3]$, $\text{pr}[X \geq 37] = 0.017$).

Feeding preference – four-choice trials

In this experiment, a fourth choice was added: a disc of a rust-free upper leaf from a rust-infected *A. alliariae* plant. Again, adults and larvae of both species discriminated among the diets (Table 2, Fig. 3). Leaf discs from healthy plants (H) were preferred over those carrying a sporulating area of rust infection (IR), over those from elsewhere on a rust-infected leaf (IL), and over those from an upper leaf on a rust-infected plant (IP) (Table 4 after sequential Bonferroni correction, one comparison non-significant for *O. elongata* adults). Only *O. elongata* larvae discriminated among the different categories of leaf disc from an infected plant, preferring all types to those containing sporulating rust.

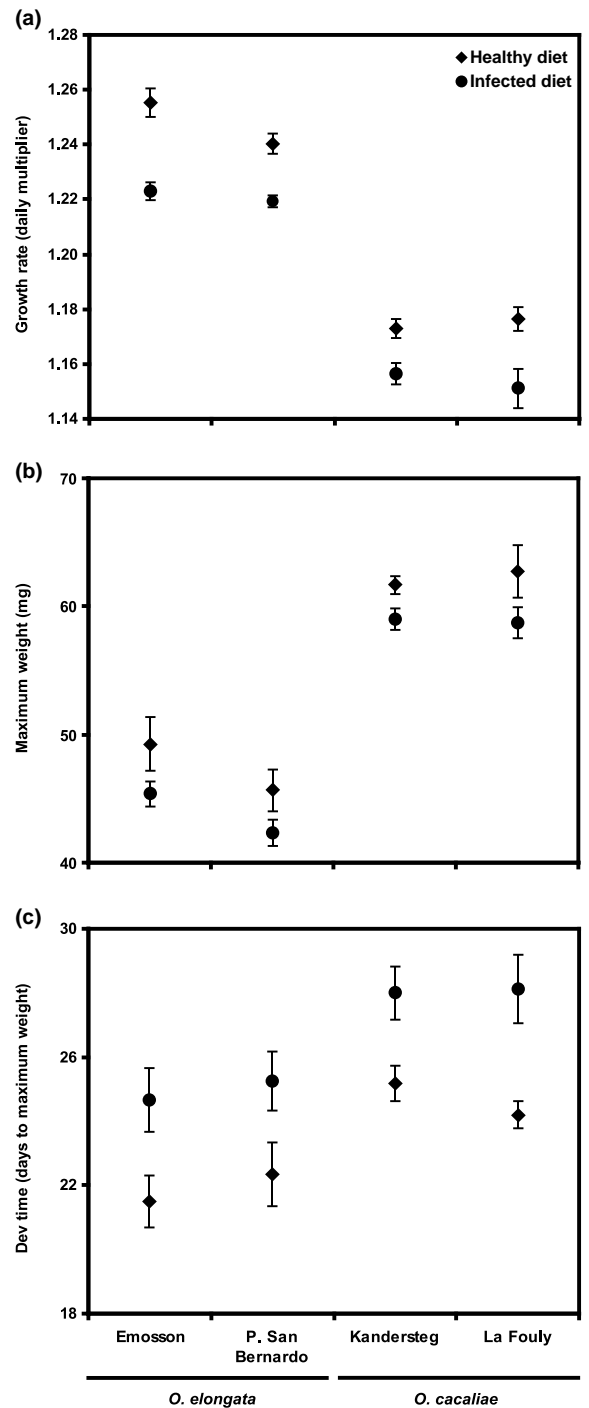


Fig. 1. Larval performance of *O. elongata* (in 2003) and *O. cacaliae* (in 2004) from four populations reared on healthy or rust-infected leaves of the host plant. The three larval parameters studied were (a) the growth rate (calculated as a daily growth multiplier, see text for details), (b) the maximum weight (in mg) reached by the larvae during their development, and (c) the number of days needed to reach the maximum weight. Graphs show means \pm SE.

