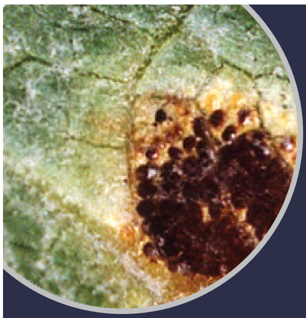

Ecological interactions between two species of leaf beetle, a rust fungus, and their host plant

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Thesis presented the 3rd April 2007 in presence of

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RESUME

Les champignons parasites et les insectes herbivores sont connus pour leur influence négative sur les populations de plantes, affectant leur reproduction, leur croissance, leur survie, et interférant dans leurs relations avec d'autres espèces. En fournissant un logement, une protection et une source de nourriture pour de nombreux organismes, les végétaux représentent un élément essentiel des écosystèmes terrestres dans lesquels ils permettent la rencontre d'organismes aussi différents que des champignons pathogènes et des insectes phytophages. Les relations triangulaires qui naissent de cette proximité peuvent être directes ou indirectes lorsque la plante hôte joue le rôle de médiateur. Les insectes peuvent se nourrir du champignon ou de l'une de ses parties, comme le mycélium ou les structures reproductrices, et de ce fait réduire l'ampleur de l'infection ou de la transmission de la maladie. En revanche, d'autres espèces sont susceptibles de véhiculer des spores infectieuses et d'inoculer de nouvelles plantes. Ici, les champignons et les insectes s'influencent directement, positivement ou négativement, mais leurs relations deviennent indirectes lorsqu'ils engendrent des perturbations chez leur hôte. Une attaque fongique est susceptible de produire des changements dans la qualité de la plante hôte, mais aussi d'y activer des résistances qui peuvent également agir sur les insectes, grâce à des mécanismes de défenses croisés. Ainsi, les plantes participent activement à ces relations en mettant en œuvre des défenses permanentes ou activables, impliquant des structures morphologiques, des substances chimiques internes ou externes, leur phénologie, ou des stratégies de tolérance.

Ce travail est centré sur l'étude des relations directes et indirectes entre la plante *Adenostyles alliariae*, la rouille *Uromyces cacaliae*, et deux chrysomèles alpines *Oreina elongata* et *Oreina cacaliae*. Dans leur environnement naturel, régit par des conditions difficiles, leur relation prend une importance particulière, principalement due à une période d'activité très courte, mais aussi à cause de la stratégie de défense des chrysomèles alpines, impliquant des composés secondaires (pyrrolizidine alcaloïdes) produits par la plante et séquestrés par ces insectes pour leur propre défense. Dans ce contexte, le nombre de conséquences possibles est accru. L'influence de chacun des protagonistes sur les deux autres fut observée à l'aide d'expériences combinées entre le terrain, le laboratoire, et l'analyse de composés chimiques. Quatre sites différents furent choisis au sein les Alpes suisses et italiennes.

Les résultats montrent que des interactions triangulaires influencent nos protagonistes, avec des effets sur leur comportement, leur phénologie, leur cycle de vie, leurs performances, leur distribution et la dynamique de leurs populations. Ces conséquences sont majoritairement négatives et les rares effets positifs ne fournissent pas d'explication valable à l'apparente continuité de ce système. Néanmoins, un mélange de défenses, d'évitement et de tolérance entre les membres de ce système semble être à la base de leur coexistence.

ABSTRACT

Independently, both fungal disease and herbivorous insects are considered to have major impacts on plant populations, affecting growth, survival, and reproduction, as well as modifying their interactions with other species. By providing habitats, protection, and food for numerous species, plants form an essential component of terrestrial ecosystems and constitute the convergent point for interactions between many groups, including plant pathogenic fungi and phytophagous insects. The three-way interactions resulting from this junction may be direct, plant-mediated, or both. Insects can feed on fungal mycelia and reproductive structures, reducing the infection and its transmission, or transport infectious spores to inoculate new plants. Fungi and insects exercise an influence, positive or negative, directly on each other. The relationships can be indirect if attack transforms the host plant, such as when fungal infection induces plant resistance against fungal attack, but in doing so also induces defences against herbivores by cross-effect mechanisms, and produces changes in host plant quality. The plants participate actively through their permanent and induced defences, involving morphological structures, internal, external, and emitted chemical compounds, phenology, or tolerance.

This study focus on the direct and indirect interactions between the host plant *Adenostyles alliariae*, systemic infections of the rust *Uromyces cacaliae*, and attacks by the alpine leaf beetles *Oreina elongata* and *Oreina cacaliae*. In the harsh conditions of their high alpine habitats, their relations are likely to be particularly intense, due to the very short period of activity as well as to the specificity of the defence strategy used by the *Oreina* leaf beetles. The involvement of pyrrolizidine alkaloids (PAs), secondary compounds produced by the plant and sequestered by the leaf beetles for their own defence, increases the potential consequences of these three-way interactions beyond those typically considered. Field trials in four populations across the Swiss and Italian Alps were combined with laboratory experiments and chemical analyses to examine the influence of each protagonist on the others.

The results show that the tripartite interactions affect all three participants, with implications for their behaviour, phenology, life cycle, fitness, population dynamics, and distribution.

Negative impacts seem to prevail, while concrete positive interactions are weak and not sufficient to explain the apparent continuity of this system. Nonetheless, a mix of defences, avoidance and tolerance to the presence of the other members seems to be basis of their coexistence.

CONTENTS

INTRODUCTION

Organisms are open systems that are constantly in interaction with others and with their environment. These relationships are an essential part of ecology, because they rule the distribution and the abundance of living creatures and set up complex and captivating fields of study, with both fundamental and practical implications. If the interactions are a central pivot in ecology, the exhaustive study of the potential connections of one single individual might be theoretically infinite. In this context, the main part of early ecological studies was focused principally on one specific aspect of a species in relation with a biotic or abiotic element. Recent decades have revealed the importance of integrative approaches, where plants often play a key role in ecosystems, as agents of the primary production, providing food, habitats and protection to a significant part of the terrestrial biodiversity (Hairston *et al.* 1960; Lawton 1994; Hector *et al.* 1999). Plants form the main focus for interactions between organisms but at the same time participate actively, reacting as a function of the weather, conditions of stress, attack by pathogens or herbivores, visitation by pollinators, or competition with other plants (Paul 1989; Hatcher *et al.* 1994; Müller-Schärer & Rieger 1998; Pfunder & Roy 2000; Keary & Hatcher 2004). Nowadays, plants, and in particular crops, cannot be considered without considering associated abiotic factors, as well as ravaging insects and their natural enemies. Worldwide, pathogens and insects are known to be among the main pests on plants, reducing growth, reproduction, and survival, and consequently influencing the dynamics and distribution of plant populations, as well as agricultural output (Shah *et al.* 1995; Mack *et al.* 2000). Since pathogens, insects, and plants began to be frequently studied together, new fields concerning the complexity of nature were revealed, including the complex mechanisms of defences of plants. Due to the critical importance of agricultural ecosystems in our civilizations, fewer studies were initially carried out in natural systems, but they have systematically revealed the amazing richness of protagonists, interactions and sometimes trade-offs allowing coexistence even between enemies.

Among all the imaginable relationships and independently of the species and ecosystem involved, interactions can be classified as direct or indirect. If the organisms directly affect each other, as in predation, or parasitism, they are in direct relationships. Indirect interactions are those in which one species indirectly affects another, by modifying or altering its habitat, food, or living conditions. For example, a disease can affect a plant in many ways, which may be reflected in the success of a herbivore sharing the plant (Hatcher 1995). To further complicate the issue, ecosystems include species which act directly on some neighbours and indirectly on others. As such, human activities can be considered to represent a major source of direct and indirect interactions with thousands of living species on Earth. All these relationships create networks of numerous and diversified participants maintaining continuous ecological interactions with each other.

Within these vast networks, tripartite interactions concern groups of three species, associated by their influence on each other during a significant part of their existence. Due to the very high diversity among insects, fungi, and plants, these groups are frequently found in three-way relationships, typically with both direct and indirect contacts.

The insects are principally phytophagous and many thousands of species are able to feed on every part of plants, including roots, stems, wood, leaves, flowers, fruit, seeds, pollen, and nectar. Direct interactions are often obvious. This proximity with plants leads many insects to be vector of diseases, involving infectious fungal material sticking to body parts, or sometimes voluntary transport of the pathogen (Farrell *et al.* 2001). On the other hand, many studies describe insects specialized on a fungal diet, feeding on mycelia, spores or other structures, like the fungus-associated erotylid beetles (Coleoptera: Erotylidae) or other insects that spend their whole life cycle on fungi (Takahashi *et al.* 2005). Insects can also cultivate fungi, like the leaf cutter ants, as their food supply (Chapela *et al.* 1994; Aanen *et al.* 2002).

Fungal species often maintain direct relations with living plants in symbioses, like the mycorhizal fungi, or as diseases. Phytopathogenic fungi, like the rusts, mildews, and oidiums, can cause important agricultural damages, which have already been responsible for major effects on human populations in the past. Pathogens can handicap plant growth, generate early senescence, and cause difficulties in flowering. In addition, fungal infections can lead the plants to produce odours to attract vector herbivores (Connick & French 1991), and some species can simulate flowers to attract pollinator insects and guarantee their dispersion (Batra & Batra 1985; Roy 1993). Some fungal species are associated with insects as parasites (mycosis), or as predators, like the entomopathogenic fungi (Kepler & Bruck 2006; Roy *et al.* 2006).

In plant-insect-fungus interactions, plants suffer mainly direct effects, attacked and infected on both sides. Yet they do not passively suffer these attacks, but can act directly on their antagonists. While plants can show plasticity in their phenology in order to minimize the costs of infestation (Wise & Cummins 2006), their defences are strongly implicated in three-way interactions, involving morphological structures as well as internal and external chemical compounds and volatile substances. These defences generate costs for all the protagonists, leading to weakening, resistance, or tolerance.

Indirect relations between the three protagonists are less explicit and require detailed observations of several parameters, involving development, preferences, behaviours, and fitness, sometimes through several generations. For example, when herbivorous insects feed and lay their eggs (or larvae), the wounds may be used as entry points for spores and infectious fungal structures, indirectly increasing the infection rate.

Some of the most relevant indirect effects are the changes in host plant quality and quantity caused by both phytophagous insects and rust fungi. Rust-infected leaves can show an increase in dry and non-digestible matter (Ahmad *et al.* 1982; Lam 1985), changes in leaf nutrient contents, including carbohydrates (Farrar 1989), nitrogen, secondary plant chemical, mineral elements, fungal metabolites and toxics, and lower photosynthetic capacities (Murray & Walters 1992). On the other hand, insect attack is more typically responsible for a decrease in the quantity of host plant available and high consumption by numerous individuals can affect the survival and life cycle of the pathogen. All these effects are mediated through the host, as are the potential systemic defences of the plant which can be induced either by foliar fungi or insects, but can act sometimes on both attackers due to cross-effect mechanisms (Bostock 1999; Thaler *et al.* 2002a; Thaler *et al.* 2002b). The consequences of these interactions are not always shared, for example the herbivore may have no visible impact on fungal growth, while the fungal infection induces local plant resistance against the herbivore (Rostas & Hilker 2002).

The permanent or induced defences of the plants are closely involved in the three-way interactions, as the means used by the plants to act truly with their antagonists. In nature, plants possess numerous and diversified types of defences, including morphological structures, chemical compounds, and calls to the assistance of allied organisms.

Many plants show special structures acting to slow down or minimize herbivory, like spines and spurs against large animals, or hairs against smaller ones. While reinforced leaves can discourage some insects from feeding, the surfaces of the leaves can be covered with waxes or slippery substances, preventing them from continuing an attack. These substances belong to the formidable chemical compounds produced by the plants and used in defence, like resin and latex secreted after wounding, or internal alkaloids and terpenoids that deter herbivores. Alkaloids, found also in some fungi, are known to be potentially influenced by fungal infection (Tinney *et al.* 1998), and other studies have revealed that such secondary compounds might reduce fungal growth (Hol & Van Veen 2002). These protections may vary in intensity and depend on the state of the plant, but they are permanent. Other kinds of defence can be induced in plants following an attack by a herbivore, a pathogen, or both. In such cases, specific compounds are produced and distributed throughout the whole plant principally in the phloem. Both salicylic acid and jasmonic acid are used as signals to induce systemic defences against attackers. The salicylate pathway was initially observed in the mechanism of defence against pathogens (Mauch-Mani & Metraux 1998), whereas the jasmonate pathway seemed to be activated against herbivory (Thaler *et al.* 2001). Nowadays, these two pathways are known to have cross-effects, and attack by one of the attackers can be sufficient to induce defences against both insects and rust fungi. This cross-talk can be either synergistic or antagonistic, leading to variation in plant defences (Bostock *et al.* 2001; Thaler *et al.* 2002b; Traw *et al.* 2003). Following infestation, potential mechanisms for communication between plants have also been studied, including the emission of volatile compounds (Farmer & Ryan 1990; Staswick *et al.* 1992; Reinbothe *et al.* 1994), and their effects on the natural predators of attackers (Rostas *et al.* 2006; Ton *et al.* 2007). Indeed, the assistance of allied organisms can be crucial in the struggle against antagonists, and many plants offer food and protection to lodge their defenders, like the Bullhorn *Acacia* which lives in symbiosis with *Pseudomyrmex* ants (Janzen 1975; Heil *et al.* 2005). Otherwise, parasitoid wasps are often involved in indirect defences, where they respond to emitted signals and parasite pests (Zhang *et al.* 2004), and similar situations are found below-ground with entomopathogenic nematodes able to destroy root-eaters (Rasmann *et al.* 2005). Again, many of these interactions have been studied in agricultural systems, with much less known about natural ecosystems.

Finally, last decade showed great interest about the three-way interactions in order to use them in biological weed control. Various biological approaches may be used to control weeds, using pathogens, insects, or both. If studies showed positive effect of simultaneous attacks and infection (Friedli & Bacher 2001), many revealed antagonistic reactions when pathogens and herbivores are present at the same time on the target plants (Hatcher *et al.* 1994; Hatcher 1996; Hatcher & Paul 2000; Kluth *et al.* 2001, 2002). Therefore, these systems and their protagonists have to be manipulated, in function of the season, to become efficient.

MATERIAL & METHODS

Study organisms

This study brings a new interaction with a phytopathogenic rust to well known system of alpine plants and associated leaf beetles. The high altitude habitats in Europe cannot be compared with others ecosystems in terms of their biodiversity, but often show remarkable adaptations to survive the harsh conditions. For example, with their life cycle, host plant use, behaviour, and many other adaptations, the leaf beetles *Oreina elongata* and *Oreina cacaliae* (Coleoptera: Chrysomelidae) are considered as permanent and specialized inhabitants of mountainous places, where they feed and breed mainly on the common plant *Adenostyles alliariae* (Asterales: Asteraceae).

This host plant is frequently infected by the phytopathogenic rust *Uromyces cacaliae* DC. Unger (Uredinales: Pucciniaceae), a poorly known member of the second largest genus of rust fungi: *Uromyces*, which occur over a wide geographical range and are parasitic upon widely separated families of hosts (Bisby 1920; Gaumann 1959; Cummins & Hiratsuka 1983). There are several economically important *Uromyces* species, involving bean, pea, beet, chickpea, alfalfa, clover (Cummins & Hiratsuka 1983). *Uromyces cacaliae* is an obligatory pathogen of its host plant *A. alliariae* and produces visible telia on the under side of leaves two to three weeks after the host plants have emerged (Bisby 1920). The identification of this rust was carried out with the help of Berndt Reinhard of the University of Tübingen and Brigitte Mauch-Mani of the University of Neuchâtel.

Fig. 1. *Adenostyles alliariae*

Fig. 2. *Uromyces cacaliae*

Oreina elongata Suffrian is a small (body length 6.5 to 9.5 mm) metallic blue to anthracite beetle, found at altitudes between 1600 m and 2400 m above sea level. Its geographical distribution is patchy, with isolated populations throughout the Alps and further south into the Apennines (Freude *et al.* 1994; Margraf *et al.* 2003; Verdon *et al.* 2007). The life cycle of *O. elongata* is adapted to the high alpine environment and includes a first summer as egg and larvae, 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 (Fig. 4.).

Fig. 3. *Oreina elongata*

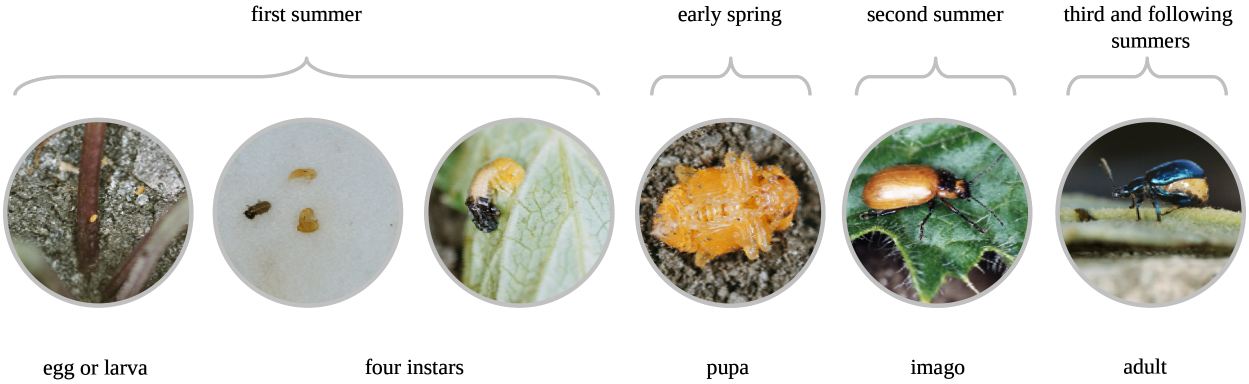

Fig. 4. Life cycle of *Oreina elongata*

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.

Both species feed and reproduce on plant species in the Asteraceae: *Adenostyles alliariae* (Gouan) A. Kerner is the focus of this study. It is a common, perennial, subalpine and alpine plant, found at a maximum altitude of 2800 m growing close to the wetlands of the subalpine stage. The flower heads include three to six pink flowers and its leaves are rather large and appear white due to the fine hairs on the lower surface. It produces secondary compounds, pyrrolizidine alkaloids (PAs) (Hartmann *et al.* 1999). Within the studied populations, both beetle species feed on *A. alliariae*, while the thistle *Cirsium spinosissimum* L. is a host for *O. elongata*, and some individuals of *O. cacaliae* begin the season on *Petasites paradoxus*, because this is one of the first plants available as the snow melts at sun-exposed sites (Kalberer *et al.* 2005).

Species of the genus *Oreina* are notable for their ability to protect themselves against predators with the help of chemical compounds. Both adults and larvae of *O. elongata* have two modes of chemical defence: pyrrolizidine alkaloids (PAs) sequestered from hosts in the genus *Adenostyles*, and autogenously synthesised cardenolides (Dobler et al. 1996). *O. cacaliae* has lost the ability to produce cardenolides and depends exclusively on PA sequestration for defence (Pasteels *et al.* 1992; 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.

Sites

The four sites were selected as a function of the presence of the host plant, the rust, and the leaf beetle species. Only the site of Piccolo San Bernardo shows the presence of both *Oreina* species. This site is near the pass and border between France and Italy, above Lac Verney (Valle d'Aosta, 2053 m). The second population of *O. elongata* is located close to Emosson Dam (Swiss Alps, Valais, 1949 m), and the site shows the longest period covered by snow, mainly due to its exposure to the north and position at the foot of a cliff. The site of Kandersteg (Swiss Alps, Ueschinental, Bern, 1481 m) is the lowest of this study, and shows a small population of *O. cacaliae*. Finally, the site of La Fouly (Swiss Alps, Val Ferret, Valais, 1587 m) is always the first accessible site, due to its open position and southern exposure (Fig. 5.).

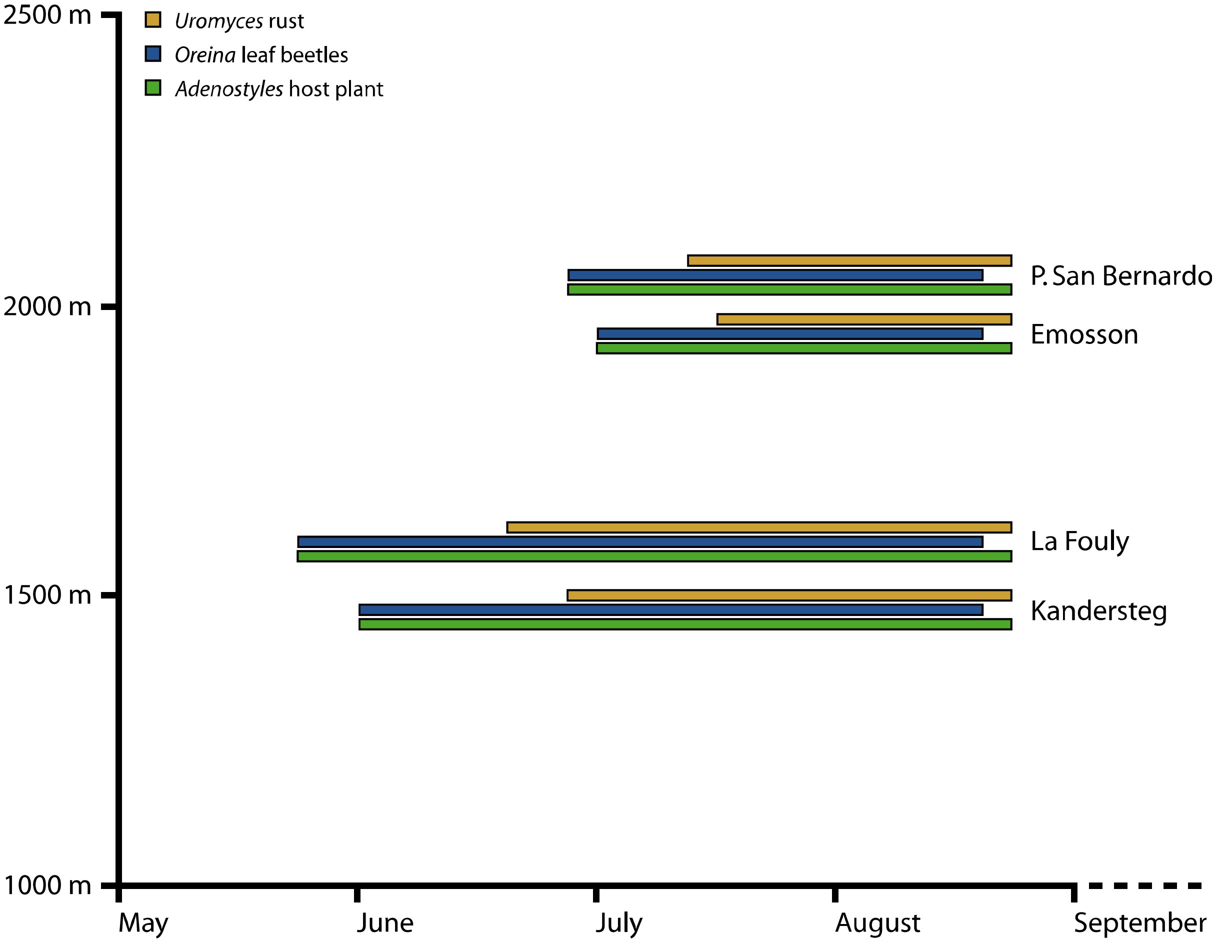

Fig. 5. Calendar of the living organisms in each site

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CHAPTER 1

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, subalpine 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 × 9 × 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 censorReg 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 lnln(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 bin[82, 1/3], pr[X ≥ 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.

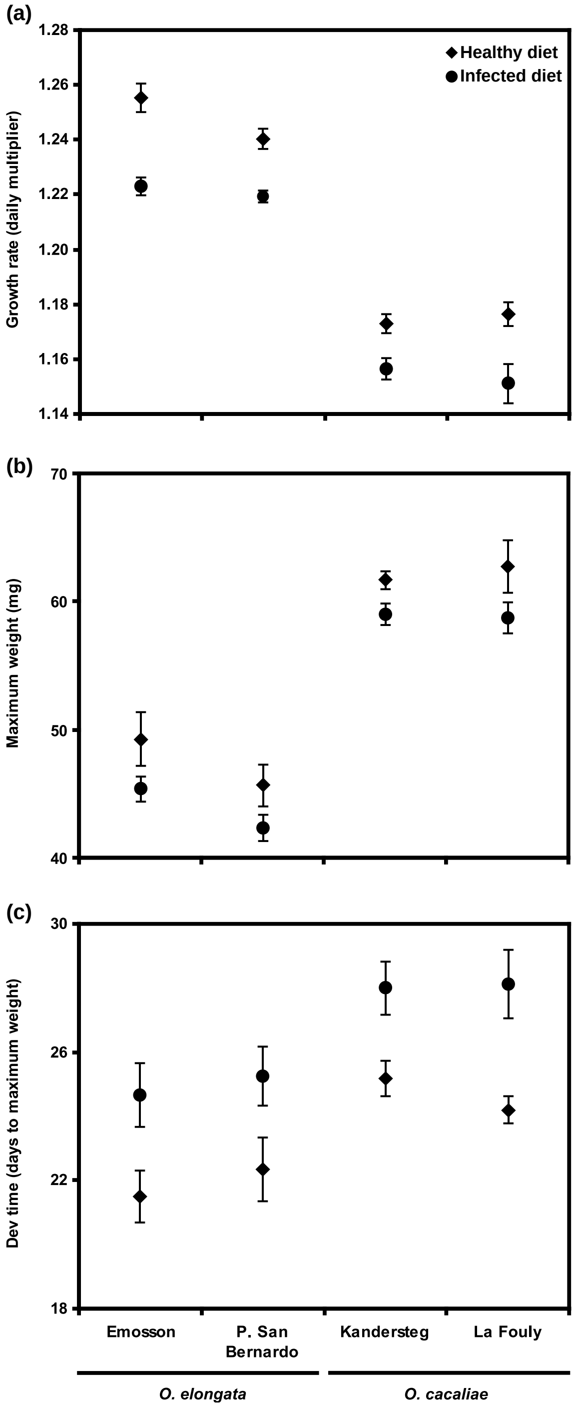

Fig. 1. Larval performance of *O. elongata* (in 2003) and *O. cacaliae* (in 2004) from four populations reared on healthy or rust-infected leaves of the host plant. The three larval parameters studied were (a) the growth rate (calculated as a daily growth multiplier, see text for details), (b) the maximum 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 ± 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)				
O. elongata adults	25	16.644	2	<0.001
O. elongata larvae	30	31.592	2	<0.001
O. cacaliae adults	11	15.297	2	<0.001
O. cacaliae larvae	16	12.473	2	0.002
Feeding preference (4 diets)				
O. elongata adults	16	23.540	3	<0.001
O. elongata larvae	63	104.861	3	<0.001
O. cacaliae adults	32	34.565	3	<0.001
O. cacaliae larvae	45	52.312	3	<0.001

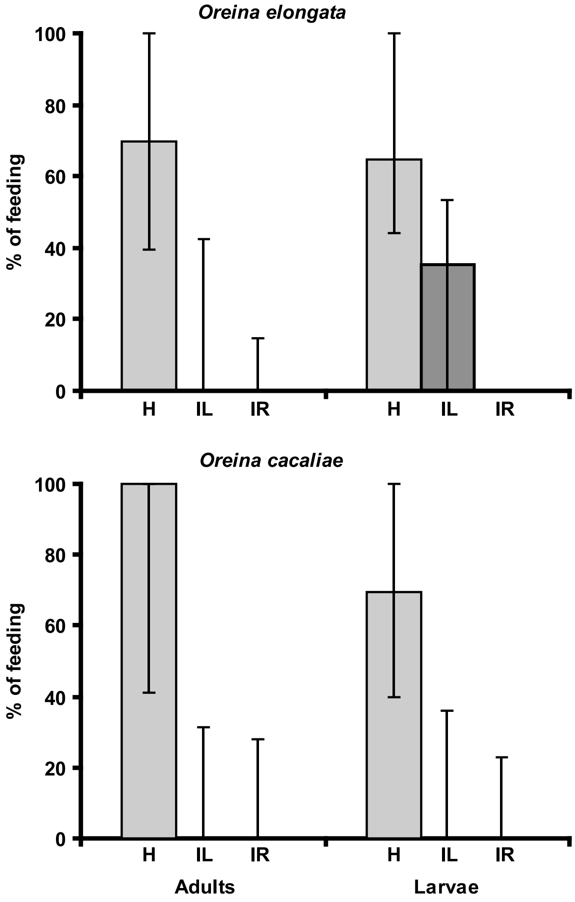

Fig. 2. Feeding preference in the three-choice experiment for adults and larvae of both species. Graphs show the proportion of total feeding on discs from healthy leaves (H), from infected leaves but cut from an area lacking the sporulating rust (IL), or from infected leaves bearing sporulating rust (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
O. elongata adults Z	**−2.972**	**−3.465**	−1.193
p-value	**0.003**	**0.001**	0.233
O. elongata larvae Z	−2.597	**−4.617**	**−3.519**
p-value	0.009	**<0.001**	**<0.001**
O. cacaliae adults Z	−2.287	**−2.869**	−0.944
p-value	0.022	**0.004**	0.345
O. cacaliae larvae Z	−2.080	−2.642	−0.664
p-value	0.038	0.008	0.507

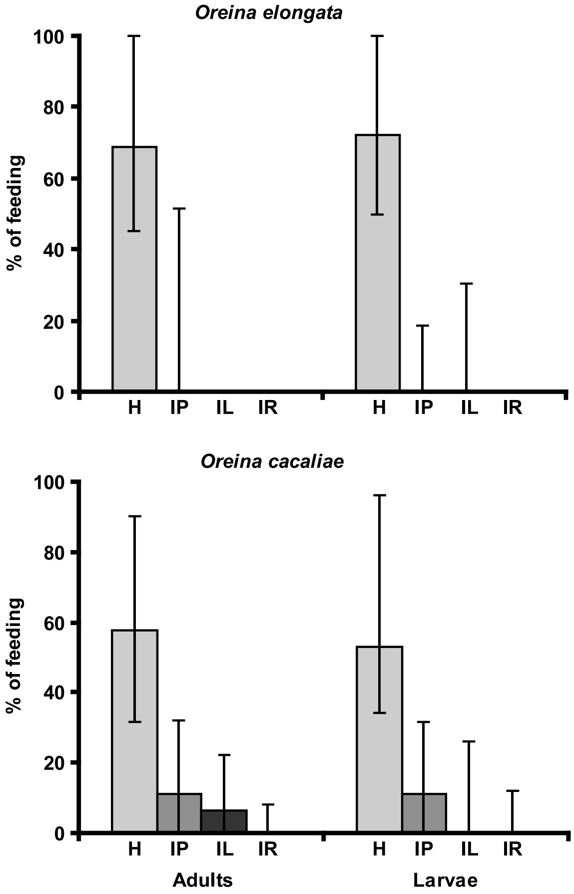

Fig. 3. Feeding preference in the four-choice experiment. Details as in Fig. 2, with the addition of a fourth choice, a leaf disc taken from an uninfected upper leaf on an infected plant (IP).

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
O. elongata 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
O. elongata 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**
O. cacaliae 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
O. cacaliae 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 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.

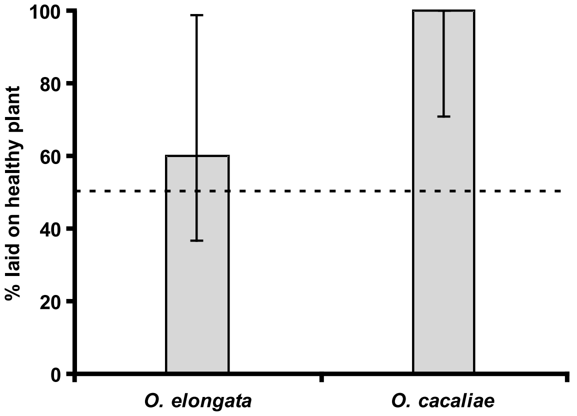

Fig. 4. Oviposition (*O. elongata*) and larviposition (*O. cacaliae*) preferences, showing the median proportions laid on healthy leaves. Error bars give the 25th and 75th percentiles.

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

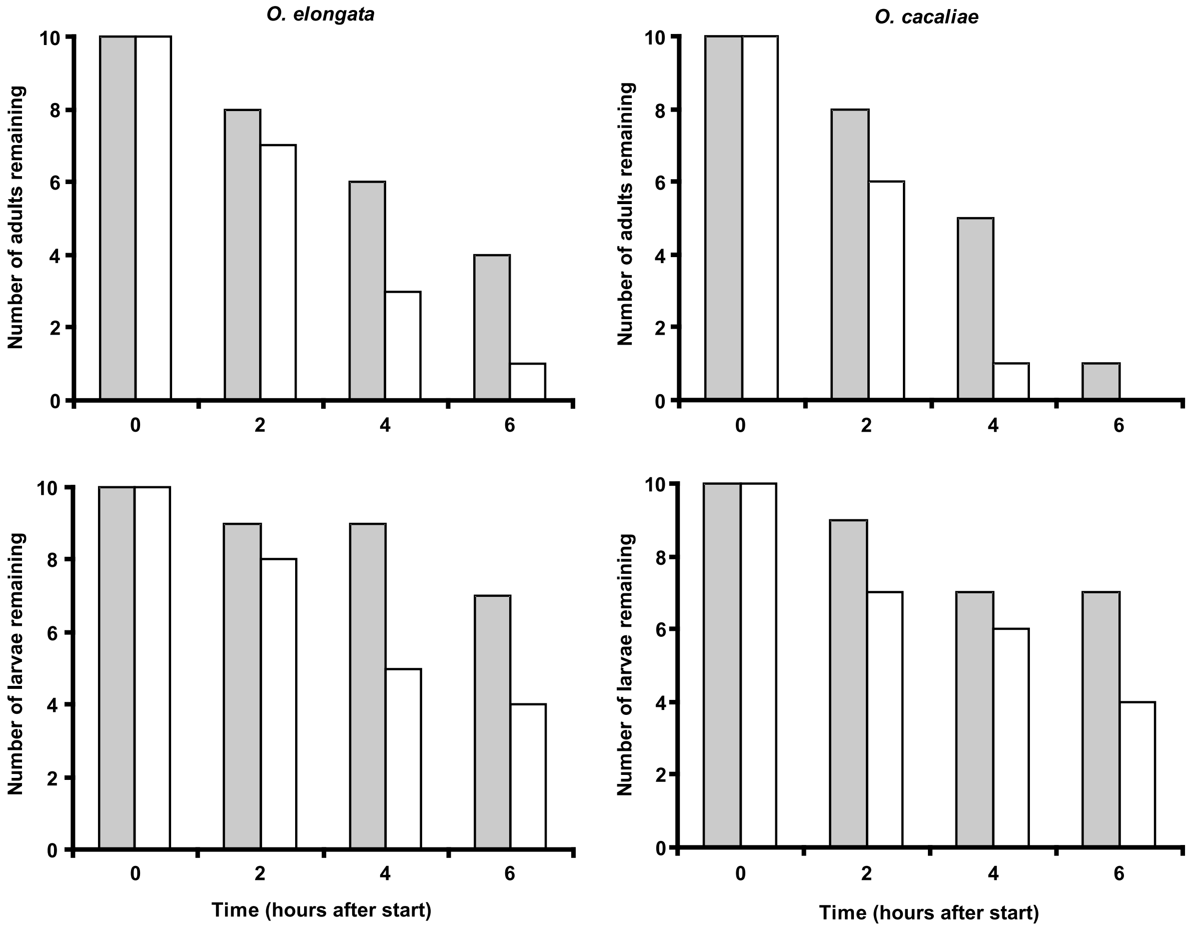

Fig. 5. Dispersal behaviour in the field. Bars show the number of adults or larvae still remaining two, four and six h after having been placed individually on healthy (shaded bars) or rust-infected plants (open bars).

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 × 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 × plant	6	193.149	0.062	1	0.803
Stage × plant	7	193.037	0.112	1	0.738
Species × stage	8	192.209	0.828	1	0.363
Species × stage × 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.

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CHAPTER 2

How should juveniles react to low quality food? A paradoxical response by two species of alpine leaf beetle

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Keywords: phenotypic plasticity, life-history traits, trade-off, Chrysomelidae, *Oreina*, *Uromyces cacaliae*, rust fungus, *Adenostyles alliariae,* host-plant quality, growth rate.

Abstract

Ranking of hosts by their quality plays a central role in research on the evolution of host-plant specialisation and speciation in insects. Here we provide a counter-intuitive example in which growth is faster on poor quality hosts. The alpine leaf beetles *Oreina elongata* and *O. cacaliae* share their host plant with the rust *Uromyces cacaliae.* Larvae reared on infected *Adenostyles alliariae* show reduced growth rate, reduced maximum weight, and longer development time. However, they normally respond in an adaptive manner to the rust's arrival. When switched from healthy to infected leaves, larvae accelerate growth and reduce development time, but pupate at lower body weight. This represents a novel plant-insect-fungus interaction in which infection forms the cue to trade-off developmental parameters to complete growth within the short alpine summer. It is also a novel mode of developmental plasticity, which leads to a paradoxical negative correlation between growth rate and host-plant quality.

Introduction

Not all food is equal. For phytophagous insects, plants differ in quality as a source of food, both when comparing species and at the intraspecific level (Jaenike 1990). The variation is typically manifested as differences in the rate of growth on different hosts, with reduced growth on poor quality hosts. This effect on growth is, after all, how we can define food as high or low quality from the point of view of the organism. Our ability to rank plants in this way plays a critical role in research on the evolution of host-plant specialisation, host-race formation, and speciation (Jaenike 1990; Berlocher & Feder 2002; Dres & Mallet 2002; Rundle & Nosil 2005).

There is, however, another possible reaction to low quality food. Many species do not grow at their maximum possible rate under normal conditions. This is despite the theoretical advantages of a shortened juvenile period and increased adult body size, and is thought to arise as a result of the developmental costs of rapid growth (Stearns 1992; Arendt 1997; Nylin & Gotthard 1998; Metcalfe & Monaghan 2001; Roff 2002). Whatever the reason, this leaves open the possibility to accelerate growth under various environmental cues such as photoperiod, in order to complete development after a delay or for larvae produced late in the season (Nylin & Gotthard 1998; Metcalfe & Monaghan 2001). Food quality provides another possible cue, particularly in species such as parasites, parasitoids or many phytophagous insects, where the ovipositing female chooses at the host, plant or patch level the only food source available to larvae. Escape from deteriorating conditions is impossible, and exposure of larvae to poor quality food can indicate that their circumstances will only get worse from that point onwards. An adaptive response may therefore be to accelerate development, producing a paradoxical negative relationship between food quality and growth rate.

Here we report exactly this behaviour in two species of alpine leaf beetle in response to rust attack of their host plant. The beetles, *Oreina elongata* Suffrian and *O. cacaliae* Schrank share their host plant, *Adenostyles alliariae* (Gouan) A. Kern., with the rust fungus *Uromyces cacaliae* (DC.) Unger. The life cycles of all four species are tightly bound in their high altitude alpine environments, where they have only two to three months each year when the habitat is free of snow. Rust infection of the host plant has a negative effect on larval growth in both beetle species (Röder *et al.* 2007). However, in the field the rust is not present for the entire growth period, but becomes more common as the season progresses. We test how larvae respond to this situation, comparing their performance on healthy, infected and switched diets (changing from healthy to infected leaves part-way through development). This reveals a novel three-way interaction in which the beetles seem to use the arrival of the rust fungus as a signal that the season is drawing to a close and are able to accelerate their growth. The diet treatment was then combined with a photoperiod treatment mimicking early and late season conditions, which is already known to affect larval development (Margraf *et al.* 2003), in order to explain the difference between the infected and switched diets. The results demonstrate a highly flexible form of developmental plasticity in which larvae are able to modify their pre-diapause strategy in response to either cue. The response to food quality is distinct from compensatory (or catch-up) growth, in which individuals grow rapidly when conditions improve after a period of nutritional deficit (Metcalfe & Monaghan 2001), and also differs from previous demonstrations of plasticity in reaction to photoperiod (Nylin & Gotthard 1998). It represents a novel phenomenon in which growth is accelerated while still exposed to the harsh conditions, in order to reach a critical developmental stage for over-wintering. As a result of this plasticity, larval growth rate cannot be considered a good indicator of host plant quality.

Methods

Study organisms

Oreina elongata and *Oreina cacaliae* are closely related, metallic blue leaf beetles found exclusively in mountain environments (Lohse & Lucht 1994). *O. cacaliae* is the larger of the two species and has a wider geographical and altitudinal range, with isolated populations at altitudes of 800 to 2300 m across the European mountains, whereas *O. elongata* is restricted to the Alps and Apennines in habitats between 1600 m and 2400 m above sea level (Lohse & Lucht 1994; Margraf *et al.* 2007). In addition, the species differ in their mode of reproduction, host-plant use and chemical defence. *O. elongata* feeds on three species of *Adenostyles* and on the thistle *Cirsium spinosissimum*, tending to lay its eggs on *Cirsium* where available (Ballabeni *et al.* 2001; Verdon *et al.* 2007), whereas females of *O. cacaliae* deposit larvae directly on the host plant, *A. alliariae*. Leaf beetles of the genus *Oreina* are well known for their chemical defence, in the form of pyrrolizidine alkaloids (PAs) sequestered from the host plant and cardenolides synthesized autogenously (Dobler *et al.* 1996). *O. elongata* can protect itself with both methods, whereas *O. cacaliae* is not able to produce cardenolides (Dobler *et al.* 1996). In the studied populations, *O. cacaliae* can be qualified as a specialist that spends the whole reproductive season on *A. alliariae*, while the adults and larvae of *O. elongata* move repeatedly between *A. alliariae* and *C. spinosissimum*, a host that is not attacked by the rust (Gotthard *et al.* 2005). Both species face extremely short reproductive seasons of just two to three months while their habitats are free of snow, and have extended life cycles. They hibernate as larvae at the end of their first summer, pupate within the soil in spring, and spend a second summer as a non-reproductive adult before producing offspring only in the following year.

Mature adults of the two species were collected from four sites across the Alps: *O. elongata* from the dam at Emosson (Swiss Alps, Valais, 46° 03' 55.1" N, 06° 55' 24.9" E, altitude 1949 m), and from the Piccolo San Bernardo Pass on the border between France and Italy (Italian Alps, Valle d'Aosta, 45° 41' 40.7" N, 06° 53' 05.9" E, altitude 2053 m); and *O. cacaliae* from Kandersteg (Swiss Alps, Ueschinental, Bern, 46° 28' 26.7" N, 07° 38' 52.7" E, altitude 1481 m) and La Fouly (Swiss Alps, Val Ferret, Valais, 45° 56' 11.2" N, 07° 05' 38.1" E, altitude 1587 m). For each population about fifteen individuals of each sex were gathered during the first week of June or in early July as a function of altitude. Adults were housed in 19x9x8 cm plastic boxes with holes for aeration, and kept at the

University of Neuchâtel (Switzerland) in a cooled incubator (Model 250P, LMS Ltd, Sevenoaks, UK) with a fixed 12:30 h day-length and constant temperature of 6^{o}C. Every two days the food was changed, the boxes cleaned, and the eggs (*O. elongata*) or first instar larvae (*O. cacaliae*) removed.

Leaves of *Adenostyles alliariae* were obtained directly from natural populations. This plant is a common, perennial, subalpine and alpine plant, found at a maximum altitude of 2800 m growing on damp soils. It produces secondary compounds, PAs, as a defence against herbivory (Hartmann *et al.* 1999). *A. alliariae* is the exclusive host of *Uromyces cacaliae* (DC.) Unger, a rust that produces spores from patches (telia) on the underside of leaves around two weeks after infection (Bisby 1920). Healthy and naturally rust-infected leaves (with visible telia) were brought back twice weekly from La Fouly or Emosson, where fungal infections appear respectively in mid-June and mid-July (as a result of differences in altitude and exposure). The leaves were transported in a cooled box and kept in a dark, slightly humid fridge at 6^{o}C for a maximum of two days until used.

Larval growth on healthy, rust-infected or switched diets

For two populations each of *O. elongata* and *O. cacaliae,* larval growth rate, maximum weight and development time were compared when reared on healthy or rust-infected leaves, and on a diet switched mid-season from healthy to rust-infected leaves. The experiment was begun with newly hatched (*O. elongata*) or laid (*O. cacaliae*) larvae. They were reared individually in Petri dishes (5.5 cm diameter, 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 the three diets: healthy, infected with visible fungal telia, or healthy initially but then switched to rust-infected leaves after eight days. 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 (diameter 9.5 cm, depth 4.5 cm) with a layer of damp soil, where they were fed and weighed until they buried into the soil to hibernate. Experiments were carried out at the University of Neuchâtel in a cooled incubator maintained with 15:00 h day-length and temperatures varying gradually from 6.5°C (night) to 20.0°C (day). Six (*O. elongata*) or five (*O. cacaliae*) larvae of each population were reared on each diet.

Growth rate was calculated as a daily growth multiplier, estimated for two separate periods for larvae under all treatments (pre-switch, from the start until day eight; and post-switch, from day eight until the maximum weight). For each larva, the successive weights 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 0.765 and 1). 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 (in mg) was used for analysis. Development time was taken as the number of days needed to reach this maximum weight. The two growth rates, the maximum weight, and the 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. Where terms with more than two levels were significant, Tukey's HSD tests were used to determine where the differences lay. Only the post-switch growth rate required a transformation (reciprocal) to make the data conform to assumptions of normality and homogeneity of variation.

Larval growth under different diets and day-length regimes

Larval growth rate, maximum weight and development time were compared on the same three diet treatments but under two different day-length regimes. The experiment was performed with larvae from the two populations of *O. elongata* only. Trials began with 10 larvae of each population on each combination of diet and day-length regime. The larvae were reared as described above, except they were assigned randomly at the start to one of two incubators running different day-length conditions. The first incubator provided the day-length regime "early", with conditions of 5 July at the beginning of the experiment (15:36 h day-length with temperatures oscillating between 7^{o}C at night and 17^{o}C during the day). Temperatures stayed the same during the entire experiment but the light period decreased each day by 1 to 3 minutes. The second incubator simulated the day-length regime "late", with initial photoperiod typical of 14 August (14:12 h day-length) and a decrease of 3 to 4 minutes per day. Temperatures were identical to those in incubator one. The information on day-length was obtained from the meteorological station of the city of Sion (Swiss Alps, Valais).

Growth rate was again calculated as a daily growth multiplier separately for the pre-switch and post-switch periods (giving linear regressions with r^2 values of between 0.763 and 1), and maximum weight and development time were measured as described above. The two growth rates, maximum weight, and development time were analysed in separate ANOVAs with terms for day-length, diet, population, and all the two- and three-way interactions. No transformation was needed to make the data conform to assumptions of normality and homogeneity of variation. All statistical analyses were carried out using JMP 7.0 (SAS Institute, NC, USA).