Table 2. Friedman ranks tests on the results from the feeding preference experiments. In the three-diet trials, individuals were given a choice between a leaf disc bearing a patch of sporulating rust, a disc from an infected leaf but lacking the sporulating rust, and a disc from a healthy plant. The four-diet trials followed the same method but added a leaf disc from an upper leaf with no sign of rust attack on an infected plant. Each species and life stage was analysed individually.

| | n | χ^2 | DF | p-value |
|------------------------------|----|----------|----|---------|
| Feeding preference (3 diets) | | | | |
| <i>O. elongata</i> adults | 25 | 16.644 | 2 | <0.001 |
| <i>O. elongata</i> larvae | 30 | 31.592 | 2 | <0.001 |
| <i>O. cacaliae</i> adults | 11 | 15.297 | 2 | <0.001 |
| <i>O. cacaliae</i> larvae | 16 | 12.473 | 2 | 0.002 |
| Feeding preference (4 diets) | | | | |
| <i>O. elongata</i> adults | 16 | 23.540 | 3 | <0.001 |
| <i>O. elongata</i> larvae | 63 | 104.861 | 3 | <0.001 |
| <i>O. cacaliae</i> adults | 32 | 34.565 | 3 | <0.001 |
| <i>O. cacaliae</i> larvae | 45 | 52.312 | 3 | <0.001 |

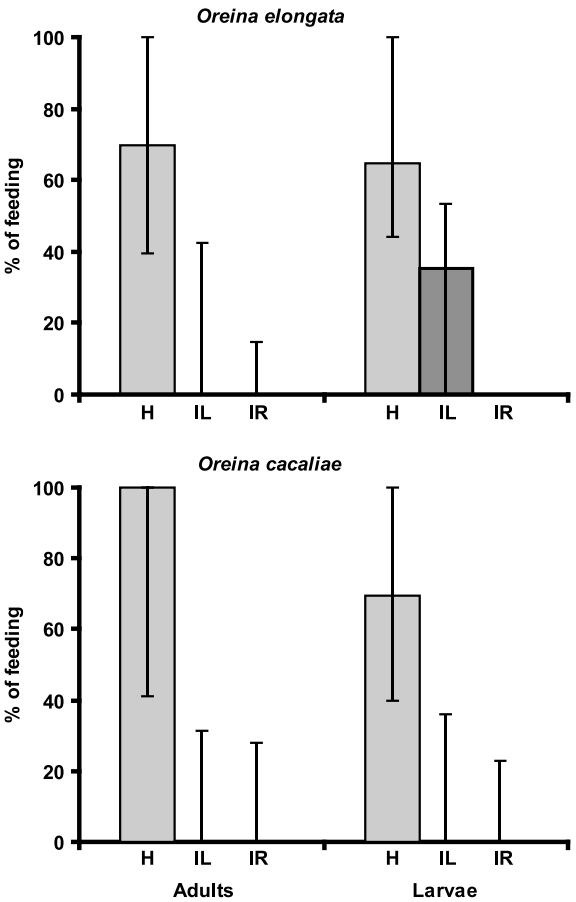


Fig. 2. Feeding preference in the three-choice experiment for adults and larvae of both species. Graphs show the proportion of total feeding on discs from healthy leaves (H), from infected leaves but cut from an area lacking the sporulating rust (IL), or from infected leaves bearing sporulating rust (IR). Bars show the median feeding per individual with error bars showing the 25th and 75th percentiles.