Results

Larval growth on healthy, rust-infected or switched diets

In the pre-switch period, there was a significant effect of diet on growth rate (Table 1). Larvae reared on healthy leaves and on the switched diet (given healthy leaves up to that point) did not differ in Tukey's HSD tests, but they both grew more rapidly than larvae on the rust-infected diet (Fig. 1). There were also significant differences between the two species (with faster growth in *O. elongata*) and between the two populations in *O. elongata*. The interaction terms were not significant.

In the post-switch period, there was again a significant effect of diet on growth rate. Overall, larvae reared on the switched diet (now feeding on rust-infected leaves) grew more rapidly than those on the healthy diet (Fig. 1). Larvae on the rust-infected diet had the lowest growth rate. The species differed (with faster growth in *O. elongata*), and the species by diet interaction was also significant because of overlap between the growth rate of *O. cacaliae* on the switched diet and *O. elongata* on the healthy and rust-infected diets.

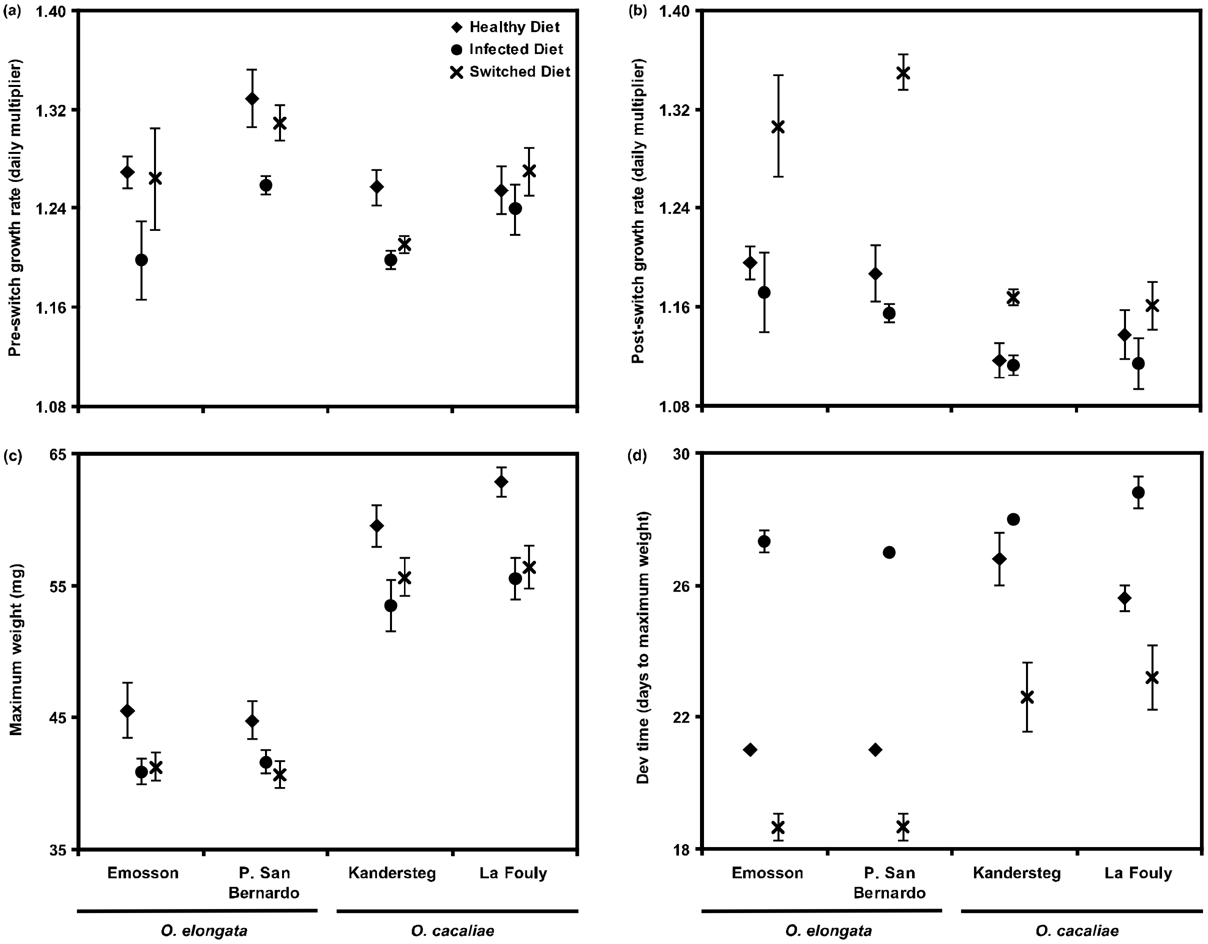

Fig. 1. Larval performance of *O. elongata* and *O. cacaliae* from two populations reared on healthy, rust-infected, or switched diets. The four larval parameters studied were the (a) pre-switch and (b) post-switch growth rates (calculated as daily growth multipliers, see text for details), (c) the maximum weight (in mg) reached by the larvae during their development, and (d) the number of days needed to reach the maximum weight. Graphs show means ± standard errors.

There was a significant effect of diet on the maximum weight reached by larvae (Table 1). Larvae on the healthy diet reached a significantly greater weight, whilst those on the rust-infected and switched diets did not differ from each other (Fig. 1). There was also a difference between the species, with heavier larvae in *O. cacaliae*.

The development time of larvae depended on their diet, with significantly shorter development in larvae on the healthy diet than in those reared on rust-infected leaves, but the most rapid development in those on the switched diet (Table 1 and Fig. 1). The species differed significantly, with longer development time in *O. cacaliae*. The species by diet interaction was also significant, because of overlap between the development time of *O. elongata* on the infected diet and *O. cacaliae* on healthy leaves.

Source	DF	SS	F	P value
pre-switch daily growth multiplier				
species	1	0.0180	6.934	0.011
diet	2	0.0343	6.604	0.003
pop[species]	2	0.0357	6.867	0.002
diet*species	2	0.0046	0.876	0.422
diet*pop[species]	4	0.0055	0.525	0.718
error	54	0.1403		
post-switch daily growth multiplier				
species	1	0.0664	206.780	<0.001
diet	2	0.0638	99.283	<0.001
pop[species]	2	0.0002	0.303	0.740
diet*species	2	0.0143	22.213	<0.001
diet*pop[species]	4	0.0031	2.420	0.060
error	54			
maximum weight				
species	1	3578.105	318.679	<0.001
diet	2	365.566	16.279	<0.001
pop[species]	2	31.635	1.409	0.253
diet*species	2	21.694	0.966	0.387
diet*pop[species]	4	12.161	0.271	0.896
error	54	606.308		
development time				
species	1	206.869	149.344	<0.001
diet	2	541.337	195.403	<0.001
pop[species]	2	0.144	0.052	0.949
diet*species	2	46.671	16.846	<0.001
diet*pop[species]	4	6.289	1.135	0.350
error	54	74.800		

Table 1. ANOVAs on the four larval performance parameters for the two populations of *O. elongata* and *O. cacaliae* when reared on healthy, infected, or switched diets. The population and population by diet terms were nested within species.

Larval growth under different diets and day-length regimes

As in the first experiment, there was a significant effect of diet on growth rate during the pre-switch period (Table 2). Growth rates on the healthy and switched diets were similar, and more rapid than those on the rust-infected diet (Fig. 2). There was also an overall effect of day-length, with faster growth under the conditions of late season photoperiod, and a difference between populations, with faster growth in larvae from Piccolo San Bernardo. The interaction between population and day-length was significant, with the difference between day-length treatments being significant for larvae from Emosson but not for those from Piccolo San Bernardo.

In the post-switch period there was a significant effect of diet, consistent with that in the first experiment (Table 2). Larvae grew more rapidly on the switched diet than on the healthy diet, and both grew more rapidly than those reared on the rust-infected leaves (Fig. 2). There was an overall effect of day-length, with faster growth under the late season photoperiod, and a difference between populations, now with faster growth in larvae from Emosson. The diet by day-length interaction was also significant. Within each day-length regime the diets differed significantly: larvae reared on the switched diet grew more rapidly than those on the healthy diet, and those on infected leaves more slowly. However, the effect of day-length regime differed within the three diet treatments, with larvae on the late season regime growing more rapidly than the early season regime when on the healthy diet, but with no significant difference between day-length regimes when on the switched and rust-infected diets.

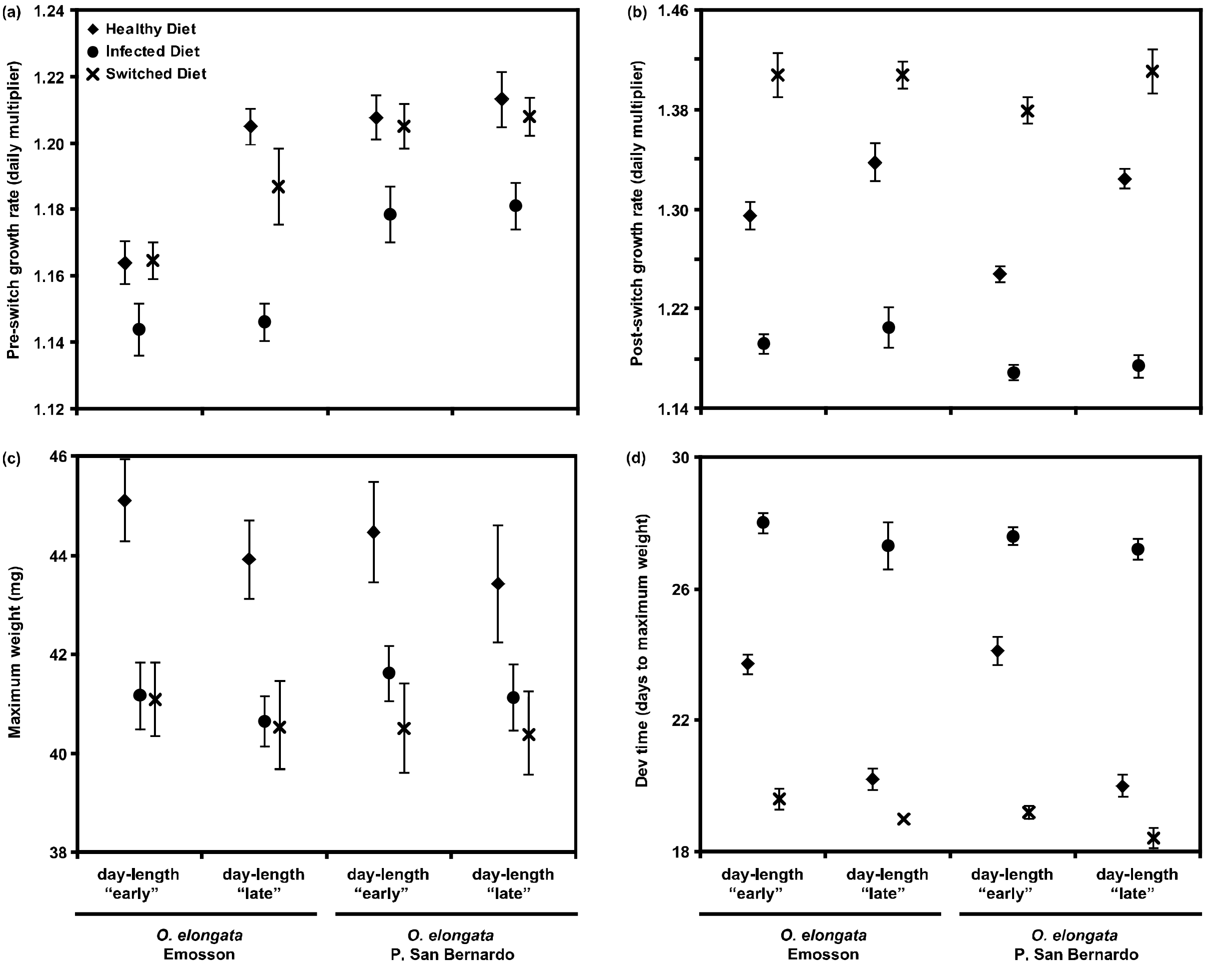

Fig. 2. Larval performance of *O. elongata* from two populations reared on healthy, rust-infected, or switched leaves of the host plant and under two different day-length regimes (early and late summer photoperiod). The four larval parameters studied were the (a) pre-switch and (b) post-switch growth rates (calculated as daily growth multipliers, see text for details), (c) the maximum weight (in mg) reached by the larvae during their development, and (d) the number of days needed to reach the maximum weight. Graphs show means ± standard errors.

For maximum weight only the diet term was significant, with equal weights on infected and switched diets and a greater larval weight on the healthy diet, as in the first experiment (Table 2 and Fig. 2).

Development time was affected by both diet (with shorter development on the switched than on the healthy diet, and longest on the infected diet) and by day-length regime (with shorter development under late season than under early season photoperiod). The diet by day-length interaction was also significant. Within each day-length regime the three diets differed, with significantly shorter development on the switched diet and significantly longer development on the infected diet, both in comparison with the healthy diet. The interaction was seen when comparing day-length regimes within each diet, with development significantly shorter under late than early regimes on the healthy diet, but with no difference between the two day-length regimes for larvae on the switched and infected diets.

Source	DF	SS	F	P value
pre-switch daily growth multiplier				
daylength	1	0.0049	9.143	0.003
diet	2	0.0281	26.374	<0.001
pop	1	0.0279	52.486	<0.001
diet*daylength	2	0.0022	2.059	0.133
pop*daylength	1	0.0025	4.740	0.032
diet*pop	2	0.0004	0.378	0.686
diet*pop*daylength	2	0.0016	1.510	0.226
error	108	0.0575		
post-switch daily growth multiplier				
daylength	1	0.0240	16.173	<0.001
diet	2	0.9372	315.209	<0.001
pop	1	0.0163	10.979	0.001
diet*daylength	2	0.0151	5.077	0.008
pop*daylength	1	0.0027	1.844	0.177
diet*pop	2	0.0017	0.580	0.562
diet*pop*daylength	2	0.0026	0.889	0.414
error	108	0.1606		
maximum weight				
daylength	1	12.4808	1.847	0.177
diet	2	302.1112	22.350	<0.001
pop	1	0.7208	0.107	0.745
diet*daylength	2	3.4145	0.253	0.777
pop*daylength	1	0.3101	0.046	0.831
diet*pop	2	5.9465	0.440	0.645
diet*pop*daylength	2	0.1972	0.015	0.986
error	108	729.9490		
development time				
daylength	1	85.0083	68.978	<0.001
diet	2	1480.7167	600.742	<0.001
pop	1	1.4083	1.143	0.288
diet*daylength	2	67.3167	27.311	<0.001
pop*daylength	1	0.2083	0.169	0.682
diet*pop	2	1.8167	0.737	0.481
diet*pop*daylength	2	1.0167	0.413	0.663
error	108	133.1000		

Table 2. ANOVAs on the four larval performance parameters for the two populations of *O. elongata* when reared on healthy, infected or switched diets, and under early or late season photoperiods.

Discussion

High altitude environments in temperate areas provide harsh conditions for insect life, and the arrival of a fungal antagonist adds another challenge. Here we show that *Oreina* leaf beetles possess highly flexible responses during their larval period to deal with changing hazards under the extreme time constraints of the alpine summer.

Infection by *Uromyces cacaliae* clearly reduces the quality of *Adenostyles alliariae* as a host plant (Röder *et al.* 2007). In the first experiment, larvae of both species reared on rust-infected leaves showed reduced growth rates (the daily multiplier over the whole growth period was reduced by 2.8% on average), lower maximum weights (reduced by 9.9%), and longer development times (increased by 17.7%). This negative effect of the rust may be a direct result of chemical compounds produced by the fungus, or an indirect consequence of changes to host chemistry or the induction of defence in the plant (Hatcher 1995; Röder *et al.* 2007).

Remarkably, switching larvae from the healthy diet to rust-infected leaves increased their growth rate. In comparison with those on a healthy diet, larvae of both species reared on the switched diet showed higher growth rates (by 7.1% in the post-switch period), lower maximum weights (by 8.8%, not significantly different from that on the infected diet), and shorter development times (by 11.9%). This switched diet was designed to mimic the natural situation as rust infection develops during the season. The earliest larvae of the summer may complete their development on rust-free plants, but most witness the arrival of the rust during their growth period. Our result suggests that they use the sudden arrival of the rust as a signal that the season is drawing to a close and that conditions will continue to deteriorate. This induces a rapid change in their pre-diapause strategy, causing them to increase their growth rate, but also shorten the development period at the expense of their final weight, to complete the larval period before the rust infection worsens or the first snow falls.

Oreina elongata is known to be able to adjust individual growth in relation to seasonal time horizons (Margraf *et al.* 2003). When reared under a photoperiod typical of conditions late in the summer season they show an increased growth rate, reduced development time, and normal final weight. This ability was used in our second experiment in an attempt to explain the dramatic difference between the behaviour of larvae on the infected and switched diets. Larvae of *O. elongata* were reared under the three diet treatments crossed with two day-length regimes, typical of early and late in the breeding season. Within each day-length regime the larvae showed the same pattern as in the first experiment, with an increased growth rate and reduced development time on the switched diet, the opposite result on the infected diet, and reduced maximum weight on both those diets. The comparison between the two day-length regimes within each diet treatment revealed a significant increase in growth rate and decrease in development time in response to the late season photoperiod when on the healthy diet, but no effect on the switched or infected diets.

Taken together, the experiments suggest several conclusions. Firstly, as has been suggested before (Margraf *et al.* 2003), larvae reared on healthy leaves do not grow at their maximal rate. This suggests the existence of costs of rapid growth, leading to stabilising selection on growth rate, and indirectly on body size. It also leaves room for acceleration in larvae developing late in the season, after weather-induced delays, or when encountering rust infection. Secondly, the difference between the infected and switched diets may arise because larvae reared from the day of hatching on infected leaves are unable to accelerate their growth. Larvae on the infected diet showed no response to photoperiod, suggesting that their exposure to the rust from the start of life left them physically unable to modify growth rates in response to the normal stimuli. Further work is needed to determine if acceleration of growth is achieved by increasing consumption or by physiological means, but the difference between these two diets suggests the latter mechanism is perhaps more likely. Finally, the response to the arrival of the rust in the switched diet is more dramatic than that to late season light conditions. Larvae on the switched diet already seem to be growing at their maximum rate and show no response to photoperiod, whereas larvae on the late season day-length regime still show a significant increase in growth rate on the switched diet.

The three growth parameters are related and the larvae seem to trade-off one against another, and do so differently depending on the conditions. On the infected diet the larvae appear to compensate for their reduced growth rate by enduring a longer development time in order to reduce the impact on their maximum weight. In contrast, larvae reared on the switched diet use their accelerated growth to produce a short development time at the expense of a reduced maximum weight. The fact that maximum weight is similar on the two diets suggests there may be a minimal weight that would allow hibernation and pupation. On the infected diet larvae must extend the development period in order to reach this weight, while on the switched diet they can stop growth at that point in order to shorten the development time. The response to late season photoperiod is less extreme, with accelerated growth and shortened development, but no effect on maximum weight. Perturbations of their normal final weight and development time are both likely to bear costs for larvae. Slower development may affect larval survival by exposing them to predation and bad weather for a longer period, whereas a reduction in larval weight is likely to lead to higher mortality during the winter, as well as to affect future reproductive success by reducing adult body size (Williams 1999; Fordyce & Shapiro 2003; Laine 2004; Blanckenhorn 2005). Larvae must therefore strike a delicate balance between the two life history parameters, and the optimal trade-off may differ according to the perceived threat to future larval development posed by the onset of rust infection or the advancing season.

The apparent trade-off between development time and final weight, and the negative correlation between larval growth rate and final weight is seen at many levels within the genus *Oreina*. It is seen here in the comparison between the species, with higher growth rate, shorter development time, and lower weight in *O. elongata* than in *O. cacaliae*. It is also seen in comparisons between populations within *O. elongata*, where those from Bosco Gurin and Mattmark in Switzerland show this same suite of changes in the three traits in comparison with other populations (Margraf *et al.* 2007). And it arises in the form of developmental plasticity in the response to the switched diet. It may represent a fundamental survival-fecundity trade-off, with future fecundity (probably related to body size) sacrificed in order to complete development within the extremely short alpine summer.

Both *O. elongata* and *O. cacaliae* suffer negative effects when reared on rust-infected leaves, and they show a suite of behaviours in their dispersal and feeding preference that would allow them to avoid infected plants (Röder *et al.* 2007). However, widespread infection of their host-plant patch or development late in the season will often leave them with no alternative but to feed on rust-infected hosts. The plasticity of growth rate that we demonstrate here may then allow them to cope with the presence of a fungal antagonist and successfully complete development. More generally, the result warns against the assumption of a simple correlation between host quality and larval growth rate, and demonstrates that poor quality hosts may paradoxically lead to rapid development.

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CHAPTER 3

Does alpine leaf beetle attack affect the probability of rust infection, and do they both cause changes in leaf chemistry and performance in the host plant *Adenostyles alliariae*?

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Abstract

Plants, by providing habitats, protection, and food, play a pivotal role in the interactions between many organisms. The three-way interactions between herbivorous insects, phytopathogenic fungi and their host plants are known to generate broad consequences for the ecology and dynamics of all the protagonists. Under infection and attack, plants suffer mainly direct impacts, but go on to modify conditions for the two other parties.

This study aims to evaluate the consequences for the host plant, *Adenostyles alliariae,* of attack by the alpine leaf beetles *Oreina elongata* and *O. cacaliae* and of infection by the rust *Uromyces cacaliae*. Overall, the growth and flowering of rust-infected plants were reduced. The changes in carbon, hydrogen, nitrogen, and pyrrolizidine alkaloid (PA) concentrations, as well as in the C/N ratios through time, were similar in healthy and infested plants, suggesting little effect of attack on host plant chemistry. Earlier findings of poor larval performance on rust-infected hosts may therefore be a result of fungal metabolites or induced defences rather than simple shifts in the concentrations of elements in the plant. The probability of infection by the rust did not show any relationship with previous attacks by the leaf beetles or with the concentrations of elements or PAs. Used in the defences of the host plant, the PAs were related to carbon levels, which may be a product of the investment strategy of the plant. All these results suggest that there are some costs to *A. alliariae* host plants of attack by rust and leaf beetles, and that the three protagonists participate in a complex web of direct and indirect interactions.

Introduction

Plants play a fundamental role in terrestrial environments, providing the link between the sun's energy, the mineral environment and the biological community (Crooks 2002; Zavaleta *et al.* 2003). From the point of view of many bacteria, animals, fungi, and even other plants, they provide habitat, protection, hunting areas, and food supplies, and consequently diversified contact points between living creatures. This sets up a network of direct and indirect interactions, with far-reaching consequences for the ecology and evolution of all the protagonists (Strauss 1991; Lill & Marquis 2001). Whilst plants are not obligatorily involved with many of these protagonists, they must constantly deal with the challenges posed by herbivorous animals, leaf-, root- and bark-miners, viruses, parasites and pathogens. Among all the likely relations between plants and other organisms, plant-insect-fungus three-way interactions show the complexity of direct and indirect connections (Hatcher 1995; Kluth *et al.* 2001; Kruess *et al.* 2004; Mondy & Corio-Costet 2004). Plants suffer mainly the direct effects, with consumption and infection often leading to a reduction in growth, survival, or reproduction, and changes in the quantity of plant chemical constituents, including organic nitrogen, carbohydrates, secondary plant chemicals, and mineral elements (Farrar 1989; Hatcher *et al.* 1994a, 1995; Tinney *et al.* 1998). They are not just a passive source of food and habitats, but also play a role as mediator between herbivorous insects and rust fungi, leading to indirect effects between the two consumers. Plants possess many forms of defence, involving morphology (hairs, spines), sticky or slippery substances (resins, waxes), repellent compounds (terpenoids, alkaloids), attractiveness to natural enemies of herbivores (tritrophic interactions), phenological strategy, and induction of systemic resistance against either insects or pathogens (Coley & Barone 1996; Mauricio & Rausher 1997; Mauch-Mani & Metraux 1998; Thaler *et al.* 2002). These systems of defence in the plants make these truly tripartite interactions.

Here we examine the three-way interactions between two species of alpine leaf beetle, *Oreina elongata* and *O. cacaliae*, the rust fungus *Uromyces cacaliae*, and their host plant, *Adenostyles alliariae*. Their high altitude habitats provide harsh conditions and extreme time stress during the breeding season, which is likely to intensify the interactions between the three groups of organisms. We consider the relationship from the point of view of the host plant, investigating the consequences of beetle attack and rust infection for leaf chemistry and for the growth and reproduction of the plant. Field experiments were used to compare healthy control plants with those subjected to natural rust infection and experimental beetle attack, both individually and simultaneously. The data allowed us to answer a number of questions:

> Does beetle or rust attack alter host leaf chemistry, in the form of carbon, hydrogen, nitrogen and pyrrolizidine alkaloid (PA) content, or C/N ratios?
>
> Does beetle or rust attack affect plant growth and the probability of flowering?

Can changes in leaf chemistry explain the reduced performance of larvae reared on rust-infected plants?

Does the probability of rust attack depend on the presence of leaf beetles or the initial host plant chemistry?

Does the level of investment in PAs, the secondary defence compounds produced by *A. alliariae*, depend on the plant's carbon, nitrogen and hydrogen budget?

Materials and Methods

Study organisms

The leaf beetles *Oreina elongata* Suffrian and *Oreina cacaliae* Schrank (Coleoptera: Chrysomelidae) are commonly found in medium to high mountain habitats in Europe (Lohse & Lucht 1994). *O. elongata* is a small (body length 6.5 to 9.5 mm), metallic blue beetle, found at altitudes between 1600 m and 2400 m above sea level. Its distribution is patchy, with isolated populations throughout the Alps and further south into the Apennines (Lohse & Lucht 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 studied populations, host plant use is restricted to two plant species in the Asteraceae: *Adenostyles alliariae* (Gouan) A. Kerner and the thistle *Cirsium spinosissimum* (L.) (in 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.* 2001). 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* is a closely related species and 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 (Lohse & Lucht 1994). The life cycle of *O. cacaliae* 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 plants of the genus *Senecio*, and others briefly begin the season on *Petasites paradoxus* (Asteraceae), because this is one of the first plants to grow as the snow melts at sun-exposed sites (Kalberer *et al.* 2005). *O. cacaliae* has lost the ability to produce cardenolides and depends exclusively on the sequestration of PAs 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.

The shared host plant, *Adenostyles alliariae,* is a common, perennial, subalpine 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).

Uromyces rusts occur over a wide geographical range and are parasitic upon many families of hosts (Bisby 1920), but *Uromyces cacaliae* (DC.) Unger (Pucciniceae) is an obligatory pathogen of *Adenostyles alliariae*. It produces spores from patches on the underside of leaves around two weeks after infection (Bisby 1920) and seems to strongly affect the host plant at the end of the summer. Both larvae and adults of *Oreina* species avoid host plants infected by the rust (Röder *et al.* 2007).

For this study, host plants, rust, and *Oreina* leaf beetles were found and used directly in the field at three sites: Emosson (Swiss Alps, Valais, 1949 m), La Fouly (Swiss Alps, Val Ferret, Valais, 1587 m), and Piccolo San Bernardo (Italian Alps, Valle d'Aosta, 2053 m).

Experimental design

The experiments were started on 2 June 2005 at La Fouly, 22 June at Emosson, and 23 June at Piccolo San Bernardo. Within the studied populations, 204 plants of *A. alliariae* were chosen at random (Emosson = 80, La Fouly = 80, Piccolo San Bernardo = 44). All were newly-emerged, healthy plants with two leaves and no flowers. Their heights were measured, and a leaf disc of 2 cm diameter was cut from the lowest leaf of each plant using a cork borer. The plants were then randomly assigned to one of two treatments. For half of the plants, larvae of *Oreina* leaf beetles were briefly collected, settled on the plant (one per plant) and watched until they had fed at least once on the lowest leaf of each plant. They were then allowed to leave or to stay on the plant. The second half of the plants did not undergo any manipulation. Back at the University of Neuchâtel (Switzerland), the 204 leaf discs were dried, placed individually in 2 ml plastic tubes, crushed mechanically to obtain leaf powder (Retsch MM 300, frequency: 30 s^{-1}, 2 min), and stored in the dark until analysis.

After 14 days, the 196 remaining plants were surveyed again, recording their height, and whether or not they had produced flowers and new leaves. They were classified into four categories: rust-infected, leaf-beetle attacked, rust and leaf-beetle attacked, and non-attacked plants used as controls. A second leaf disc was taken from the lowest leaf of each plant, and another from the leaf directly above. These discs were treated in exactly the same was as those from the first survey.

Carbon, hydrogen, nitrogen analysis

A total of 65 plants were selected for chemical analysis (Emosson = 28, La Fouly = 28, P. San Bernardo = 1 showing rust; 3 = beetle attack; 3 = infestation by both attackers; 2 = no attack), making a total of 195 samples (lowest leaf initially, lowest leaf after two weeks, and upper leaf after two weeks). The carbon (C), and nitrogen (N) contents of the leaves were determined by mass spectrometry with a Carlo Erba EA 1108 elemental analyzer, treating around 2 mg of leaf powder per sample. Although the hydrogen (H) levels are not important in this study, data were obtained directly with the same analyzer and they were included in the analysis. The C/N ratio was also calculated for each sample.

Pyrrolizidine alkaloid extraction and analysis

Samples from the same 65 plants were analysed for their PA content using combined Gas Chromatography-Flame Ionization Detection (GC-FID). For each sample, around 40 mg of the leaf powder was weighed on an electronic balance and agitated thoroughly in an Eppendorf tube with 0.5 ml of acidified methanol (0.1% HCl) and 1 mg of another alkaloid, heliotrine, added as an internal standard (Latoxan). The tube was centrifuged (1 min at 13’000 rpm) and the supernatant transferred into a 7 ml brown glass vial. This washing and centrifugation was carried out three times, with heliotrine added only on the first occasion. The methanol was then evaporated under nitrogen flow, 0.6 ml of sulphuric acid (H_2SO_4 1N) added, then 1.2 ml of ether was added and the upper of the two layers of liquid removed and discarded. After adding a spatula tip of zinc, the samples were agitated for two hours with a magnetic stirrer, then left to stand for 15 min. The extract was made basic (pH = 11) with 0.425 ml of NH_3 solution 25%, then transferred to a preconditioned (with 9 ml dichloromethane) 15 ml Extrelut NT3 column (Merck). The vial was washed with 3 ml of dichloromethane and transferred again to the column, before the PAs were recovered from the column by washing with 9 ml of dichloromethane. The dichloromethane was evaporated at 35° C in a Rotavapor (RE 120, Büchi), and the extract was finally recovered from the flask by washing three times with 0.5 ml of dichloromethane, followed by storage in a 2 ml brown vial at -76° C until analysis.

For analysis, 50 µl of the extract solution was mixed with 10 µl of heptadecane (200 ng/µl), used as an extra standard with fixed concentration. PAs were quantified by GC-FID, injecting 1 µl of each sample in splitless mode into an Agilent Technologies 6850 gas chromatograph equipped with a HP-1MS column (0.25 µm film thickness, 0.25 mm ID, 30 m, J&W Scientific). Helium at constant pressure (17.93 psi) was used as carrier gas. Following injection, the column temperature was maintained at 150° C for 3 min, then increased to 300° C at 6° C/min, followed by 7 min at 300° C. The total run time was 35 min and the temperature of the detector was 250° C. Seneciphylline and senecionine, the two main PAs present in *A. alliariae*, were identified by comparison with the chromatogram obtained with pure seneciphylline (94%) and senecionine (Carl-Roth). Quantification was carried out by dividing the peak area of the plant alkaloids by the area of the heliotrine internal standard (of which 1 mg was added at the start of the extraction), then dividing by the sample mass to give the PA concentration in the leaf in mg/g dry weight.

Statistical analysis

The growth of the plants was expressed as a percentage of their original size and then analysed in an ANOVA with terms for population, leaf beetle (attacked or not), and rust (infected or not), and all their two- and three-way interactions. A square root transformation was used to make the data conform to assumptions of normality and homogeneity of variance.

The flowering data were analysed using a logistic regression (with binomial response of flower production, yes or no). The numbers of new leaves produced were compared using a quasi likelihood analysis based on a Poisson distribution, which takes into account the under-dispersion of the data (mean > variance). In both cases the model included terms for population, leaf beetle, rust, and all their two- and three-way interactions. The association between the production of at least one new leaf and the production of flowers was analysed in G-test of independence.

The changes in C, H, N, and PA content (in mg/g dry leaf) between the first and second samples from the lower leaf and between the first sample and the upper leaf were analysed in separate repeated measures ANOVAs (using the MANOVA framework in JMP 6.0). The same was done with the C/N ratios. The change was included as a term for time, interacting with terms for population, leaf beetle, rust, and all their two- and three-way interactions. For both analyses this allowed a method to control for the initial variation in chemistry between plants, and for the lower leaf it also represented the real change in chemistry. A square root transformation was used to make the PA data conform to assumptions of normality and homogeneity of variation.

The levels of C, H, N, and PAs in the upper leaves at the end of the trial were analysed in separate ANOVAs with terms for population, leaf beetle, rust, and all the possible interactions. The ratios of C/N were compared with the same method. A square root transformation was used to make the PA data conform to assumptions of normality and homogeneity of variation, while the C/N data needed a logarithmic transformation. The population of P. San Bernardo was excluded for the analysis of the PA concentration because of the loss of some data-points during the chemical analysis.

The initial concentrations of C, H, N, and PAs, as well as the initial C/N ratios, were analysed in separate ANOVAs with the same terms as in the previous analysis. A logarithmic transformation was used to make the initial PAs and C/N data conform to assumptions of normality and homogeneity of variation, while no transformation was needed for the C, H, and N values.

The initial amounts of PAs in the plants were correlated separately with their initial contents in C, H, and N. The ANCOVAs included terms for population, the concentration of the tested element (C, H, N), and the population by element interaction.

All the ANOVAs, MANOVAs, and ANCOVAs were carried out using JMP 6.0 (SAS Institute, NC, USA), whilst the logistic regression and Poisson regression were performed using S-PLUS 7.0 (Insightful Corporation 2005).

Results

The effects of rust and leaf beetle attack on the host plant

On average, plants measured 22.9 cm at the start of the experiment, and had grown to 44.2 cm by the end. The growth of rust-infected plants was lower than that of the healthy plants (by 28.3%). The rust by beetle interaction was significant, with plants attacked by leaf beetles and those doubly infested showing equivalent growth, lower than that of healthy plants but higher than that of rust-infected plants (Table 1 and Fig. 1).

Source	DF	SS	F	P value
growth				
pop	2	45.007	2.1953	0.114
leaf beetle	1	0.2265	0.0221	0.882
rust	1	54.339	5.3009	0.022
rust*leaf beetle	1	63.978	6.2412	0.013
pop*leaf beetle	2	4.1724	0.2035	0.816
pop*rust	2	3.5905	0.1751	0.840
pop*rust*leaf beetle	2	4.3483	0.2121	0.809
error	184	1886.2		

Table 1. ANOVA on the growth of the plants for the three populations of *A. alliariae*. Healthy plants (control) were compared to rust-infected, leaf-beetle attacked, and doubly infested plants.

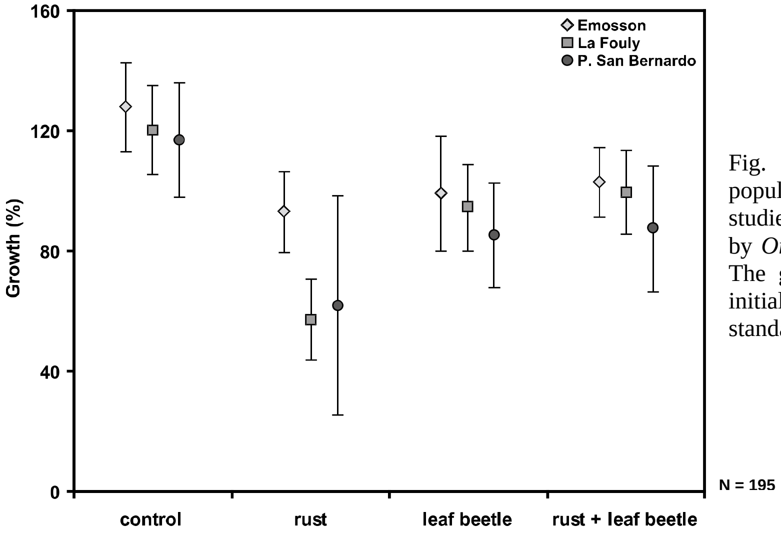


Fig. 1. The growth of *A. alliariae* from three populations and under different threats. The plants studied were healthy (control), rust-infected, attacked by *Oreina* leaf beetles, or infested by both attackers. The growth was calculated as a percentage of the initial height of the plant. The graph shows means ± standard errors.

The probability of flowering during the two weeks of the experiment differed between the populations, with a higher proportion of plants from P. San Bernardo producing flowers (35% on average), followed by those from Emosson (31.8%) and La Fouly (14.1%). Rust-infected plants had a lower probability of flowering than the other groups (by 21% on average). The population by leaf beetle by rust interaction was significant, with in general a smaller effect of rust infection in plants that had already been attacked by beetles, but with variation in this effect between populations (Table 2 and Fig. 2).

Source	DF	Deviance	Resid. DF	Resid. Dev.		P (Chi)
flowering						
null			195	228.80		
pop	2	14.378	193	214.42		< 0.001
leaf beetle	1	0.0006	192	214.42		0.981
rust	1	5.6803	191	208.74		0.017
pop*leaf beetle	2	0.4408	189	208.30		0.802
pop*rust	2	1.5475	187	206.75		0.461
rust*leaf beetle	1	0.3318	186	206.42		0.565
pop*rust*leaf beetle	2	8.2485	184	198.17		0.016
number of leaves					F	P (F)
null			195	29.472		
pop	2	2.2501	193	27.222	7.4317	< 0.001
leaf beetle	1	0.3555	192	26.866	2.3476	0.127
rust	1	0.0415	191	26.825	0.2742	0.601
pop*leaf beetle	2	0.4026	189	26.422	1.3296	0.267
pop*rust	2	0.2414	187	26.181	0.7974	0.452
rust*leaf beetle	1	0.4350	186	25.746	2.8733	0.092
pop*rust*leaf beetle	2	0.1131	184	25.633	0.3735	0.689

Table 2. Logistic regression on the probability of flowering (binomial data), and quasi-likelihood analysis based on a Poisson regression model for the number of new leaves produced during the two weeks.

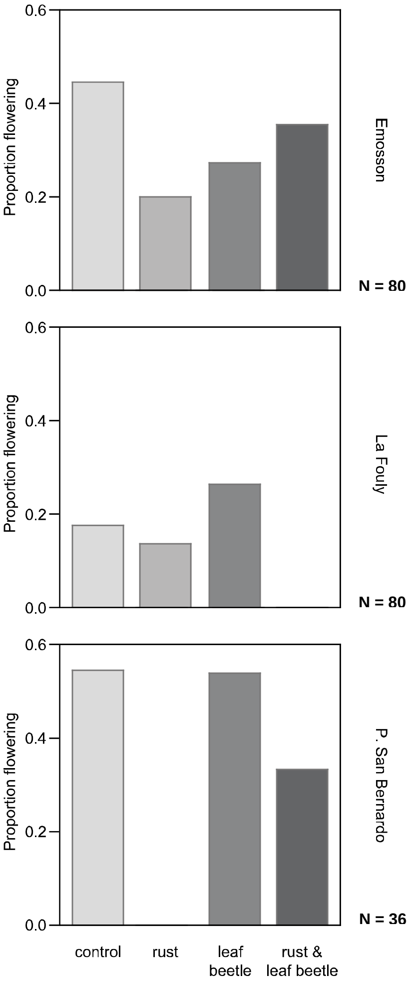
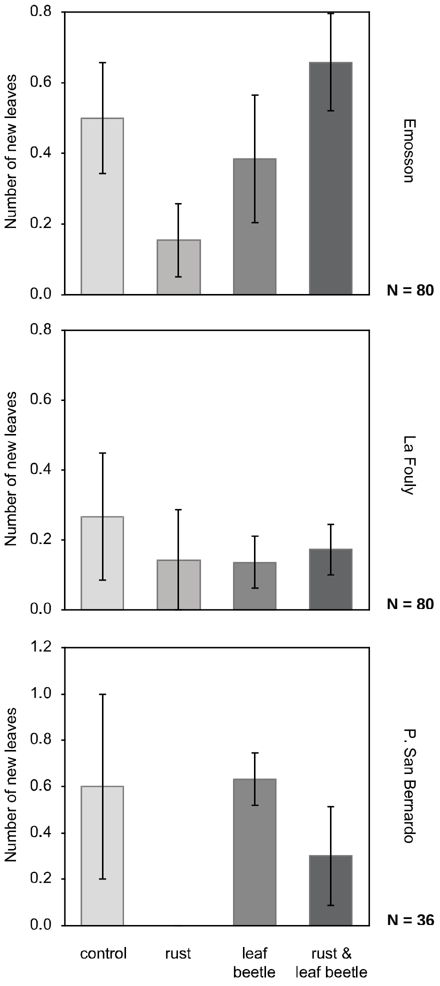


Fig. 2. Proportion of plants from the three populations producing flowers during the two weeks of the trials and under different treatments. Graphs show the proportion of flowering from healthy (control), rust-infected, *Oreina* leaf beetle attacked, or simultaneously infested plants.



The numbers of leaves produced during the two weeks showed differences between populations in a similar pattern to that observed for the flowers. New leaves were produced by 37.3 % of the plants from P. San Bernardo, 36.1% from Emosson, and 13.8% from La Fouly. Only at P. San Bernardo was there any hint of an effect of rust infection, and only the population term was significant in the model (Table 2 and Fig. 3). There was a significant association between the production of new leaves and the production of flowers during the experiment (G-test of independence, $G_1 = 83.727$, $p < 0.001$) (Table 3).

Fig. 3. Numbers of new leaves produced by plants from the three populations during two weeks and under different threats. Graphs show the number of new leaves produced by healthy (control), rust-infected, *Oreina* leaf beetle attacked, or simultaneously infested plants.

count	no flowering	flowering
no additional leaf	127	11
additional leaves	16	42

Table 3. Data table for the number of plants flowering and the number producing new leaves during the experiment.

Over the two weeks of the experiment, the concentrations of C, H, N, and the C/N ratios in the lowest leaves of the plants changed significantly (C by -0.6%, H by -1.7%, N by -15.5%, and C/N by + 16.8% on average). For C, H, and N, there were also differences among the populations in their response: plants from Emosson and P. San Bernardo showed losses of all elements with time, whereas those from La Fouly increased slightly their levels of C and H and showed no change in N (Table 4 and Fig. 4). There was no effect of rust or beetle attack. PA concentrations also decreased with time (by 24.3 % on average). Both the time by population by leaf beetle and time by population by rust terms were significant, with no consistency in the effect of beetle or rust attack on the size of the reduction in PA content with time when compared across populations (Table 4 and Fig. 5). For the C/N ratios, the time by rust term was significant, with a stronger increase in the rust-infected than in the control plants (by 12.2%) (Table 4).

	Lowest leaves					Upper leaves				
Source	DF_1	DF_2	Value	F	P value	DF_1	DF_2	Value	F	P value
carbon										
time	1	53	0.1794	9.5090	**0.003**	1	53	0.3601	19.087	**< 0.001**
time*pop	2	53	0.1788	4.7390	**0.013**	2	53	0.0716	1.8980	0.160
time*leaf beetle	1	53	0.0352	1.8644	0.178	1	53	0.0256	1.3564	0.249
time*rust	1	53	0.0091	0.4829	0.490	1	53	0.0002	0.0122	0.913
time*rust*leaf beetle	1	53	0.0023	0.1221	0.728	1	53	0.0055	0.2921	0.591
time*pop*leaf beetle	2	53	0.0410	1.0863	0.345	2	53	0.0364	0.9645	0.388
time*pop*rust	2	53	0.0340	0.9018	0.412	2	53	0.0182	0.4812	0.621
time*pop*rust*leaf beetle	2	53	0.0397	1.0523	0.356	2	53	0.3182	8.4336	**< 0.001**
hydrogen										
time	1	53	0.0877	4.6475	**0.036**	1	53	0.0209	1.1064	0.298
time*pop	2	53	0.1986	5.2617	**0.008**	2	53	0.0561	1.4860	0.236
time*leaf beetle	1	53	0.0311	1.6473	0.205	1	53	0.0132	0.6972	0.408
time*rust	1	53	0.0010	0.0537	0.818	1	53	0.0030	0.1567	0.694
time*rust*leaf beetle	1	53	0.0002	0.0126	0.911	1	53	0.0021	0.1138	0.737
time*pop*leaf beetle	2	53	0.0859	2.2753	0.113	2	53	0.0123	0.3261	0.723
time*pop*rust	2	53	0.0347	0.9193	0.405	2	53	0.0004	0.0101	0.990
time*pop*rust*leaf beetle	2	53	0.0129	0.3429	0.711	2	53	0.1512	4.0070	**0.024**
nitrogen										
time	1	53	1.2979	68.788	**< 0.001**	1	53	1.4568	77.212	**< 0.001**
time*pop	2	53	0.7650	20.273	**< 0.001**	2	53	0.8033	21.288	**< 0.001**
time*leaf beetle	1	53	0.0004	0.0235	0.879	1	53	0.0046	0.2413	0.625
time*rust	1	53	0.0668	3.5393	0.065	1	53	0.0565	2.9922	0.090
time*rust*leaf beetle	1	53	0.0095	0.5169	0.475	1	53	0.0031	0.1621	0.689
time*pop*leaf beetle	2	53	0.0487	1.2899	0.284	2	53	0.0521	1.3807	0.260
time*pop*rust	2	53	0.0384	1.0173	0.369	2	53	0.0224	0.5928	0.556
time*pop*rust*leaf beetle	2	53	0.0142	0.3750	0.689	2	53	0.1795	4.7574	**0.013**
PAs										
time	1	51	0.5744	29.296	**< 0.001**	1	47	0.1226	5.7596	**0.020**
time*pop	2	51	0.0211	0.5368	0.588	2	47	0.0201	0.9460	0.336
time*leaf beetle	1	51	0.0020	0.1021	0.751	1	47	0.0001	0.0045	0.947
time*rust	1	51	0.0473	2.4103	0.127	1	47	0.0080	0.3751	0.543
time*rust*leaf beetle	1	51	0.0313	1.5974	0.212	1	47	0.0016	0.0768	0.783
time*pop*leaf beetle	2	51	0.1405	3.5826	**0.035**	2	47	0.0017	0.0776	0.782
time*pop*rust	2	51	0.1255	3.2009	**0.049**	2	47	< 0.001	0.0000	0.999
time*pop*rust*leaf beetle	2	51	0.1005	2.5924	0.087	2	47	0.0222	1.0437	0.312
C/N										
time	1	53	0.9767	51.764	**< 0.001**	1	53	0.8504	45.069	**< 0.001**
time*pop	2	53	0.5284	14.004	**< 0.001**	2	53	0.6358	16.850	**< 0.001**
time*leaf beetle	1	53	0.0001	0.0041	0.949	1	53	0.0092	0.4858	0.489
time*rust	1	53	0.1225	6.4929	**< 0.014**	1	53	0.0275	1.4586	0.233
time*rust*leaf beetle	1	53	0.0095	0.5020	0.482	1	53	0.0104	0.5532	0.460
time*pop*leaf beetle	2	53	0.0295	0.7829	0.462	2	53	0.0144	0.3822	0.684
time*pop*rust	2	53	0.0881	2.3343	0.107	2	53	0.0222	0.5885	0.559
time*pop*rust*leaf beetle	2	53	0.0076	0.2006	0.819	2	53	0.2369	6.2782	**0.004**

DF_1 = numerator degrees of freedom; DF_2 = denominator degrees of freedom

Table 4. Repeated measures ANOVAs for the changes (time terms) in leaf chemistry between the first sample from the lower leaf and the sample two weeks later, and between the first sample and that from the upper leaf.