Table 3. Pairwise Wilcoxon signed ranks tests on the data from the three-choice feeding preference experiment. Sample sizes are identical to those in Table 2. Individuals were given a choice between a leaf disc bearing a patch of sporulating rust (IR), a disc from an infected leaf but lacking the sporulating rust (IL), and a disc from a healthy plant (H). Values shown in bold are significant after sequential Bonferroni correction to provide a table-wide significance level of 5%.

| | H–IL | H–IR | IL–IR |
|-----------------------------|----------------|----------------|----------------|
| <i>O. elongata</i> adults Z | – 2.972 | – 3.465 | –1.193 |
| p-value | 0.003 | 0.001 | 0.233 |
| <i>O. elongata</i> larvae Z | –2.597 | – 4.617 | – 3.519 |
| p-value | 0.009 | < 0.001 | < 0.001 |
| <i>O. cacaliae</i> adults Z | –2.287 | – 2.869 | –0.944 |
| p-value | 0.022 | 0.004 | 0.345 |
| <i>O. cacaliae</i> larvae Z | –2.080 | –2.642 | –0.664 |
| p-value | 0.038 | 0.008 | 0.507 |

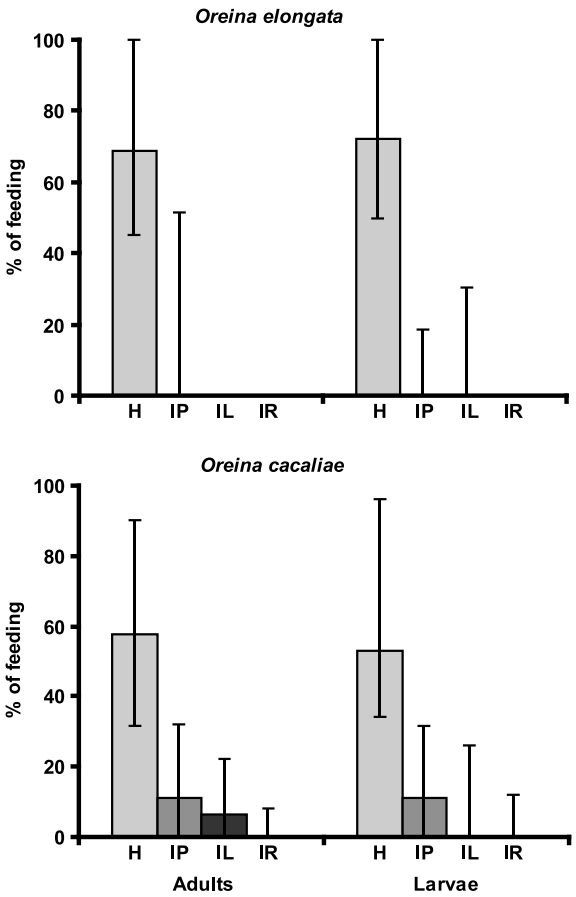


Fig. 3. Feeding preference in the four-choice experiment. Details as in Fig. 2, with the addition of a fourth choice, a leaf disc taken from an uninfected upper leaf on an infected plant (IP).

Table 4. Pairwise Wilcoxon signed ranks tests on the data from the four-choice feeding preference experiment. Sample sizes are identical to those in Table 2. Experimental details were the same as for Table 3, with the addition of a leaf disc from a rust-free upper leaf on an infected plant (IP). Values in bold are significant after sequential Bonferroni correction to provide a table-wide significance level of 5%.

| | H-IP | H-IL | H-IR | IP-IL | IP-IR | IL-IR |
|-----------------------------|------------------|------------------|------------------|--------|---------------|---------------|
| <i>O. elongata</i> adults Z | -2.401 | -3.432 | -3.318 | -1.718 | -1.352 | -0.405 |
| p-value | 0.016 | 0.001 | 0.001 | 0.086 | 0.176 | 0.686 |
| <i>O. elongata</i> larvae Z | -5.486 | -6.355 | -6.380 | -0.049 | -2.950 | -2.931 |
| p-value | <0.001 | <0.001 | <0.001 | 0.961 | 0.003 | 0.003 |
| <i>O. cacaliae</i> adults Z | -3.347 | -3.707 | -4.670 | -0.182 | -2.549 | -2.495 |
| p-value | 0.001 | <0.001 | <0.001 | 0.855 | 0.011 | 0.013 |
| <i>O. cacaliae</i> larvae Z | -4.252 | -5.012 | -4.873 | -0.998 | -2.117 | -1.922 |
| p-value | <0.001 | <0.001 | <0.001 | 0.318 | 0.034 | 0.055 |

Overall, 48 of the 156 individuals tested (31%) fed only on the healthy leaf (exact binomial probability $\text{bin}[156, 1/4], p[X \geq 48] = 0.060$).