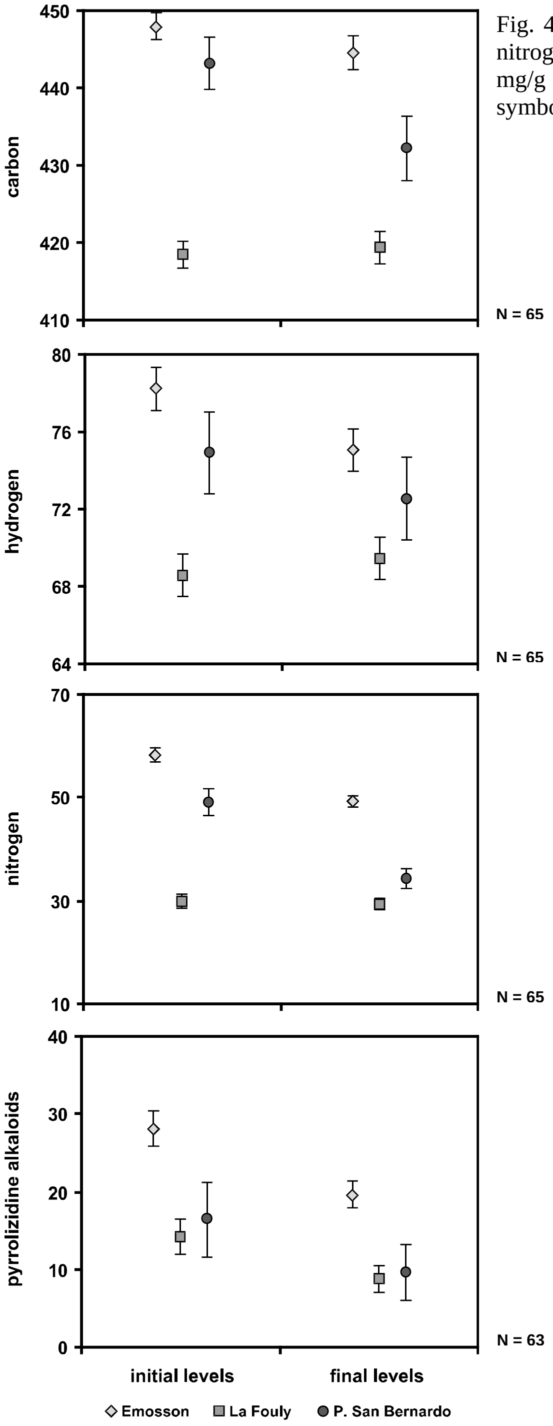


Fig. 4. Changes over two weeks in the concentrations of carbon, hydrogen, nitrogen, and pyrrolizidine alkaloids (PAs) in the lower leaves. Values are in mg/g dry leaf and the graphs show means ± standard errors, with different symbols for the three populations of *A. alliariae*.

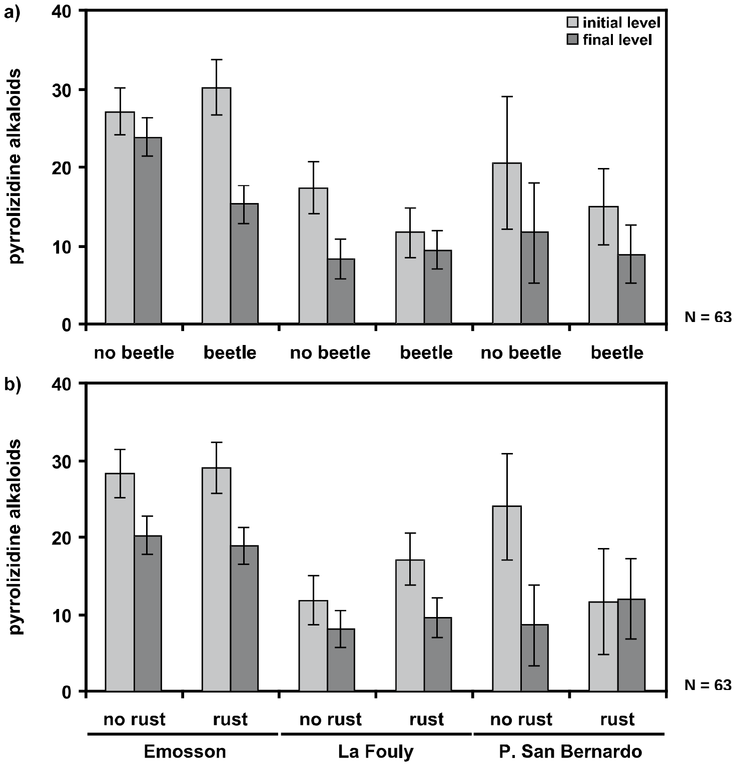


Fig. 5. Changes in the PA levels (in mg/g dry leaf) in the lower leaves, depending on (a) *Oreina* leaf beetle attack, and (b) infection by the rust *U. cacaliae*. The graphs show the means (± standard errors) of the initial and final levels.

The values measured in the upper leaves at the end of the trial, compared with the initial amounts found in the lowest leaves of plants, showed a significant decrease with time for all elements except H (C by -1.6% and N by -11.8% on average). Carbon, hydrogen and nitrogen showed significant four-way interactions, with no consistent effect of any treatment (Table 4 and Fig. 6), although the population of P. San Bernardo lost more nitrogen than the others (by 43.7% on average). The levels of PAs decreased over the two weeks (by 10.3%), but did so equally across populations and treatments (Table 4 and Fig. 7). All the C/N ratios increased with time (by 8.9% on average), but those measured in the plants from P. San Bernardo increased the most (by 48.0%, compared to 6.9% for Emosson and 2.6% for La Fouly). The significant population by rust by beetle interaction showed inconsistent effects, with leaf-beetle attacked and rust-infected plants from La Fouly having lower C/N ratios at the end than at the beginning.

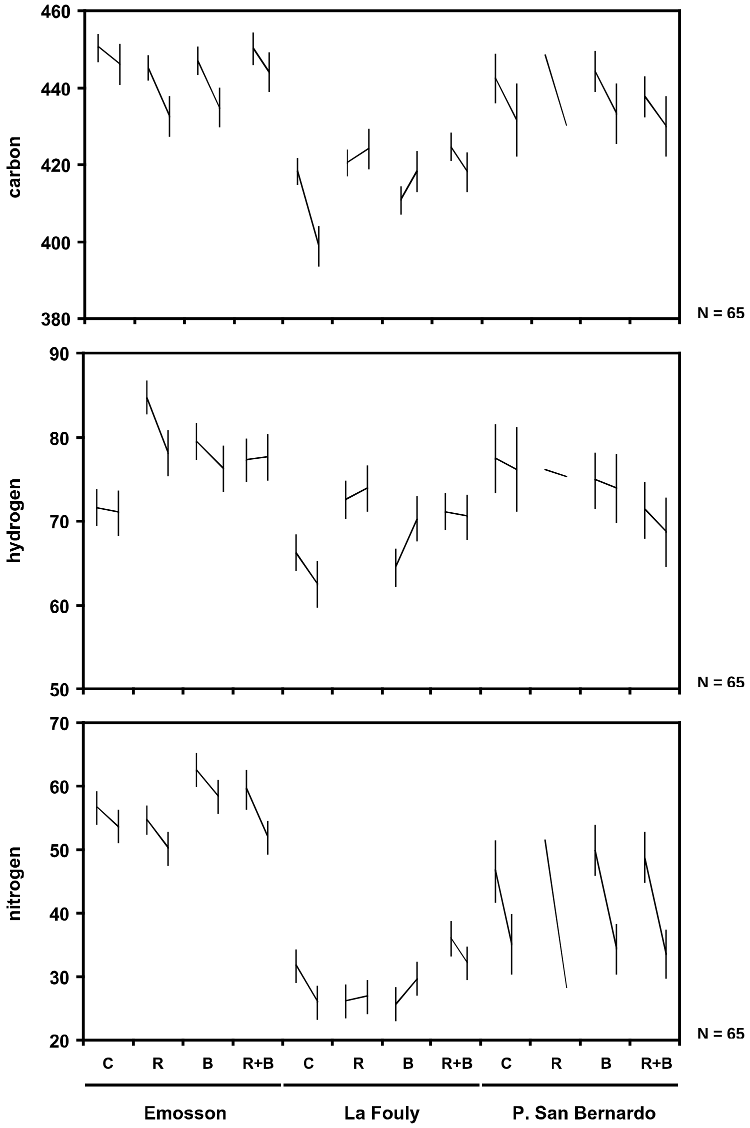


Fig. 6. Changes over two weeks in carbon, hydrogen, and nitrogen concentrations in the plants (mg/g dry leaf). Joined pairs of points show the initial levels in the lowest leaf (left) and the final levels in the upper leaf (right), giving means ± standard errors. Plants were from three populations, and had one of four treatments: healthy (C), rust-infected (R), leaf beetle attacked (B), and rust and leaf beetle infested (R+B).

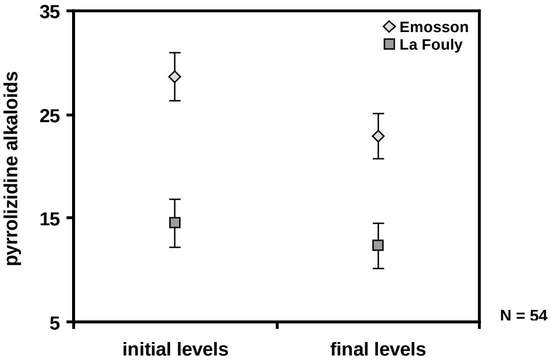


Fig. 7. Modifications of the PA levels (in mg/g dry leaf) between the initial levels in the lowest leaves and the final levels in the upper leaves for plants from Emosson and La Fouly (means ± standard errors).

Can changes in leaf chemistry explain the reduced performance of larvae reared on rust-infected plants?

The concentrations of C, H, N, and PAs measured in the upper leaves revealed either no significant or no consistent effect of rust infection across the four analyses, but differences between populations (Table 5, Fig. 8, 9, and 10). Emosson and P. San Bernardo were higher than La Fouly for carbon (by 5.9% and 3.6%), Emosson was higher than La Fouly for hydrogen (by 9.3%), and Emosson was higher than both La Fouly and P. San Bernardo for nitrogen (by 87.0% and 63.1%). Plants from Emosson also showed significantly higher levels of PAs in their upper leaves than those from La Fouly (by 81.0%) (P. San Bernardo was excluded from this analysis). The significant population by rust by beetle interaction showed inconsistent effects of the treatments, with treated plants having lower carbon levels than healthy ones at Emosson but higher levels at La Fouly, and there was a similar pattern for PAs (Table 5, Fig. 8, 9, and 10). Plants from La Fouly and P. San Bernardo also showed higher C/N ratios than those measured at Emosson (by 78.8% and 63.5%).

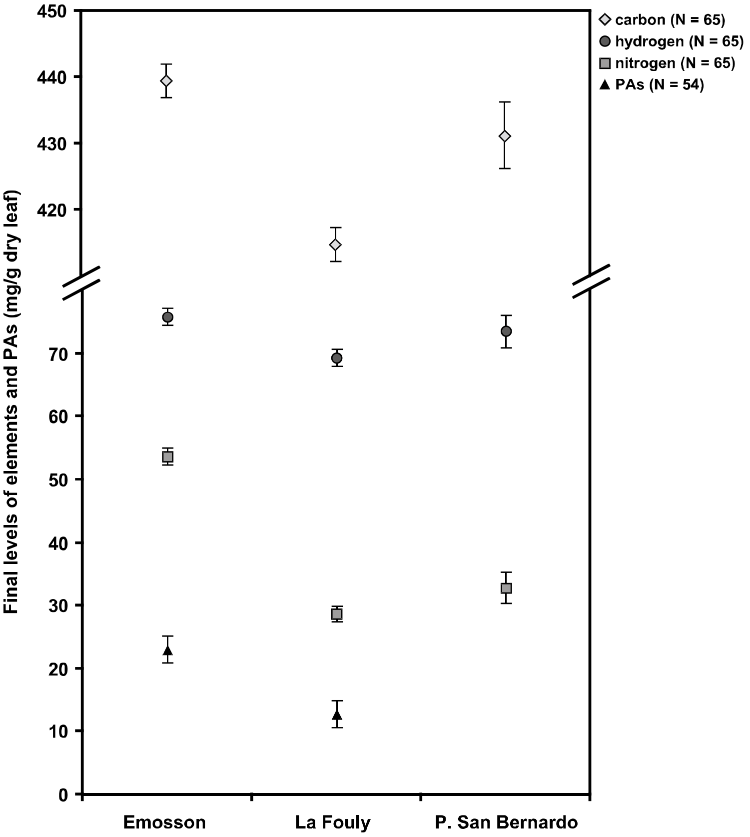


Fig. 8. Final levels of the elements (C, H, and N) and secondary defence compounds (PAs) in the upper leaves of *A. alliariae*, showing means ± standard errors.

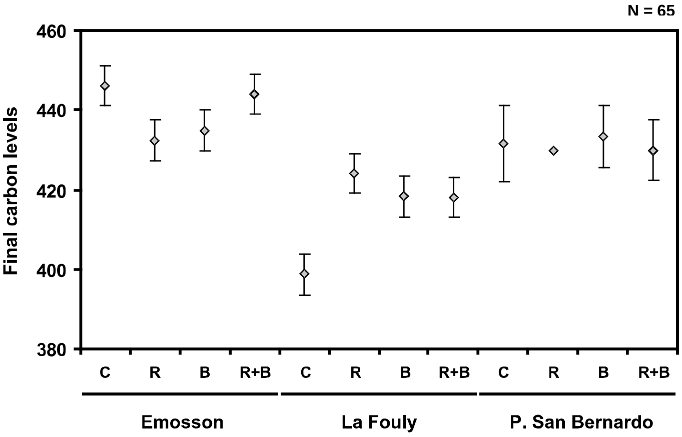


Fig. 9. Final levels of carbon in the upper leaves of the three populations of *A. alliariae* under the four treatments (in mg/g dry leaf, means ± standard errors).

Source	DF	SS	F	P value
carbon				
pop	2	8575.7	23.701	< 0.001
leaf beetle	1	73.192	0.4046	0.527
rust	1	73.641	0.4070	0.526
rust*leaf beetle	1	6.1109	0.0338	0.855
pop*leaf beetle	2	159.65	0.4412	0.646
pop*rust	2	873.22	2.4133	0.099
pop*rust*leaf beetle	2	2065.7	5.7089	0.006
error	53	9588.6		
hydrogen				
pop	2	588.84	5.9884	0.005
leaf beetle	1	0.0705	0.0014	0.970
rust	1	57.125	1.1619	0.286
rust*leaf beetle	1	137.86	2.8041	0.100
pop*leaf beetle	2	72.124	0.7335	0.485
pop*rust	2	117.55	1.1955	0.311
pop*rust*leaf beetle	2	33.675	0.3425	0.712
error	53	2605.7		
nitrogen				
pop	2	9132.8	101.57	< 0.001
leaf beetle	1	123.12	2.7386	0.104
rust	1	60.745	1.3512	0.250
rust*leaf beetle	1	7.5792	0.1686	0.683
pop*leaf beetle	2	9.7670	0.1086	0.897
pop*rust	2	165.24	1.8377	0.169
pop*rust*leaf beetle	2	36.871	0.4101	0.666
error	53	2382.7		
PAs				
pop	1	19.940	13.082	0.001
leaf beetle	1	1.5352	1.0072	0.321
rust	1	0.0055	0.0036	0.952
rust*leaf beetle	1	1.3818	0.9065	0.346
pop*leaf beetle	1	1.1590	0.7604	0.388
pop*rust	1	1.1643	0.7639	0.387
pop*rust*leaf beetle	1	6.2406	4.0941	0.049
error	47	71.642		
C/N				
pop	2	4.9965	74.118	< 0.001
leaf beetle	1	0.0881	2.6133	0.112
rust	1	0.0288	0.8540	0.360
rust*leaf beetle	1	0.0128	0.3793	0.541
pop*leaf beetle	2	0.0132	0.1955	0.823
pop*rust	2	0.0574	0.8516	0.433
pop*rust*leaf beetle	2	0.0542	0.8041	0.453
error	53	1.7865		

Table 5. ANOVAs on the C, H, N, and PA concentrations and C/N ratios of the upper leaves of the plants after two weeks.

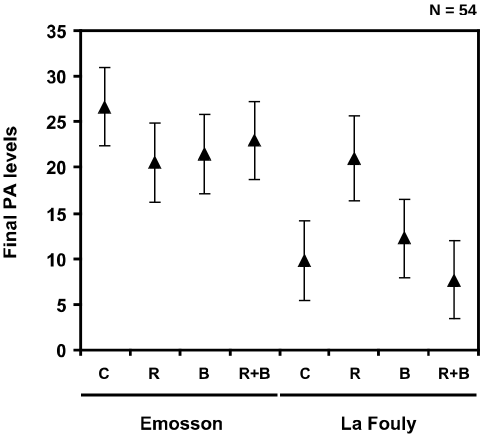


Fig. 10. Final levels of pyrrolizidine alkaloids (PAs) in the upper leaves of two populations of *A. alliariae* under the four treatments (in mg/g dry leaf, means ± standard errors).

Does the probability of rust attack depend on the presence of leaf beetles or on the initial host plant chemistry?

Among all the effects involving a rust term for the initial C, H, N and PA concentration, only the population by rust by beetle interaction for nitrogen was significant, with no consistent effect across the treatments and populations (Table 6, Fig. 11, and Fig. 12). The populations differed significantly in all four analyses, with the highest levels at Emosson in all cases, most strikingly for nitrogen (with 95.9% higher concentrations than La Fouly, on average) and for PAs (with levels 97.7% higher than for La Fouly). Overall there seemed to be no effect of beetle damage on the probability of rust infection, for there was no association between the initial beetle treatment and later rust infection (G-test of independence, $G_1 = 1.213$, $p = 0.2708$).

Source	DF	SS	F	P value
carbon				
pop	2	13047	79.796	< 0.001
leaf beetle	1	24.527	0.3000	0.586
rust	1	67.385	0.8242	0.368
rust*leaf beetle	1	24.205	0.2961	0.589
pop*leaf beetle	2	64.690	0.3956	0.675
pop*rust	2	255.79	1.5643	0.219
pop*rust*leaf beetle	2	221.75	1.3562	0.266
error	53	4333.1		
hydrogen				
pop	2	1239.3	18.977	< 0.001
leaf beetle	1	9.4877	0.2906	0.592
rust	1	96.487	2.9551	0.091
rust*leaf beetle	1	47.370	1.4508	0.234
pop*leaf beetle	2	83.791	1.2831	0.286
pop*rust	2	118.92	1.8210	0.172
pop*rust*leaf beetle	2	109.63	1.6787	0.196
error	53	1730.5		
nitrogen				
pop	2	11420	124.63	< 0.001
leaf beetle	1	53.668	1.1714	0.284
rust	1	5.5010	0.1201	0.730
rust*leaf beetle	1	15.922	0.3475	0.558
pop*leaf beetle	2	37.295	0.4070	0.668
pop*rust	2	73.792	0.8053	0.452
pop*rust*leaf beetle	2	367.79	4.0137	0.024
error	53	2428.3		
PAs				
pop	2	7.8327	15.187	< 0.001
leaf beetle	1	0.1569	0.6084	0.439
rust	1	0.3491	1.3538	0.250
rust*leaf beetle	1	0.1173	0.4550	0.503
pop*leaf beetle	2	0.7329	1.4210	0.251
pop*rust	2	1.3280	2.5748	0.086
pop*rust*leaf beetle	2	0.9582	1.8578	0.166
error	51	13.152		
C/N				
pop	2	15.222	88.271	< 0.001
leaf beetle	1	0.1224	1.4199	0.239
rust	1	0.0092	0.1066	0.745
rust*leaf beetle	1	0.1666	1.9318	0.170
pop*leaf beetle	2	0.0190	0.1099	0.896
pop*rust	2	0.0624	0.3620	0.698
pop*rust*leaf beetle	2	1.2391	7.1858	0.002
error	53	4.5697		

Table 6. ANOVAs on the initial levels of C, H, N, and PAs and the C/N ratios of the lowest leaves.

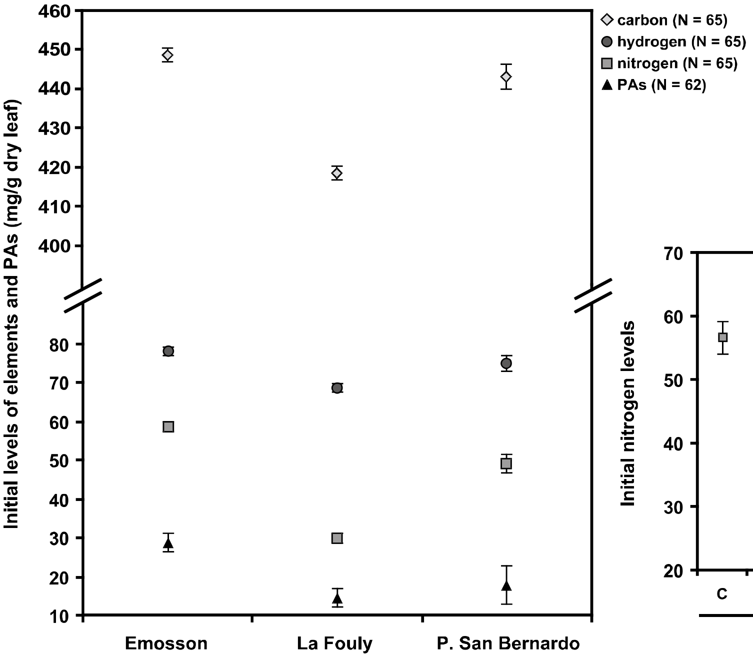


Fig. 11. Carbon, hydrogen, nitrogen, and pyrrolizidine alkaloid (PA) concentrations measured in lower leaves at the beginning of the summer season. All the plants from the three populations were healthy, recently emerged plants. The graph shows means ± standard errors.

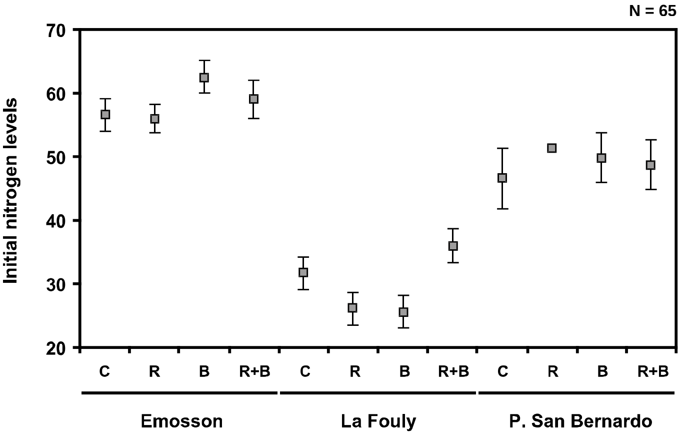


Fig. 12. Nitrogen levels at the beginning of the summer in *A. alliariae* plants from three populations (in mg/g dry leaf), under the four treatments. The graph shows means ± standard errors.

Does the level of investment in PAs depend on the plant's carbon, nitrogen and hydrogen budget?

The initial concentration of PAs in the leaves was positively correlated with the initial carbon level (Table 7 and Fig.13). The population by carbon interaction was also significant, with a steeper slope for plants from Emosson. In contrast, the initial concentration of PAs was not dependant on the initial level of hydrogen or nitrogen.

Source	DF	SS	F	P value
PAs - carbon				
pop	2	22.365	0.1375	0.872
carbon	1	472.68	5.8118	0.019
pop*carbon	2	772.44	4.7487	0.012
error	56	4554.5		
PAs - hydrogen				
pop	2	1266.2	9.8031	< 0.001
hydrogen	1	57.640	0.8925	0.350
pop*hydrogen	2	31.094	0.2407	0.787
error	54	3487.5		
PAs - nitrogen				
pop	2	594.45	4.639	0.014
nitrogen	1	80.933	1.263	0.266
pop*nitrogen	2	21.777	0.170	0.844
error	54	3460.0		

Table 7. ANCOVAs for the initial concentration of PAs, with terms for populations and separate models for the levels of C, H, and N in the leaves.

Discussion

In nature, all living creatures must cope with both abiotic parameters, like weather conditions, and biotic factors, like their relationships with other organisms. Plants, being unable to flee from unfavourable conditions, have developed numerous adaptations against disadvantageous situations, like defences against both phytophagous organisms and pathogens, or multiple strategies to deal with hot or cold temperatures, drought or flooding, and limitations on mineral and nutritive elements (Kalapos *et al.* 1996; Huner *et al.* 1998; Rostas *et al.* 2003; Hall 2004).

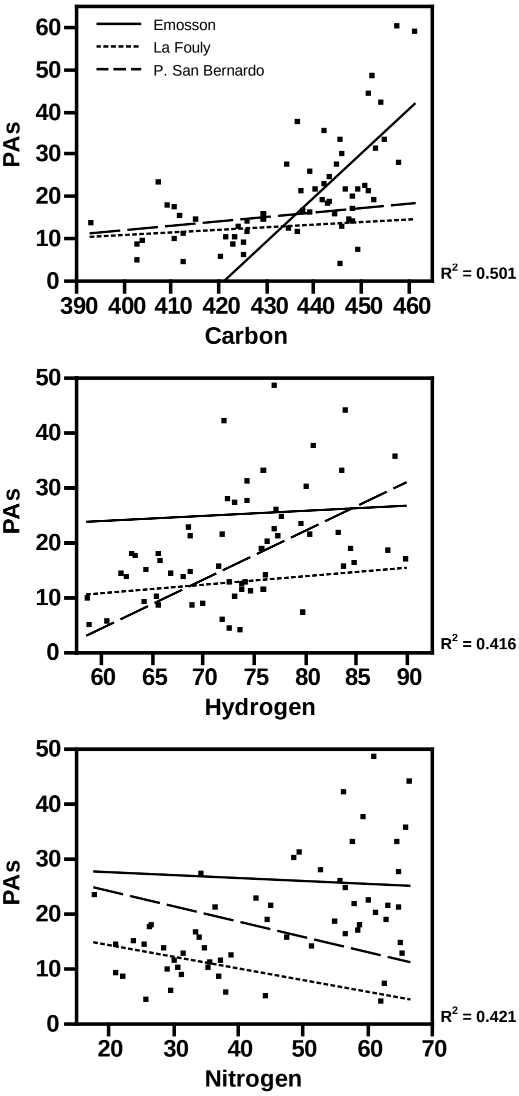


Fig. 13. The relationships between the levels of carbon, hydrogen, and nitrogen and the concentrations of pyrrolizidine alkaloids (PAs) (all measures in mg/g dry leaf) for the three populations of *A. alliariae*.

High altitude environments in Europe provide difficult conditions for plants, with great variation in temperatures, unpredictable weather conditions, strong radiation, and a very short season of activity. For a plant, the presence of rust fungi and specialized herbivorous leaf beetles clearly adds another hazard to what is already a harsh existence, in the struggle to complete their reproduction and life cycle during the two to three months in which their habitat is free of snow. Here we show that the host plant *A. alliariae* is affected in some aspects by the individual or simultaneous presence of the phytopathogenic rust *U. cacaliae* and two species of *Oreina* leaf beetles. Individually, the rust has the strongest effect on plant development, reducing growth and altering the timing of flowering. Such impacts can be observed in other equivalent systems (Hatcher *et al.* 1994b), and leads to the prediction that herbivory combined with infection will have additive effects (Hatcher *et al.* 1994b; Hatcher 1996). Surprisingly, such additive effects are not seen in plants attacked by both the rust and *Oreina* leaf beetles, suggesting that only one of these antagonists, probably the first attacker, seems to be responsible for the magnitude of the harm. The results for plant growth and flowering, in which the rust has a large effect when alone but the impacts of beetle attack and the combined attack are similar, suggest that perhaps the initial beetle damage induces defences in the plant that limit subsequent damage by the rust. In this context, the method used to sample the plants at the beginning of the experiments might have induced some defences and influenced the magnitude of the responses. Nonetheless, as all the plants suffered the same sample, the comparisons between them remain valid.

In the field, wounding by *O. elongata* and *O. cacaliae* leaf beetles and infection by the fungus seem to be independent processes. Adults of *Oreina* species rarely transport fungal spores (washing beetles caught in the field shows that less than 5% of *O. elongata* individuals and less than 9% of *O. cacaliae* carry spores) and both adults and larvae avoid rust-infected host plants (Röder *et al.* 2007), minimizing their role as a potential vector. In addition, there seemed to be no effect of beetle damage on the probability of rust infection, for there was no association between the initial beetle treatment and later rust infection.

Since rust attack was obtained by natural infection and not inoculation, rust infection cannot be considered as an experimental treatment in the statistical analyses. The rust term therefore represents a combination of the real effects of rust infection on host plant performance and chemistry, and the reverse, the effect of plant characteristics on the probability of rust attack and establishment.

The records of flowering during the two-week period of the experiment are best seen as a measure of effects on the timing of flowering, rather than an absolute effect that can halt reproduction. The experiments were all started at the same relative point in the season, when newly emerged plants had produced their second leaf. The results suggest some plasticity in the phenology of flowering related to the altitude and resulting length of the favourable season (Stinson 2004). The overall frequency of flowering during the period of the experiment was perfectly correlated with altitude across the three populations, with the highest values at P. San Bernardo, where the summer season is shortest, and the lowest values at La Fouly. The effects of the rust on the timing of flowering do not seem to be a consequence of changes in general leaf chemistry, because the concentrations of the elements (C, H, and N) and defence compounds (PAs) in the leaves vary greatly and are present in same range in attacked and healthy plants. In contrast, the lowest leaves of the rust-infected plants showed higher C/N ratios than the healthy plants, but in all categories, the leaves showed decreases in their C and N levels. Although in some fungal infections both C and N fall with infection (Mainer & Leath 1978) the relative changes can lead to a higher C/N ratio. Studies suggest that an elevated C/N ratio should create a more efficient plant (Drake *et al.* 1997), but opposite results have also been shown, as well as great variation in the allocation of the benefits in different parts of the plant (Poorter & Nagel 2000). In addition, this study does not reveal a higher C/N ratio in the rust-infected upper leaves. This suggests that perturbations of the normal growth and delays in flowering are not the result of losses due to the infection or infestation, but may be a product of fungal metabolites, or a side-effect of the induction of plant defences. These shifts in phenology are likely to bear costs for the plants. Slower development will expose plants to predation and disease for a longer period before reproducing, and it also places them at risk of not being able to complete their life cycle before the first snow arrives, for many sites are free of snow for a maximum of 2-3 months each year. This might be especially critical for the populations living at the highest altitudes, since *A. alliariae* plants need between three to six weeks to flower, so they are already on a very tight developmental schedule. When added to the threats of a delayed spring and unpredictable cold periods during the summer, the rust clearly represents a real challenge to successful reproduction in these plants.

Our previous work has shown that *Oreina* larvae fed on rust-infected upper leaves have poor larval performance in comparison with those reared on healthy upper leaves (Röder *et al.* 2007), suggesting a reduction in food quality due to the rust infection (Hatcher *et al.* 1994a; Laine 2004). Nevertheless, these consequences do not seem to result from reductions in the main leaf constituents. Neither infection by the rust nor attacks by the leaf beetles were responsible for a reduction in the nutritional content of the upper leaves, with no significant (H and N) or consistent (C) effect of either type of attack. Here, only the lowest leaves of the rust-infected plants showed an increase in the C/N ratio, which is known to reduce the nutrient quality of the leaf for insects (Drake *et al.* 1997) and may be deleterious to larvae of the spruce budworm, *Choristoneura occidentalis* (Clancy 1992). The PAs, used by many plants of the Asteraceae family in defence against herbivores, might provide a possible explanation, but as for the elements, these compounds were found in similar concentrations in the infested and healthy plants. PAs are known also to affect fungal growth, but their concentration was not increased in plants suffering rust infection (Hol & Van Veen 2002). Like the effect on the performance of the plant itself, the negative effect of the fungus on larval growth may therefore result from the induction of defences, or from direct interactions arising from fungal compounds that act as toxins for the larvae.

Whereas the beetle attack was a manipulated factor in this experiment, the rust treatment relied on natural infection. Plants that were eventually attacked by the rust did not differ from the others in their initial concentrations of C, H, N, and PAs, or C/N ratios, suggesting that the probability of infection is not related to host plant chemistry. The probability and timing of rust infection is probably dependent on a number of factors, including weather conditions, sun exposure, the over-wintering of infectious fungal spores in the ground near to the future shoots (Frantzen & Müller-Schärer 1999), wounds and entry point on the leaves, and the level of induced defences.

The investment in PAs by the plants was correlated with their initial carbon content, raising the possibility of a trade-off between plant growth and defences, even though a correlation with nitrogen content was initially expected, PAs being N-based compounds (Frischknecht *et al.* 2001). For the same level of carbon, plants from Emosson seemed to invest more in PAs, perhaps correlated with stronger herbivory pressure in this site. These plants might be expected to show lower growth than those of the other populations (Herms & Mattson 1992), but this was not the case (Fig. 1). This higher investment in defences without obvious costs might be explained by the high level of carbon (340 to 440 mg/g dry leaf) compared to the concentrations of PAs (5 to 60 mg/g dry leaf). An insignificant fraction of the carbon budget of the plant can greatly increase the concentration of PAs. Overall, the most striking differences in the studied parameters were found between populations rather than between treatments. Obviously, altitude and orientation play a role, but the size of the population, density, and the presence or absence of different host plants might have an influence. The heterogeneity of the soil can also play as well a role in the distribution of soil organic matter (Hook *et al.* 1991).

This study seems to suggest that both the rust fungus and the phytophagous insects do not induce great fitness costs and that the host plant shows a level of tolerance to attack (Simms & Triplett 1994). Nonetheless, the investigations were carried out during a period of only two weeks early in the season, which was necessary to find enough healthy plants of *A. alliariae* and to observe their evolution into the four desired treatments. The level of damage contrasts with that seen at the end of the summer when all the senescent plants are infected and dramatically attacked, suggesting critical impacts on populations of plants. At this point, the results on host plant chemistry may lead us to set aside our hypothesis that the rust *Uromyces cacaliae* modifies the food plant quality and so indirectly affects the larval performance and the ecology of both *Oreina elongata* and *Oreina cacaliae*, whereas they raise new questions about the implications of induced defences and the production of fungal metabolites.

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CHAPTER 4

A field test for induced defences against leaf beetles and phytopathogenic rust in the alpine plant *Adenostyles alliariae*

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Abstract

In nature, living creatures are permanently connected with their environment and with other organisms. These relationships rule the distribution, the ecology, and the dynamics of all protagonists. Some relationships are particularly intense, and the three-way interactions between phytopathogenic rusts, specialized herbivorous insects, and their host plants provide perfect examples of the richness and the consequences that such triangular relations imply. These interactions can be direct or plant-mediated, but in either case plants play a central role and frequently bear the costs of simultaneously harbouring several antagonists. In the field, plants are under constant threat from herbivores and pathogens, and they have developed many structures and substances to deal with these attackers, often only deploying these strategies after damage. In this study we focus on the induced responses of the host plant *Adenostyles alliariae*, and their ecological consequences for the plant, the alpine leaf beetles *Oreina elongata* and *Oreina cacaliae,* and the phytopathogenic rust *Uromyces cacaliae*. Plants were artificially induced using chemical elicitors in the field and then monitored for one month under natural conditions, revealing an effect of the induction of jasmonic and salicylic acid pathways on beetle and rust attack. Overall, chemically-induced plants showed a lower probability and later incidence of beetle and fungal attack, with signs of cross-talk between the responses. However, the short-term experiments did not provide any evidence of an effect on plant performance, when measured by leaf consumption, plant growth, and flowering. In the context of the very short season of activity in high alpine environments, these results suggest that *Adenostyles* plants are forced to use two different strategies, initially using defences in order to gain crucial time free of attackers, allowing quick growth and flowering, then tolerating attack during the second part of the summer.

Introduction

As the major part of the terrestrial biomass, the primary producer of food supplies, the residence for most living creatures, and the intersection between mineral elements and organic compounds, plants occupy a central place in nature (Hector *et al.* 1999). Plant life often has to cope with harsh and unpredictable conditions, like cold and hot temperatures, drought or excess of water, wind and poor mineral quality of the soil, and competition with other plants. In addition, they are under constant threat of attack by herbivorous animals and pathogenic micro-organisms, like phytophagous insects and rust fungi (Paul 1989; Hatcher 1995; Friedli & Bacher 2001; Kluth *et al.* 2001). The antagonists must often share the same host plant, leading to the possibility of both direct and plant-mediated interactions. This sets up a network of possible interactions affecting the fitness and the ecology of all the protagonists and leading to adaptations, tolerance strategies, and arms races. Some insect species are specialists that feed on fungal parts, obtaining food supplies and reducing the fungal infection (Hatcher & Paul 2000; Takahashi *et al.* 2005), whereas many other herbivores will wound the leaves and inoculate the plant with fungal infectious material (Bacher & Friedli 2002). The phytopathogenic fungi are known to affect the reproduction, development, and survival of plant populations (Hatcher 1995), and some have been tested as potential biocontrol agents against invasive weeds (Müller-Schärer & Frantzen 1996; Grace & Müller-Schärer 2003). Whilst some rusts may provide beneficial compounds in the diets of herbivorous insects, leading to higher survival and performance (Mondy & Corio-Costet 2004), others produce repellent compounds or toxins affecting both their feeding preferences and fitness (Laine 2004). Although plants can be harmed or protected by both insects and fungi, they do not simply passively play host to these antagonists and show adaptations and strategies to defend themselves and participate truly in these three-way interactions (Rostas *et al.* 2003; Simon & Hilker 2003). Plant defences were generally assumed to be constitutive and independent of damage, but it has been demonstrated that many traits and processes that defend plants against herbivores and pathogens show changes following attack (Karban & Baldwin 1997). Plant defences are highly varied, with plasticity in phenology to avoid the attackers, morphological structures to prevent transmission, or the production of extremely diversified substances. Some of these chemical compounds are used on the plant surface, like resins and waxes, or found inside the plant, like the alkaloids and the terpenoids (Hol & Van Veen 2002), or even emitted to call the assistance of allied partners, above and below ground (Rasmann *et al.* 2005). Few studies describe the production of organic volatile compounds from plants involved in triangular relationships with phytopathogenic rust and herbivorous insects. They are usually implicated in activation or inhibition of fungal germination, or attractiveness to other herbivores and vector insects used in cross fertilization (Connick & French 1991; French *et al.* 1993; Kepler & Bruck 2006; Mendgen *et al.* 2006), although recent studies revealed modifications in herbivore-induced plant volatiles (Rostas *et al.* 2006) and the emission of volatile organic compounds susceptible to prime nearby plants for enhanced induction of defence upon future insect attack (Ton *et al.* 2007). On the other hand, plant induced defences have raised a great deal of interest during the last decade. There is a hierarchy of terminology, with the changes in plants following damage or stress referred to as induced responses (Bradshaw & Hardwick 1989), those that reduce herbivore survival, reproductive output, or preference for a plant termed induced resistance, and those shown to currently reduce the negative fitness consequences of attacks on plants called induced defences (Karban & Baldwin 1997). Nowadays, some mechanisms and compounds involved in plant responses following damage are well identified, like the jasmonate (JA) and salicylate (SA) signalling pathways generating resistance to herbivores and pathogens (Mauch-Mani & Metraux 1998; Bostock 1999; Kessler & Baldwin 2002). Initially, these two processes seemed to be independent, but they are now known to interact, with cross-talk between the pathways sometimes leading to synergistic and sometimes to antagonistic responses (Thaler *et al.* 2002a; Thaler *et al.* 2002b; Traw *et al.* 2003). It is likely that neither of these pathways is wholly specific to herbivory or disease, with plants seeming to use more complex signals than independent linear pathways (Genoud & Metraux 1999), and at the whole plant level, the costs and benefits of dual induction of these pathways can be related to the attacking species or environmental factors (Thaler *et al.* 2002a).

In their high alpine environments, the host plant *Adenostyles alliariae* shows by the end of the summer a high proportion of leaves consumed by the leaf beetle species *O. elongata* and *O. cacaliae*, and the arrival of the phytopathogenic rust *Uromyces cacaliae* in mid summer seems associated with rapid senescence of the plant. As part of the likely three-way interactions occurring between these participants, we here test for inducible natural defences in *A. alliariae*. Artificial induction of potential JA and SA pathways in the field was used to answer several questions:

> Do *Adenostyles alliariae* plants have a system of induced resistance and defence?
>
> Do artificially induced plants show differences in the probability, intensity, and timing of attack by *Oreina* leaf beetles and infection by the rust?
>
> If induction deters attackers, are induced plants more successful in growth and reproduction?

Materials and Methods

Study organisms

The host plant *Adenostyles alliariae* (Gouan) A. Kerner is a common, perennial, subalpine and alpine plant, found at a maximum altitude of 2800 m. It grows preferentially on damp soils in the region of the timber-line. Their particularity is the production of secondary compounds, pyrrolizidine alkaloids (PAs) (mainly seneciphylline, senecionine, and spartioidine), used as a defence against herbivory (Hagele & Rowell-Rahier 1999; Hartmann *et al.* 1999). Plants ingested by mammals induce liver disease, but until the beginning of the last century they were used by the inhabitants of the Alps as a pectoral tea (Roeder 1995). *A. alliariae* is a regular host plant of the alpine leaf beetles *Oreina elongata* Suffrian and *Oreina cacaliae* Schrank (Coleoptera: Chrysomelidae). *O. elongata* is a small (body length 6.5 to 9.5 mm), metallic blue beetle, found at altitudes between 1600 m and 2400 m above sea level. Its distribution is patchy, with isolated populations throughout the Alps and further south into the Apennines (Lohse & Lucht 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. Within the populations studied, host plant use is restricted to two species in the Asteraceae: *Adenostyles alliariae* and the thistle *Cirsium spinosissimum* (L.) (in some other sites they use *A. glabra* and *A. leucophylla*). Eggs are laid on the leaves of hosts 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.* 2001). Both adults and larvae have two modes of chemical defence: pyrrolizidine alkaloids (PAs) sequestered from the three *Adenostyles* species, and autogenously synthesised cardenolides (Dobler et al. 1996). *Oreina cacaliae* is a closely related species and 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 (Lohse & Lucht 1994). The life cycle of *O. cacaliae* 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 plants of the genus *Senecio*, and others briefly begin the season on *Petasites paradoxus* (Asteraceae), because this is one of the first plants to grow as the snow melts at sun-exposed sites (Kalberer et al. 2005). *O. cacaliae* has lost the ability to produce cardenolides and depends exclusively on the sequestration of PAs for defence (Dobler et al. 1996). *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. The study sites of Emosson (Swiss Alps, Valais, altitude 1949 m) and La Fouly (Swiss Alps, Val Ferret, Valais, 1587 m), inhabited by *O. elongata* and *O. cacaliae* respectively, show *A. alliariae* populations infected with the rust *Uromyces cacaliae* (DC.) Unger (Pucciniaceae). *Uromyces* rusts occur over a wide geographical range and are parasitic upon many families of hosts (Bisby 1920; Gaumann 1959; Cummins & Hiratsuka 1983). *U. cacaliae* is an obligatory pathogen of *Adenostyles alliariae*. It produces spores from patches on the underside of leaves around two weeks after infection (Bisby 1920).

Field experiment and treatments

The two populations were studied for one season in consecutive years. For each population, 80 plants of *A. alliariae* were chosen at random, just after their emergence. The plants from La Fouly were selected on 27 May 2006, while the experiment began on 28 June 2005 at Emosson, due to the higher altitude of this site. All were newly emerged, healthy plants with two leaves and no flowers. Their initial heights were measured, and they were randomly assigned to one of the seven possible treatments, mixed throughout a single patch.

For the BTH treatment (1), 20 plants were treated with acibenzolar-s-methyl (BTH), provided as Bion solution (60 mg/l) with 50 % active ingredient (Syngenta, Basel, Switzerland). The effect of this compound on the plant is similar to that of salicylic acid, used as a signal to induce the SA-dependent defence pathways of mechanisms of resistance against fungal infection (Friedrich *et al.* 1996; Inbar *et al.* 1998). On the first day of the experiment, the plants were individually sprayed four times with 0.5 ml of solution, and a repetition of this treatment was performed one week later, as suggested by the manufacturer.

(2) For the methyl jasmonate treatment, 20 plants were treated with methyl jasmonate, a derivative of jasmonate acid used by the plants to activate induced defences against herbivory (Preston *et al.* 2002). Since methyl jasmonate is a volatile, field conditions might influence the efficiency of the treatment, so to minimize evaporation the methyl jasmonate 95% (Aldrich) was mixed in pure lanolin (Riedel-de Haën) in order to obtain droplets of lanolin (20 µl) containing 150 µg of methyl jasmonate (Held & Baldwin 2005). The droplets were formed with a syringe the day before their use and kept in a plastic box in a refrigerator at 6° C until taken to the field. The lowest leaf of each plant was treated, applying half of one droplet to the upper surface and half to the lower by gently spreading with a spatula. This covered an area of around 2 cm^2 in each case, representing about 2% of the leaf surface.

(3) 20 plants were treated simultaneously with both compounds. The droplets of lanolin were applied first, immediately followed by the spray of the Bion solution.

Control plants were split among the final four treatments: (4) five were sprayed with water, the carrier substance for the BTH; (5) five were treated with one droplet of pure lanolin, the carrier for the methyl jasmonate; (6) five were treated with pure lanolin and sprayed with water; and finally, (7) five plants were left with no treatment.

Over a period of one month, the plants were monitored weekly, recording their height, the number of leaves, whether or not they were flowering, and whether or not they showed signs of beetle and rust attack. At the end of the experiment, all the plants were cut at their base and brought back to the laboratory. The leaves were dried and scanned (Epson Expression 1640 XL, Seiko Epson Corp. Japan) and their total area and area lost to beetle consumption measured using Scion Imaging software (Scion Corporation, Maryland, USA).

Statistical analysis

The overall frequencies of attack by beetles and infection by fungus at the end of the experiment were analysed in separate logistic regressions on the binomial (yes/no) data. The models included terms for population (2 levels), treatment (seven levels) and their interaction. Significant differences were observed between the treatments for both attack and infection, so the analyses were repeated after grouping the treatments to give a factor with two levels (induced/control). For the attack by beetles, the analysis was repeated a third time, grouping the treatments into three levels (BTH/methyl jasmonate alone and with BTH/control).