Oviposition and larviposition preference

Females of *O. elongata* did not discriminate between healthy and rust-infected leaves for oviposition (33/21/3 preferred healthy/infected/ties, sign test $p = 0.134$), whereas females of *O. cacaliae* showed a strong preference for healthy leaves (15/0/2, sign test $p < 0.001$) (Fig. 4).

Dispersal rates from healthy and infected plants in the field

Individuals left significantly more rapidly from rust-infected plants than they did from healthy plants (Fig. 5, Table 5). The effect of stage was also significant (adults left more rapidly than larvae), but the two species did not differ and none of the interaction terms were significant.

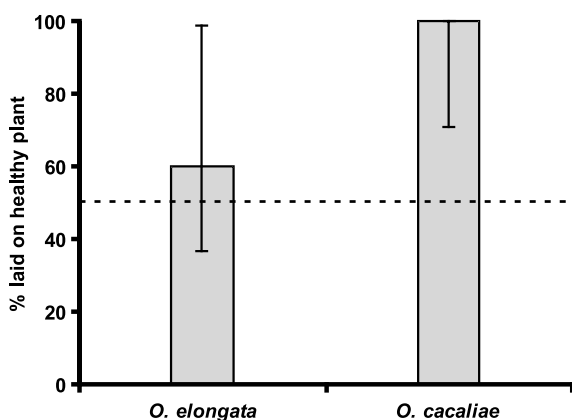


Fig. 4. Oviposition (*O. elongata*) and larviposition (*O. cacaliae*) preferences, showing the median proportions laid on healthy leaves. Error bars give the 25th and 75th percentiles.

Discussion

High altitude environments in Europe provide harsh conditions for insect life, with low and unpredictable temperatures, patchy and unreliable food supplies, and a very short season of activity (Danks 1992). The leaf beetles *Oreina elongata* and *O. cacaliae* must deal with these pressures throughout their life-cycle as permanent inhabitants of alpine habitats with very limited dispersal ability. Like many insect species they have developed a cold tolerance strategy with life cycles that need more than one year, prolonged dormancy, and plasticity of their growth rates (Bogacheva and Khruleva 2002, Margraf et al. 2003, Sinclair et al. 2003).

The presence of the fungus clearly adds another hazard to what is already a harsh existence. Here we show that larval performance is significantly affected by the presence of the phytopathogenic rust *Uromyces cacaliae*, suggesting detrimental effects of a reduction in plant nutritional quality or the production of fungal metabolites (Hatcher 1995). Another species of *Uromyces* attacking *Rumex* spp. has a similar effect, increasing the development time and reducing the pupal weight of larvae of the leaf beetle *Gastrophysa viridula* (Hatcher et al. 1994b). Exposure to phytopathogenic fungi need not always be harmful, for larvae of the herbivorous moth *Lobesia botrana* have a lower mortality rate and faster development time in the presence of a fungus (Mondy and Corio-Costet 2004). However, for *Oreina* the presence of the rust is disadvantageous: when reared on rust-infected leaves, larvae of both species showed reduced growth rates (the daily multiplier was reduced by nearly 2% on average), longer development times (by 13.8%), and lower maximum weights (by 6.5%). These three parameters are related, and it seems that the beetles trade-off development time against maximum weight to some extent. To compensate for the reduced daily growth rate (which would have a multiplicative effect each day) they appear to endure a longer development time in order to reduce the impact on their maximum weight. Perturbations of their normal final weight and development