For each treatment, the successive proportions of the plants remaining non-attacked at each survey were analysed using parametric survival analysis, treating the act of being infested as “mortality”. The censorReg function was used in S-PLUS 7.0 (Insightful Corporation 2005), coding those individuals that remained for the full month as right-censored and all others as interval-censored (because their infestation times could only be estimated to within roughly one-week intervals). A value of 0.001 was added to data with a lower bound of zero to allow log terms to be treated. Beetle attack and rust infection were analysed separately. 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 analysed with terms for population (Emosson and La Fouly), treatment (1 to 7), and the population by treatment interaction. The order in which terms were added to the model had very little effect on their significance. S-PLUS offers 10 possible distribution families, but all gave very similar p values for the three terms in the model 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 plants that remain healthy at time t (Gross & Clark 1975; Fox 2001).

The mean percentage of the area consumed per leaf by beetles was analysed in separate ANOVAs for each population. The data for Emosson required a square root transformation and that for La Fouly needed a logarithmic transformation to conform to assumptions of normality and homogeneity of variance. The model included a treatment term with seven levels, and when significant, Tukey HSD tests were used to determine where the differences lay. The analysis was performed a second time with only four treatments considered, to examine how using this incomplete experimental design would have altered the conclusions. For this, only the three induced treatments and the final unmanipulated control were included. A very low percentage of leaf area was consumed during the experiment, but analysis of the absolute leaf area consumed gave identical results.

Growth of the plants was calculated as a daily growth rate (in cm/day) by carrying out regressions of height against time in days individually for each plant (these linear regressions gave a close approximation to the growth process, with r^2 values of between 0.61 and 0.99). The data were analysed in separate ANOVAs for each population with a term for treatment, and required no transformation.

The numbers of leaves on plants at the end of the experiment were compared using quasi likelihood analysis based on a Poisson distribution, which takes into account the under-dispersion of the data (mean > variance). The model included terms for population, treatment, and their interaction.

The probability and timing of flower production were analysed using exactly the same methods as the beetle and rust attack, using logistic regression and survival analysis.

Finally, to determine if attack by leaf beetles and infection by rust were independent processes, a G-test of independence was performed on the overall count data.

The logistic regression, survival analysis and quasi-likelihood models were carried out using S-PLUS 7.0 (Insightful Corporation 2005), whilst the ANOVAs were performed using JMP 6.0 (SAS Institute, NC, USA).

Results

Frequency, intensity, and timing of attack by Oreina leaf beetles and infection by rust

The probability of attack by *Oreina* beetles was similar in the two populations but differed according to treatment (Table 1 and Fig 1). The significant effect of treatment disappeared when grouping plants as “induced” vs “control”, but was again apparent when grouping into three categories, “BTH”, “methyl jasmonate alone and with BTH”, and “control”. Plants treated with BTH suffered a higher rate of attack than the controls, while those treated with methyl jasmonate or with both compounds were less likely to be attacked. The proportion of plants infected by the rust *U. cacaliae* differed between the populations (with higher overall rates at Emosson) but there were consistent significant differences between the treatments at both sites (Table 1 and Fig. 2). The treatment term remained significant when grouping plants into the categories “induced” and “control”, with lower infection rates in all induced plants than in the controls. The level of consumption by leaf beetles differed according to treatment in both populations, but the patterns were difficult to interpret with regard to the controls (Table 2 and Fig. 3). There was a tendency for increased consumption of BTH-treated plants, reduced consumption of methyl jasmonate plants, and an even greater reduction in the combined treatment, but in all cases the induced plants did not differ significantly from their respective controls. The results changed slightly when excluding all controls except the un-manipulated plants (Table 2 and Fig. 3). The treatment term was again significant, and the methyl jasmonate and doubly treated plants had significantly lower consumption than the control at Emosson, and the doubly treated plants did so at La Fouly.

Source	DF	Deviance	Resid. DF	Resid. Dev.	P (Chi)
leaf beetle					
null			159	184.21	
pop	1	2.0760	158	182.13	0.150
treatment	6	16.404	152	165.73	0.012
pop*treatment	6	3.9257	146	161.80	0.687
rust					P (Chi)
null			159	213.64	
pop	1	12.960	158	200.68	< 0.001
treatment	6	48.048	152	160.63	< 0.001
pop*treatment	6	8.9851	146	151.64	0.174

Source	DF	Deviance	Resid. DF	Resid. Dev.	P (Chi)
leaf beetle					
null			159	184.21	
pop	1	2.0760	158	182.13	0.150
treatment	1	2.2667	157	179.87	0.132
pop*treatment	1	1.3650	156	178.50	0.243
rust					P (Chi)
null			159	213.64	
pop	1	12.960	158	200.68	< 0.001
treatment	1	37.785	157	162.89	< 0.001
pop*treatment	1	1.0358	156	161.86	0.309

Source	DF	Deviance	Resid. DF	Resid. Dev.	P (Chi)
leaf beetle					
null			159	184.21	
pop	1	2.0760	158	182.13	0.150
treatment	2	14.479	156	167.66	< 0.001
pop*treatment	2	2.2184	154	165.45	0.330

Table 1. Logistic regressions on the probability of attack by *Oreina* leaf beetles and infection by the rust *U. cacaliae*. In the upper table the treatment term has seven levels, in the middle table the treatments were grouped into two levels (induced/control), and in the lower table the *Oreina* data were reanalysed after grouping treatment into three levels (BTH/methyl-jasmonate and doubly treated/control).

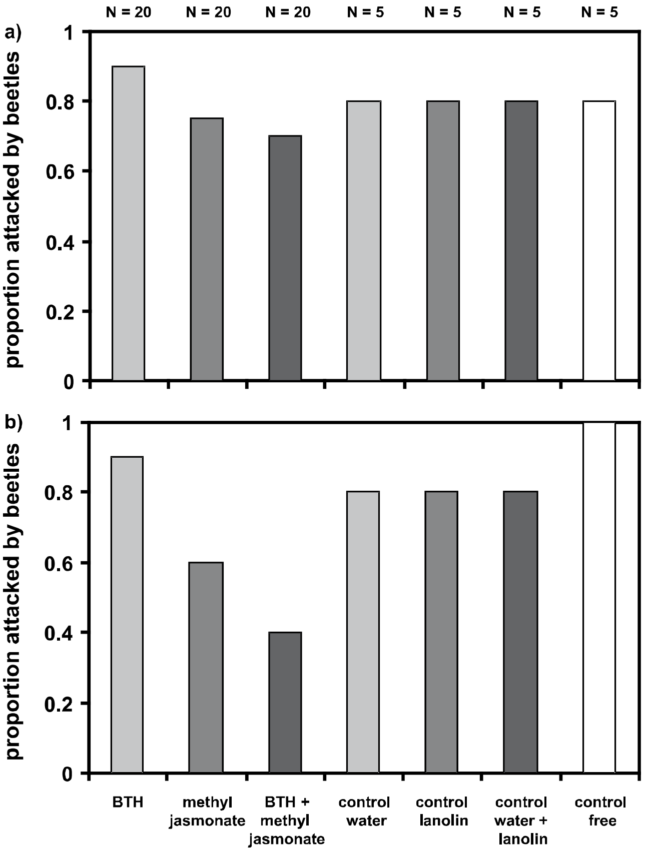

Fig. 1. Proportions of *A. alliariae* plants from (a) Emosson and (b) La Fouly attacked by the leaf beetles *O. elongata* and *O. cacaliae*. Three groups were treated with single or combined chemical inducers of plant defences, three others were used as their respective controls (the treatments and corresponding controls are shown in the same colour), and finally one group was left with no manipulation (free control in white).

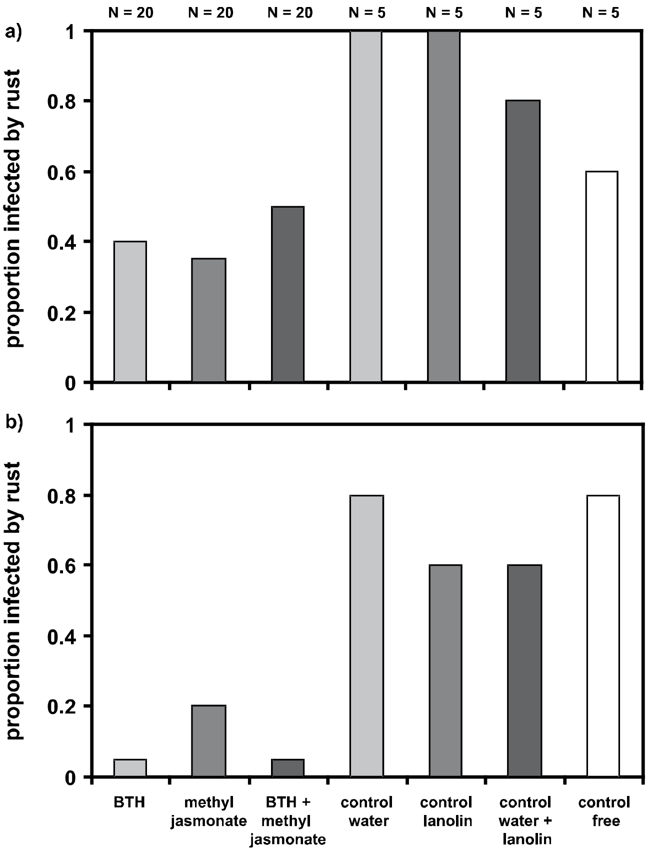

Fig. 2. Proportions of *A. alliariae* plants from (a) Emosson and (b) La Fouly infected by the rust *U. cacaliae*. Three groups of plants were treated with single or combined chemical inducers of defences, three others were used as their respective controls (the treatments and corresponding controls are shown in the same colour), and finally one group was left with no manipulation (free control in white).

Source	DF	SS	F	P value
Emosson				
treatment	6	55.124	9.1622	< 0.001
error	73	73.200		
La Fouly				
treatment	6	57.345	12.122	< 0.001
error	73	57.558		

Source	DF	SS	F	P value
Emosson				
treatment	3	52.525	15.828	< 0.001
error	61	67.477		
La Fouly				
treatment	3	51.756	20.181	< 0.001
error	61	52.148		

Table 2. ANOVAs on the percentage of leaf area consumed by beetles for the two populations of *A. alliariae* separately. The upper table compares all seven treatments, whilst in the lower table three controls were excluded to leave just four treatments (three induced treatments and the control un-manipulated plants).

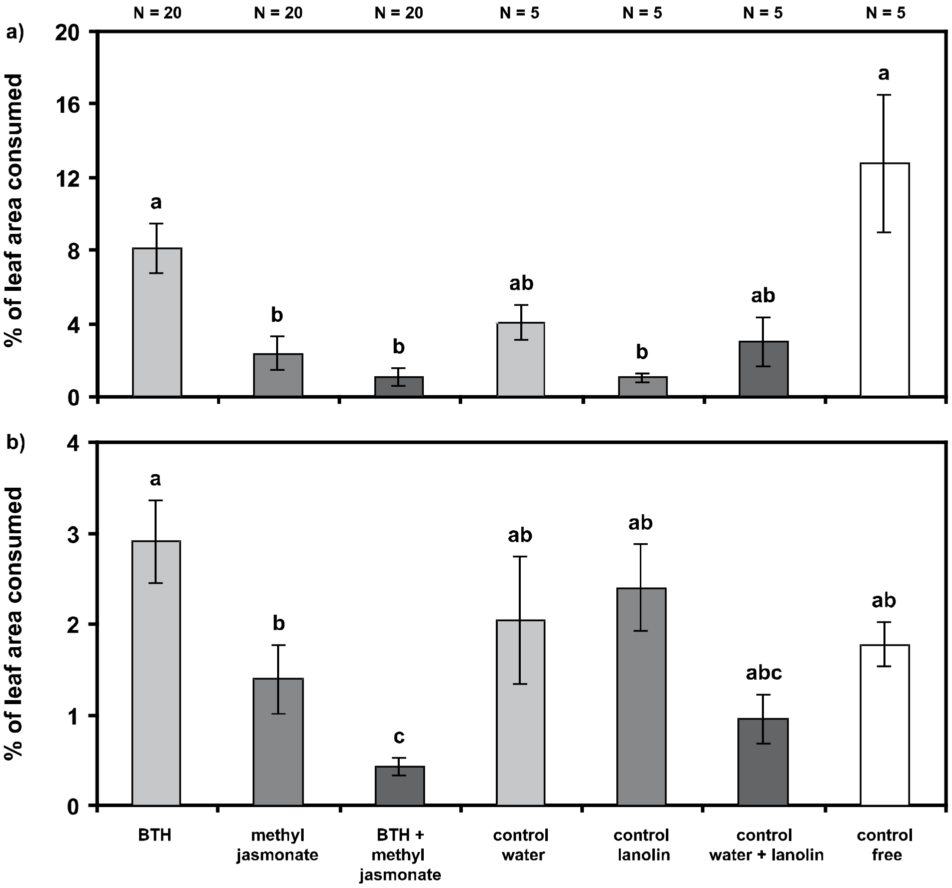

Fig. 3. *Oreina* leaf beetle consumption, for plants at (a) Emosson and (b) La Fouly under the seven treatments (see text for details). Graphs show the mean percentage area consumed per leaf ± standard errors. Bars which do not share a letter are significantly different in Tukey HSD tests within each population.

The treatments had a significant effect on the timing of beetle and rust attack in both populations (Table 3). Methyl jasmonate and doubly treated plants were attacked later by beetles, whilst the BTH treated plants were attacked more rapidly than the controls (Fig. 4). For rust infection, the plants treated with any of the chemical inducers showed later signs of disease than the controls (Fig. 5).

	Parameters	-2*LogLik	Likelihood ratio	DF	P (Chi)
leaf beetle					
null	2	488.77			
population	3	488.77	0.0003	1	0.985
treatment	9	456.70	32.073	6	< 0.001
population*treatment	15	450.66	6.0340	6	0.419
rust					
null	2	384.95			
population	3	382.34	2.6104	1	0.106
treatment	9	330.09	52.252	6	< 0.001
population*treatment	15	320.07	10.020	6	0.124

Table 3. Parametric survival analysis of the timing of leaf beetle and rust attack (beetle and rust data were analysed separately, with attack treated as “mortality”). The lines show the null model (with a single distribution location and scale parameter) and the change in log likelihood as terms for population (Emosson or La Fouly), treatment (seven levels), and the population by treatment interaction were sequentially added. The final three columns provide likelihood ratio tests of the significance of each term.

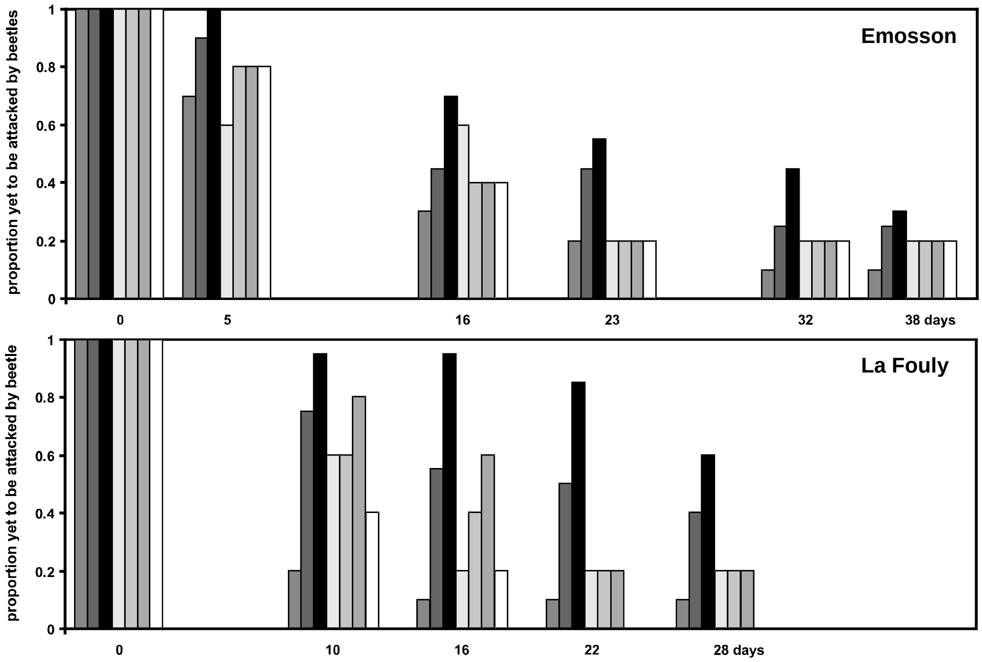

Fig. 4. Proportions of plants still free of attack by *Oreina* leaf beetles over time. For both populations, the time scales start on the first day of experiments (day 0) and continue linearly to show the timing of the attacks. The three induced groups of plants are shown with dark colours, while their control groups are paler.

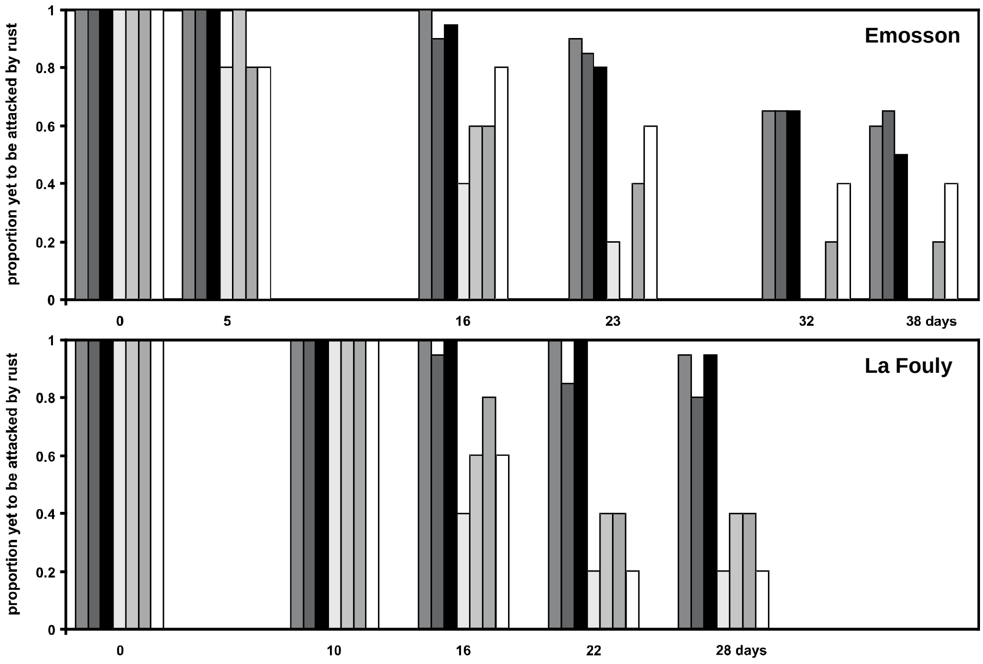

Fig. 5. Proportions of plants still free of infection by the rust *U. cacaliae* over time. For both populations, the time scales start on the first day of experiments (day 0) and continue linearly to show the timing of the infections. The three induced groups of plants are shown with dark colours, while their control groups are paler.

▨ 1 ▨ 2 ■ 3 □ 4 ▨ 5 ▨ 6 □ 7

1 = BTH (N = 20)
2 = methyl jasmonate (N = 20)
3 = BTH + methyl jasmonate (N = 20)
4 = control water (N = 5)
5 = control lanolin (N = 5)
6 = control water + lanolin (N = 5)
7 = control free (N = 5)

Are induced plants more successful in growth and reproduction?

For both populations, the growth rate did not differ significantly among treatments (Table 4 and Fig. 6) and neither did the number of leaves at the end of the experiment (Table 5 and Fig. 7). There was also no effect of treatment on the probability of flowering during the experiment (Table 6 and Fig. 8), or on the timing of flowering (Table 7 and Fig. 9), although plants at Emosson were more likely to flower and did so more rapidly.

Source	DF	SS	F	P value
Emosson				
treatment	6	1.5408	0.6574	0.684
error	73	28.516		
La Fouly				
treatment	6	0.9774	0.7537	0.609
error	73	15.776		

Table 4. ANOVAs on the growth rate (in cm/day) of *A. alliariae* plants in two populations under the seven treatments.

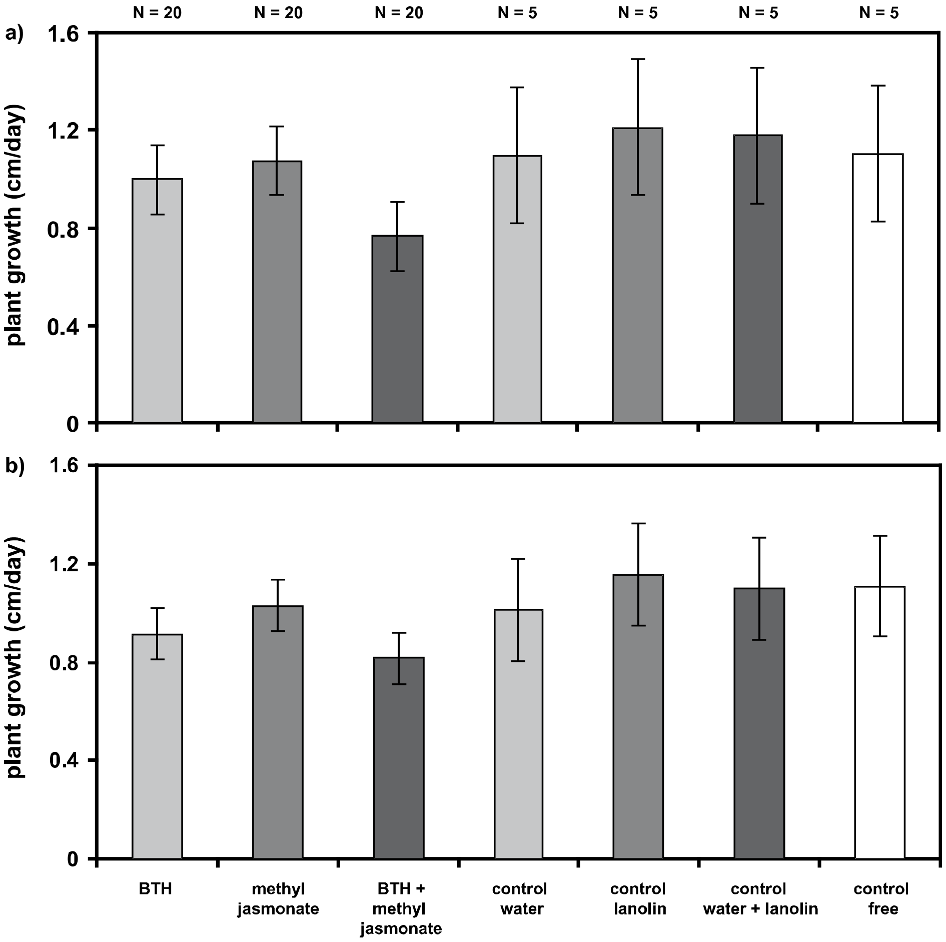

Fig. 6. The overall growth rate (in cm/day) of plants under the seven treatments at (a) Emosson and (b) La Fouly. Graphs show means ± standard errors.

Source	DF	Deviance	Resid. DF	Resid. Dev.	F	P (F)
null			159	26.617		
pop	1	0.0025	158	26.615	0.0139	0.906
treatment	6	1.0289	152	25.586	0.9554	0.458
pop*treatment	6	0.9283	146	24.658	0.8621	0.525

Table 5. Quasi-likelihood analysis based on a Poisson regression of the number of leaves on plants in two populations under the seven treatments.