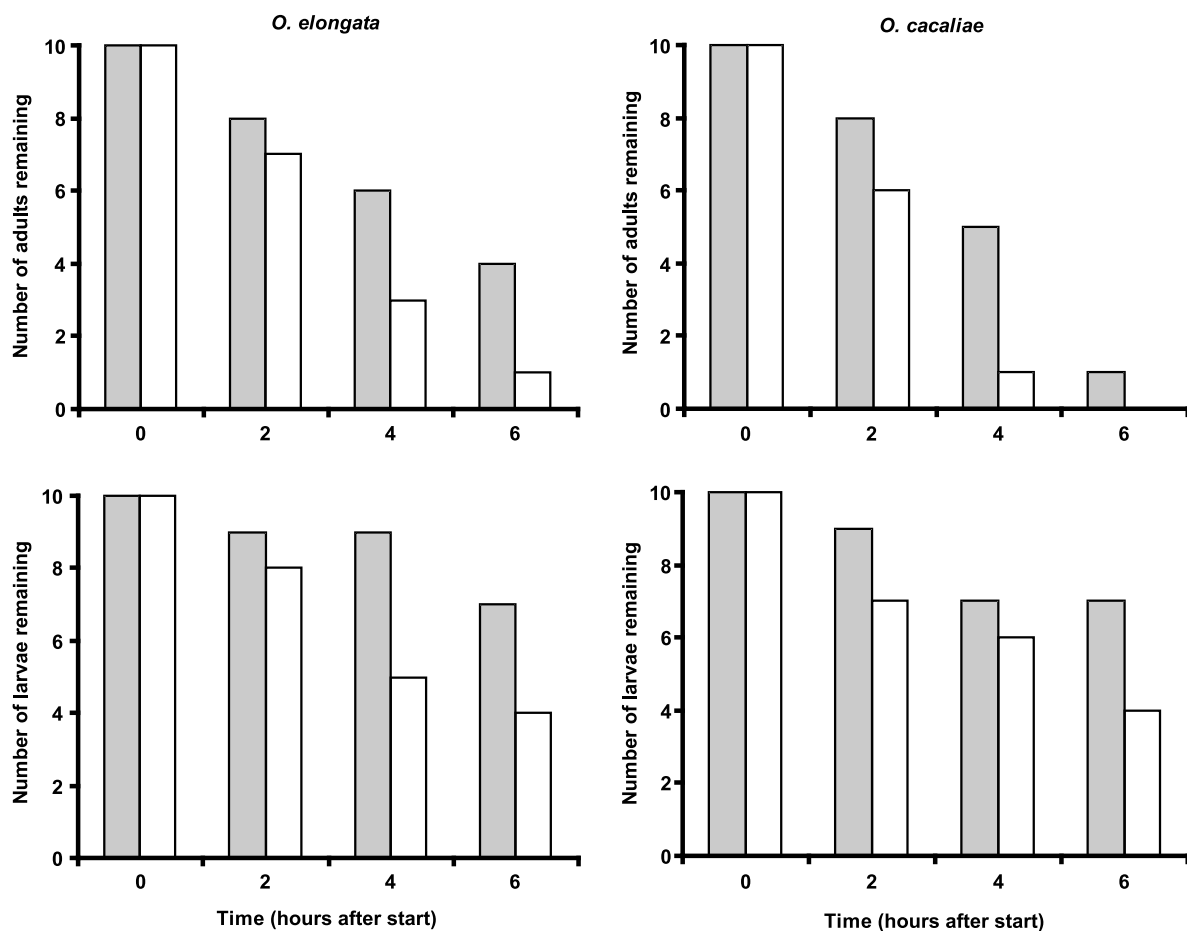


Fig. 5. Dispersal behaviour in the field. Bars show the number of adults or larvae still remaining two, four and six h after having been placed individually on healthy (shaded bars) or rust-infected plants (open bars).

time would both bear costs for larvae. Slower development may affect larval survival by exposing them to predation and parasitism for a longer period (Williams 1999). It would also place larvae at risk of not being able to complete development before the first snow arrives, because many sites are only free of snow for a maximum of 2–3 months each year. Since the eggs of

O. elongata take 15–20 days to hatch and larval growth nearly one month, those larvae laid late in the season are already on a very tight developmental schedule to be ready for hibernation (Verdon et al. 2007). Similarly, a reduction in the larval maximum weight would be likely to increase mortality during the long alpine winter (Naisbit et al. unpubl.), and affect future reproductive

Table 5. Parametric survival analysis of the dispersal experiment (in which dispersal was treated as mortality). The lines show the null model (with a single location and scale parameter) and the change in log likelihood as terms for species (*O. cacaliae* or *O. elongata*), stage (adult or larva), plant (healthy or infected), and their interactions were sequentially added. The final three columns provide likelihood ratio tests of the significance of each term.

| | Parameters | $-2 \times \text{LogLik}$ | Likelihood ratio | DF | p (χ^2) |
|---------------------------------------|------------|---------------------------|------------------|----|----------------|
| Null | 2 | 216.843 | | | |
| Species | 3 | 215.859 | 0.984 | 1 | 0.321 |
| Stage | 4 | 202.088 | 13.771 | 1 | <0.001 |
| Plant | 5 | 193.211 | 8.877 | 1 | 0.003 |
| Species \times plant | 6 | 193.149 | 0.062 | 1 | 0.803 |
| Stage \times plant | 7 | 193.037 | 0.112 | 1 | 0.738 |
| Species \times stage | 8 | 192.209 | 0.828 | 1 | 0.363 |
| Species \times stage \times plant | 9 | 192.208 | 0.001 | 1 | 0.976 |

success by reducing adult body size. Larvae must therefore manage a delicate balance between the two life history traits, and show some plasticity in their response to the challenge of rust-infection of their host plant.

The beetle species have a number of behavioural mechanisms by which they can avoid the negative effects of the rust, in their choice of leaves for feeding and oviposition, and their pattern of dispersal. Short-range avoidance of rust-infected plants was seen in the food-choice experiments. Greater discrimination could be detected in the four-choice experiment, presumably due to the mostly larger sample sizes. Adults and larvae of both species preferred leaf discs from healthy leaves over those from any part of a rust-infected plant, regardless of whether the disc included a patch of sporulating rust, was taken from elsewhere on that leaf, or was from an upper leaf with no visible sign of rust attack. Individuals discriminated strongly against all discs from infected plants, and choice among them was seen only in one group, the larvae of *O. elongata*. Whilst the behaviour may be part of a general response to low quality food, the experiment demonstrates that beetles can detect rust infection throughout the plant without relying on the visible spore patches. The number of individuals that fed only on the healthy leaf also suggests that they are able to discriminate without having to actually feed. Of course it remains uncertain if detection of the rust requires contact with the plant, or if the beetles can do so purely by olfaction.

Adults and larvae of both species also showed larger-scale avoidance of infected plants, leaving significantly more rapidly when placed on them in the field. This behaviour would structure populations by causing individuals to accumulate on healthy plants, and enhances the discrimination against rust-infected plants in the feeding and larviposition preference.

Oreina elongata and *O. cacaliae* had remarkably similar responses to rust infection in their performance and behaviour, with the only difference in their oviposition/larviposition behaviour. *Oreina cacaliae* females showed a strong preference during larviposition, thereby giving their larvae access to healthy leaves. The absence of such discrimination in *O. elongata* probably stems from its oviposition behaviour in the field, where it displays a strong preference for the thistle *C. spinosissimum*, a host which offers the eggs a refuge from predation amongst its spiny leaves (Ballabeni et al. 2001a, 2001b, Verdon et al. 2007). For the other behaviours, since adults and larvae of *O. elongata* move repeatedly between *Adenostyles* and *Cirsium* (Gotthard et al. 2005), they, like *O. cacaliae*, will benefit from means to avoid infected plants. Contact with the rust may invoke a shift to a healthy plant of either genus. *O. elongata* may persist in using *Adenostyles* despite the possibility of encountering the rust because it allows

them to combine the use of enemy-free space for their eggs on *Cirsium*, with access to slightly higher growth rates and sequestered defence chemicals from *Adenostyles* (Ballabeni et al. 2001a, Margraf et al. 2007).

Phytopathogenic fungi can clearly have far-reaching effects on coexisting herbivores. Rust infection has the potential to have an influence at all levels, from individual behaviour through to population dynamics and evolution. Detrimental effects are likely to have repercussions throughout the lifetime of an individual. Shifts in larval growth rates will alter their interactions with predators and parasitoids, while the body weight of larvae at the end of development will affect their overwinter survival and determines their size as an adult, with implications for sexual selection and fecundity (Blanckenhorn 2005). These individual-level effects will sum at larger scales, leading to an influence on the pattern of movement between patches, metapopulation survival, and population dynamics (Hatcher 1995, Laine 2004, Mondy and Corio-Costet 2004). For instance, dispersal and host choice may occur at the patch level, particularly late in the season when entire patches tend to be infected together. There are also likely to be longer-term evolutionary consequences. In addition to selection on feeding behaviour, the presence of the rust may impose selection to avoid the worst of the infection by breeding earlier in the season. Any resulting shift in the life cycle will have further consequences for all ecological relationships of the species. Phytopathogenic fungi therefore have the potential to have a profound impact on herbivorous insects, shaping their interactions with host plants and all aspects of their behaviour, ecology and evolution.

Acknowledgements – We are very grateful to Berndt Reinhard and Brigitte Mauch-Mani for identification of the rust, and to the Ordine Mauriziano and the Association Internationale du Jardin Alpin de la Chanousia who gave us the use of the Old Customs House at the Col du Petit Saint-Bernard. The project was financed by Swiss National Science Foundation grant 31-64864.01.

References

- Aanen, D. K. et al. 2002. The evolution of fungus-growing termites and their mutualistic fungal symbionts. – *Proc. Natl Acad. Sci. USA* 99: 14887–14892.
- Anonymous 2005. S-Plus 7.0 guide to statistics. – Insightful Corp.
- Ballabeni, P. et al. 2001a. Does enemy-free space for eggs contribute to a leaf beetle's oviposition preference for a nutritionally inferior host plant? – *Funct. Ecol.* 15: 318–324.
- Ballabeni, P. et al. 2001b. Spatial proximity between two host plant species influences oviposition and larval distribution in a leaf beetle. – *Oikos* 92: 225–234.

- Batra, L. R. and Batra, S. W. T. 1985. Floral mimicry induced by mummy-berry fungus exploits hosts pollinators as vectors. – *Science* 228: 1011–1013.
- Bisby, G. R. 1920. Short cycle *Uromyces* of North America. – *Bot. Gaz.* 69: 193–217.
- Blanckenhorn, W. U. 2005. Behavioral causes and consequences of sexual size dimorphism. – *Ethology* 111: 977–1016.
- Blanford, S. et al. 2005. Fungal pathogen reduces potential for malaria transmission. – *Science* 308: 1638–1641.
- Bogacheva, I. A. and Khruleva, O. A. 2002. The chrysomelid beetle *Chrysomela collaris*: adaptation to existence in mountains and the Arctic. – *Zool. Zhurnal* 81: 678–685.
- Chapela, I. H. et al. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. – *Science* 266: 1691–1694.
- Danks, H. V. 1992. Long life-cycles in insects. – *Can. Entomol.* 124: 167–187.
- Dobler, S. et al. 1996. Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus *Oreina*. – *Evolution* 50: 2373–2386.
- Farrell, B. D. et al. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). – *Evolution* 55: 2011–2027.
- Fox, G. A. 2001. Failure-time analysis. – In: Scheiner, S. M. and Gurevitch, J. (eds), *Design and analysis of ecological experiments*. Oxford Univ. Press, pp. 235–265.
- Freude, H. et al. 1994. *Die Käfer Mitteleuropas*. Vol. 14. – Goecke & Evers.
- Friedli, J. and Bacher, S. 2001. Mutualistic interaction between a weevil and a rust fungus, two parasites of the weed *Cirsium arvense*. – *Oecologia* 129: 571–576.
- Gotthard, K. et al. 2005. The evolution of larval foraging behaviour in response to host plant variation in a leaf beetle. – *Oikos* 109: 503–512.
- Gross, A. J. and Clark, V. A. 1975. Survival distributions: reliability applications in the biomedical sciences. – John Wiley & Sons.
- Hartmann, T. et al. 1999. Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles. – *J. Insect Physiol.* 45: 1085–1095.
- Hatcher, P. E. 1995. Three-way interactions between plant-pathogenic fungi, herbivorous insects and their host plants. – *Biol. Rev.* 70: 639–694.
- Hatcher, P. E. and Paul, N. D. 2000. Beetle grazing reduces natural infection of *Rumex obtusifolius* by fungal pathogens. – *New Phytol.* 146: 325–333.
- Hatcher, P. E. et al. 1994a. Interactions between *Rumex* spp, herbivores and a rust fungus-*Gastrophysa viridula* grazing reduces subsequent infection by *Uromyces rumicis*. – *Funct. Ecol.* 8: 265–272.
- Hatcher, P. E. et al. 1994b. The effect of a foliar disease (rust) on the development of *Gastrophysa viridula* (Coleoptera, Chrysomelidae). – *Ecol. Entomol.* 19: 349–360.
- Kalberer, N. M. et al. 2005. An alternative hibernation strategy involving sun-exposed ‘hotspots’, dispersal by flight, and host plant finding by olfaction in an alpine leaf beetle. – *Entomol. Exp. Appl.* 114: 189–196.
- Kluth, S. et al. 2001. Interactions between the rust fungus *Puccinia punctiformis* and ectophagous and endophagous insects on creeping thistle. – *J. Appl. Ecol.* 38: 548–556.
- Kluth, S. et al. 2002. Insects as vectors of plant pathogens: mutualistic and antagonistic interactions. – *Oecologia* 133: 193–199.
- Laine, A. L. 2004. A powdery mildew infection on a shared host plant affects the dynamics of the Glanville fritillary butterfly populations. – *Oikos* 107: 329–337.
- Margraf, N. et al. 2003. The growth strategy of an alpine beetle: maximization or individual growth adjustment in relation to seasonal time horizons? – *Funct. Ecol.* 17: 605–610.
- Margraf, N. et al. 2007. Glacial survival and local adaptation in an alpine leaf beetle. – *Mol. Ecol.* 16: 2333–2343.
- Mondy, N. and Corio-Coster, M. F. 2004. Feeding insects with a phytopathogenic fungus influences their diapause and population dynamics. – *Ecol. Entomol.* 29: 711–717.
- Rice, W. R. 1989. Analyzing tables of statistical tests. – *Evolution* 43: 223–225.
- Roy, B. A. 1993. Floral mimicry by a plant pathogen. – *Nature* 362: 56–58.
- Roy, H. E. et al. 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. – *Annu. Rev. Entomol.* 51: 331–357.
- Sinclair, B. J. et al. 2003. Insects at low temperatures: an ecological perspective. – *Trends Ecol. Evol.* 18: 257–262.
- Stout, M. J. et al. 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. – *Annu. Rev. Entomol.* 51: 663–689.
- Takahashi, K. H. et al. 2005. The relative importance of spatial aggregation and resource partitioning on the coexistence of mycophagous insects. – *Oikos* 109: 125–134.
- Verdon, A. et al. 2007. Conserved oviposition preferences in alpine leaf beetle populations despite host shifts and isolation. – *Ecol. Entomol.* 32: 62–69.
- Williams, I. S. 1999. Slow-growth, high-mortality - a general hypothesis, or is it? – *Ecol. Entomol.* 24: 490–495.