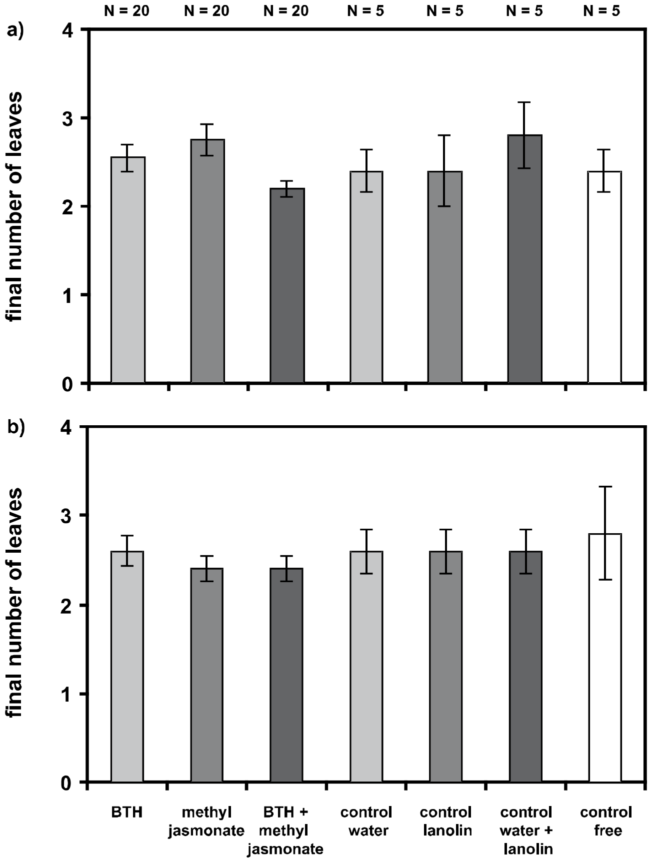

Fig. 7. Number of leaves per plant at the end of the experiments. Plants from (a) Emosson and (b) La Fouly began the experiment with two leaves, and were given one of seven treatments before being monitored over approximately one month. Graphs show means ± standard errors.

Source	DF	Deviance	Resid. DF	Resid. Dev.	P (Chi)
null			159	218.77	
pop	1	5.7709	158	213.00	0.016
treatment	6	10.745	152	202.26	0.097
pop*treatment	6	2.4382	146	199.81	0.875

Table 6. Logistic regression on the proportion of plants flowering during the month of the experiment in the two populations and under seven treatments.

	Parameters	-2*LogLik	Likelihood ratio	DF	P (Chi)
null	2	432.83			
population	3	427.29	5.5433	1	0.019
treatment	9	417.49	9.7995	6	0.133
population*treatment	15	415.36	2.1232	6	0.908

Table 7. Parametric survival analysis of the timing of flowering (in which flowering was treated as "mortality"). The lines show the null model (with a single distribution location and scale parameter) and the change in log likelihood as terms for population (Emosson or La Fouly), treatment (seven levels), and the population by treatment interaction were sequentially added. The final three columns provide likelihood ratio tests of the significance of each term.

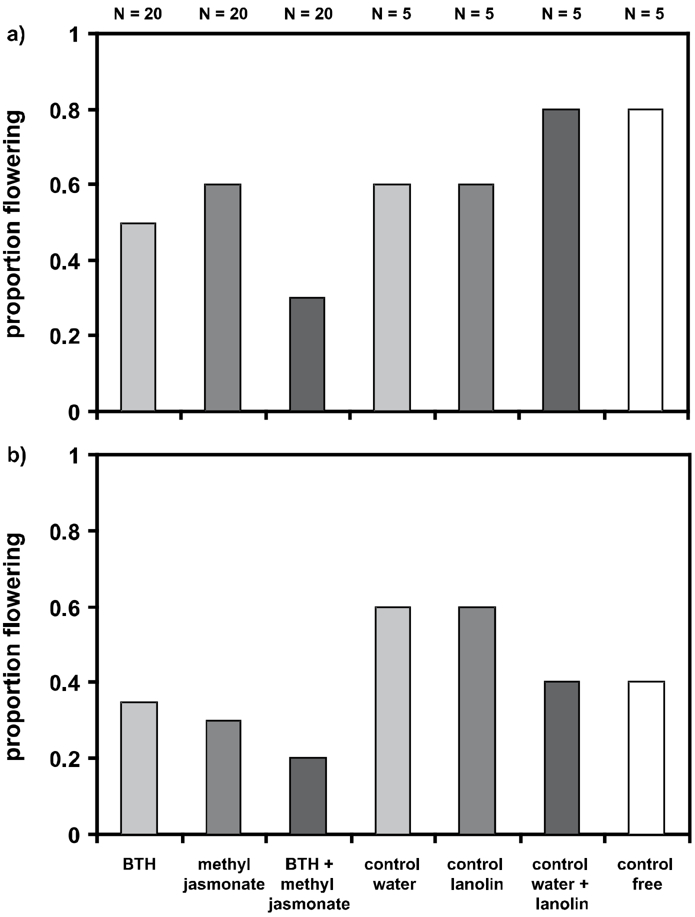

Fig. 8. Proportions of *A. alliariae* plants from (a) Emosson and (b) La Fouly producing flowers. Three groups were treated with single or combined chemical inducers of plant defences, three others were used as their respective controls (the treatments and corresponding control are shown in the same colours), and finally one group was left with no manipulation (free control in white).

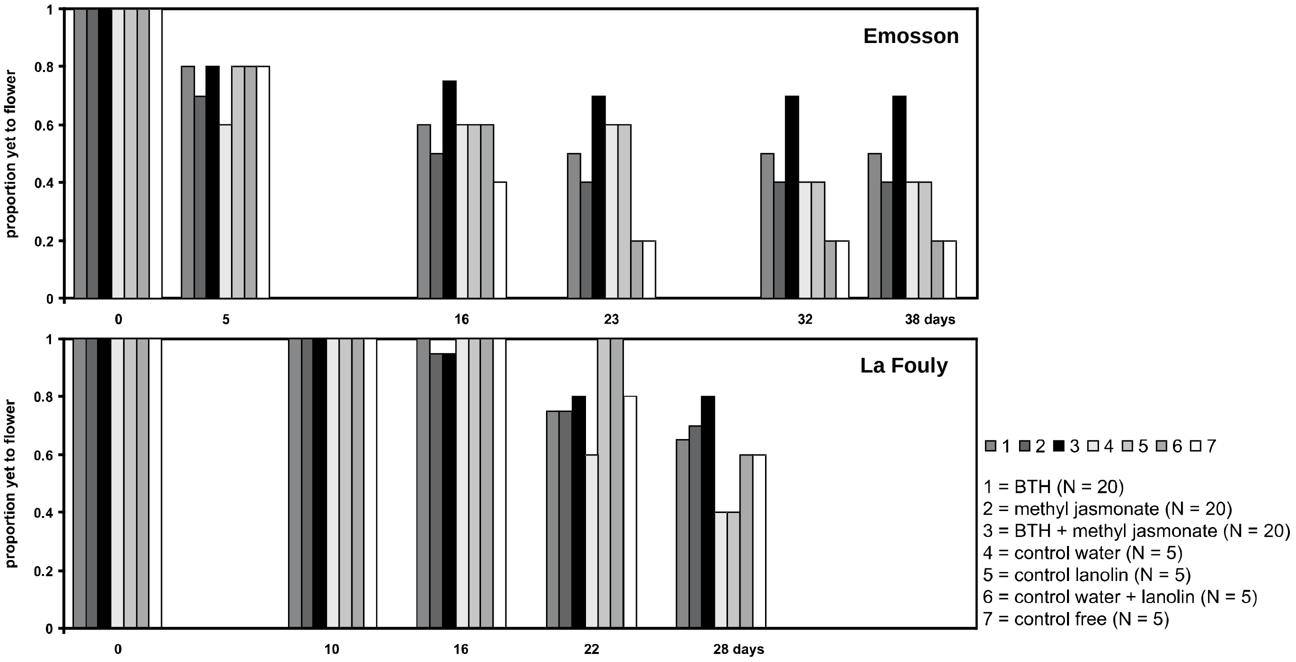

Fig. 9. Proportions of plants yet to flower over time. For Emosson and La Fouly populations, the time scales start on the first day of experiments (day 0) and continue linearly to show the timing of flowering. The three induced groups of plants are shown with dark colours, while their control groups are paler.

There was no association between attack by leaf beetles and infection by the rust (Table 8, G test of independence, $G_1 = 0.713$, $p = 0.398$).

Table 8. Data table for the analysis of the association between beetle attack and rust infection, showing the number of plants in each category.

count of plants	no rust infection	rust infection
no leaf beetle attack	28	14
leaf beetle attack	70	48

Discussion

The diversity of potential threats to plants is impressive. Large mammals can remove branches and even entire plants in a single bite, while other herbivores make less damage individually, but present a challenge due to their sheer numbers. Plants are also attacked by a diverse assemblage of parasitic micro-organisms, including viruses, bacteria, and fungi. Nonetheless, plants cover much of our planet. This simple observation implies that herbivores and pathogens are too few in number to consume all the food available, and perhaps more importantly, that plants are sufficiently defended against their potential herbivores and pathogens to avoid total consumption (Hairston *et al.* 1960). Over evolutionary time, plants have developed numerous adaptations against disadvantageous situations, in their responses to herbivores and pathogens, to extremes of hot and cold temperatures, to drought or flooding, and to a lack of mineral and nutritive elements. High altitude habitats in Europe provide some of the most demanding and variable conditions for their permanent inhabitants, with sudden shifts of temperature, unpredictable weather conditions, strong radiation, and a very short season of activity. For *Adenostyles alliariae*, the presence of a rust fungus and specialized herbivorous leaf beetles clearly adds another hazard to what is already a harsh existence. In this context, the response following attack is crucial, because individual plants that show inappropriate induced resistance may do less well than those that do not respond.

During the past decade, laboratory studies, and more rarely work in the field, have focused on the origins, the mechanisms, the costs, and the benefits of the induction of plant defences against herbivorous insects and pathogens, sometimes bridging the molecular and ecological levels (Farmer *et al.* 1992; Herms & Mattson 1992; Baldwin & Preston 1999; Halitschke *et al.* 2000; Heil 2001; Kessler & Baldwin 2002; Moore *et al.* 2003). Nonetheless, all these studies concerned the induced defences of plants and their potential cross-effects following attack or infection (Bostock 1999; Genoud & Metraux 1999; Bostock *et al.* 2001; Rostas *et al.* 2003; Taylor *et al.* 2004; Bostock 2005; Stout *et al.* 2006). In contrast, very few studies have been carried out in the field on artificial, preventive induction of defences against herbivores, pathogens, or both (Inbar *et al.* 1998; Iverson *et al.* 2001), and never in a natural system like that studied here. In this context, our results suggest the presence of induced resistance in *A. alliariae*, with effects on the leaf beetles *O. elongata* and *O. cacaliae* and on the rust *U. cacaliae*. In the field, plants artificially induced with chemical signalling compounds were less susceptible to be attacked and were attacked later in the season. If the first effect had already been observed (Iverson *et al.* 2001), the effects on the timing of attack are poorly known but particularly relevant in the alpine environment, allowing the plants to benefit from a part of the short summer season without the challenged posed by the two antagonists.

There is also evidence for interactions between the two signalling pathways. As in other studies, treatment with methyl jasmonate inhibited attack by both beetles and rust (Thaler *et al.* 2002a). In contrast, whilst BTH inhibited rust infection (Mauch-Mani & Metraux 1998; Achuo *et al.* 2004), surprisingly it promoted beetle attack. This suggests that there may be asymmetric cross-talk between defences and no simple mapping of jasmonic acid and salicylic acid defence pathways onto herbivore and pathogen attack respectively.

Despite the effects of the induction treatments on the rate of attack, there was little evidence for an effect on plant performance, when considering the leaf area consumed by beetles, growth rate, leaf production, or flowering. Similar observations have been seen before (Simms & Triplett 1994; Halitschke *et al.* 2000). There was actually a hint of a cost to the induction of defence, for although not significant, plants in the three induction treatments tended to show reduced growth and probability of flowering. Of course, a real test of the cost of induced defence and an explanation for why these defences are induced and not constitutive would require experiments in which plants are induced and then protected from attack by excluding the antagonists. In addition, perennial plants typically grow structures that allow them to adapt to living from one year to the next. These structures include bulbs, tubers, woody crowns, or rhizomes that allow them to survive periods of dormancy over cold or dry seasons during the year. In this context, longer experiments including the study of the below ground parts of *Adenostyles alliariae* would be relevant to evaluate the costs, the benefits, the defences and the tolerance strategies of the plants (Thompson 1998; Van Der Putten 2003).

At this point, *Adenostyles* host plants seem to use a strategy of tolerance during the second part of the summer. Tolerance can be considered as plant traits that decrease the negative fitness consequences of a given level of attack (Simms & Triplett 1994; Mauricio & Rausher 1997; Baldwin & Preston 1999; Eubanks *et al.* 2005). Several studies, however, suggest that tolerance of herbivory is not necessarily costly (Agrawal *et al.* 1999).

The analysis of the measures of leaf area consumed highlight the need for proper control treatments, showing a possible deterrent effect of the control lanolin and water treatments. It would therefore have been possible to draw a different conclusion if only the un-manipulated control had been included.

The differences between the populations over the month of the experiment suggest a difference in seasonality between sites. Experiments were started in late May at La Fouly and late June at Emosson, but this represented the same point in the season, when plants had emerged and produced two leaves. Over the month of the experiment, plants at Emosson were more likely to be attacked by rust, flowered more rapidly, and showed greater consumption by the beetles. Previous results have already shown differences between these two populations (see chapter 3), mainly due to their different exposition and altitude. The season appears to be compressed at the higher altitude Emosson site, where plants, leaf beetles, and rust fungi have only two months to complete their life cycle, whereas activity in La Fouly can start in late May and end in mid-September.

Despite the interaction of the defences against beetles and rust, there was no association overall between rust infection and beetle attack. This suggests that, at least at the time-scale of this month-long experiment, there were no negative effects of one attacker inducing defences that would repel a second attack, nor positive effects either from wounding by beetles promoting the establishment of the rust, or weakening of defences by the first attacker.

Our results suggest that *Adenostyles alliariae* possesses inducible resistance involving the jasmonic acid and salicylic acid pathways which seem to be capable of reducing the rate of beetle and rust attack in the field. Perhaps because of the short time scale, we were unable to demonstrate a concrete fitness benefit, but instead a remarkable tolerance by the host plant during the major part of the summer. Moreover, over the longer term, the defences might be critical in repelling enemies and allowing reproduction under the time stress of the alpine environment.

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SUMMARY & CONCLUSION

Summary

Chapter one

The first chapter describes the results of field and laboratory experiments carried out to investigate the effect of rust infection on the larval performance (growth rate, maximum weight, and development time), food preference, oviposition and larviposition choice, and dispersal behaviour of *O. elongata* and *O. cacaliae*.

Chapter two

In the second chapter, I describe laboratory experiments to investigate a paradoxical response of *Oreina* larvae to a diet in which they were shifted from healthy to infected leaves mid-way through development. Their response, to accelerate growth, mimics that seen when exposed to late season photoperiod.

Chapter three

In chapter three, the impacts on their host plant of attack by leaf beetles and infection by rust are studied. Two-week field experiments were used to investigate changes in leaf chemistry (C, H, N, and PAs) and performance (growth, number of leaves, and flowering) of the host plant following infestation.

Chapter four

The fourth chapter describes the result of artificial induction of defences in the field to test for induced responses to beetle and rust attack in *A. alliariae*.

Effects of the rust U. cacaliae on O. elongata and O. cacaliae leaf beetles

Both leaf beetle species seem to be the most affected organisms in this triangular system. Larvae reared on rust-infected leaves show a decrease in growth rate and maximum weight, as well as an increase in the number of days needed to complete their larval development. All these effects are likely to provoke disadvantages for the larvae and their future adult life, affecting survival and reproductive success. In order to minimize these consequences, both larvae and adults avoid rust-infected plants in the field. The means by which they select the suitable host plant remains unclear, but they neither have to feed on the rust-infected plant, nor to be exposed to telia and spores to choose among different leaves. In addition, females of *O. cacaliae* show preferences to lay their larvae on healthy plants. If forced to lay eggs on *A. alliariae*, the females of *O. elongata* do not show the same behaviour, with no preference. Nonetheless, they are known to prefer to lay eggs on the thistle *C. spinosissimum* in the field, which would avoid any contact of their eggs with the rust. All these observations suggest strong indirect effects of the rust on the various steps of the life cycle of the two *Oreina* species, increasing the difficulty of survival in the stressful conditions of high alpine environments.

Further experiments revealed a surprising influence of the arrival of the rust on the pre-diapause development of the larvae. Larvae fed on healthy leaves then switched onto rust-infected leaves of the host plant actually showed an acceleration of their growth rate and a reduced development time, but then pupated at a lower body weight. This paradoxical reaction to poor quality food reveals that plasticity of their growth rates can be an extremely flexible strategy, allowing a response to a variety of cues under the time stress encountered in high altitude habitats.

Effect of the two leaf beetle species on the rust U. cacaliae

Asymmetric consequences are frequently observed in three-way interactions and this study seems to show that the *Oreina* leaf beetles have very little influence on the rust *Uromyces cacaliae*. The transport of infectious fungal material by the beetles is rare and unlikely to be a major source of transmission. In addition, both tests concerning the association between the attacks by the beetles and infection by the rust suggested that these processes are independent in the field. *Oreina* leaf beetles are also unlikely to have direct negative effects on the phytopathogenic fungus, as they avoid rust-infected plants.

Effects of the leaf beetles and the rust on their host plants

At the end of the summer, populations of *A. alliariae* plants are dramatically infested by both herbivores and rust. These attackers have undoubtedly a role in their rapid senescence at the end, but during the rest of the season, the plants may not be as strongly affected. Nonetheless, the rust seems to induce some disadvantages, like the reduction in growth and flowering. Overall, the changes in carbon, hydrogen, nitrogen, and pyrrolizidine alkaloid (PA) concentrations through time were similar in healthy and infested plants (by beetles, rust, or both), suggesting little effect of attack on host plant chemistry. Earlier findings of poor larval performance on rust-infected hosts may therefore be a result of fungal metabolites or induced defences rather than simple shifts in the concentrations of elements in the plant. The probability of infection by the rust did not show any relationship with the concentrations of elements or PAs. Used in the defences of the host plant, the PAs were related to carbon levels, suggesting a variable investment strategy by the plant. All these results suggest that there are some costs to *A. alliariae* of attack by rust and leaf beetles, but that they are remarkably tolerant.

The investigation of induced responses in *A. alliariae* revealed some participation of pathways activated by JA and SA mimics. Induced plants in the field faced reduced attack by beetles and by rust, and there was evidence for cross-talk between the systems. However, there was little evidence for an overall effect on plant fitness during the short-term experiment.

Maybe further investigations in emission of volatile compounds as well as in production of fungal metabolites and toxins would be necessary to explain some of these interactions.

Conclusion

Both *Oreina elongata* and *Oreina cacaliae* leaf beetles are strongly affected by the presence of the rust *Uromyces cacaliae*, but they have developed strategies to avoid it where possible, involving detection, displacement, oviposition or larviposition choice, and the use of multiple host plants. The arrival of the fungal antagonist can not be positive for these herbivores, but larvae of both species show a remarkable use of the arrival of the pathogen and combine this signal with their natural plasticity of growth in order to escape likely worsening conditions. The rust does not seem to derive advantages from the presence of the beetle, with infection of *A. alliariae* plants seemingly independent of beetle attack. The host plants survive despite the presence of their antagonists, probably by virtue of early emergence, fast growth and flowering, some induced defences, and tolerance.

These varied mechanisms all the three protagonists to coexist and participate in a complex web of direct and indirect interactions with consequences at different levels of the ecology of all parties, from individual behaviour and reproductive success through to population dynamics and fitness.

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HOBBIES

- Scuba diving, alpinism, sport
- Nature, entomology and herpetology
- Photography
- Travel

PROFESSIONAL EXPERIENCE

PhD thesis – Ecological interactions between two species of leaf beetle, a rust fungus, and their host plant	*Neuchâtel, Switzerland*	2003 – 2007
Biology teacher – Lycée Denis-de-Rougemont	*Neuchâtel, Switzerland*	2005 – 2007
Teaching assistant – Practicals in entomology	*Neuchâtel, Switzerland*	2004 – 2006
Science teacher – Lycée Jean-Piaget, ESCN	*Neuchâtel, Switzerland*	2004 – 2005
Scuba diving guide – Red Sea Diving College	*Sharm El Sheikh, Egypt*	2003
Insects curator – Natural History Museum of Neuchâtel	*Neuchâtel, Switzerland*	2003
Scientific assistant – Plant Survival National Centre of Competence in Research	*Neuchâtel, Switzerland*	2003
Master thesis – First study of the coleopteran biodiversity of Shipstern Nature Reserve in Belize, Central America	*Sarteneja, Belize*	2002 – 2003
Biology teacher – Ecole des Métiers de la Terre et de la Nature, CPLN	*Cernier, Switzerland*	2002 – 2007
Shop assistant (sale & management) – Terres d'Aventures (travelling, outdoor, trekking equipment)	*Geneva, Switzerland*	1999 – 2002

Gregory Röder, biologist

EDUCATION

PhD in chemical and evolutionary ecology	*Neuchâtel, Switzerland*	2003 – 2007
Master in sport, physical education teacher	*Neuchâtel, Switzerland*	2004 – 2006
PADI instructor development course, scuba instructor	*Hurghada, Egypt*	2004
Master in biology, options in ecology and systematics	*Neuchâtel, Switzerland*	1998 – 2003
Secondary school certificate, scientific type	*Geneva, Switzerland*	1994 – 1998

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PUBLICATIONS

Röder G, Rahier M and Naisbit R E (in preparation). A field test for induced defences against leaf beetles and phytopathogenic rust in the alpine plant *Adenostyles alliariae*

Röder G, Rahier M and Naisbit R E (in preparation). Does beetle attack increase probability of fungal infection and cause changes in leaf chemistry and performance in the host plant *Adenostyles alliariae* ?

Röder G, Rahier M and Naisbit R E (in revision). How should juveniles react to low quality food ? A paradoxical response by two species of alpine leaf beetle

Röder G, Rahier M and Naisbit R E (2007). Coping with an antagonist: the impact of a phytopathogenic fungus on the development and behaviour of two species of alpine leaf beetle. Oikos. 116: 1514-1523

Roeder G (2003). Coleopteran biodiversity of Shipstern Nature Reserve in Belize, with a comparison of the fauna of two tropical forest types. Open access: www.shipstern.org/documents/showFile.asp?ID=1952

CONFERENCES

Biology 06 – February 2006 – *Geneva, Switzerland* – Poster – A paradoxical response by alpine beetles to poor quality food: rust infection as a signal that winter is coming

Biology 05 – February 2005 – *Basel, Switzerland* – Poster – Interactions between a foliar disease (rust) and the herbivorous leaf beetles *Oreina cacaliae* and *Oreina elongata* (Coleoptera: Chrysomelidae) on their host plant *Adenostyles alliariae*

APPENDIX

Poster 1

Interactions between a foliar disease (rust) and the herbivorous leaf beetles *Oreina elongata* and *Oreina cacaliae* (Coleoptera: Chrysomelidae) on their alpine host plant *Adenostyles alliariae*

Poster presentation in Biology05, Annual meeting of the Swiss Zoological, Botanical and Mycological Societies, Basel, February 2005.

Poster 2

A paradoxical response by alpine beetles to poor quality food: rust infection as a signal that winter is coming

Poster presentation in Biology06, Annual meeting of the Swiss Zoological, Botanical and Mycological Societies, Geneva, February 2006.

Interactions between a foliar disease (rust) and the herbivorous leaf beetles *Oreina elongata* and *Oreina cacaliae* (Coleoptera: Chrysomelidae) on their alpine host plant *Adenostyles alliariae*

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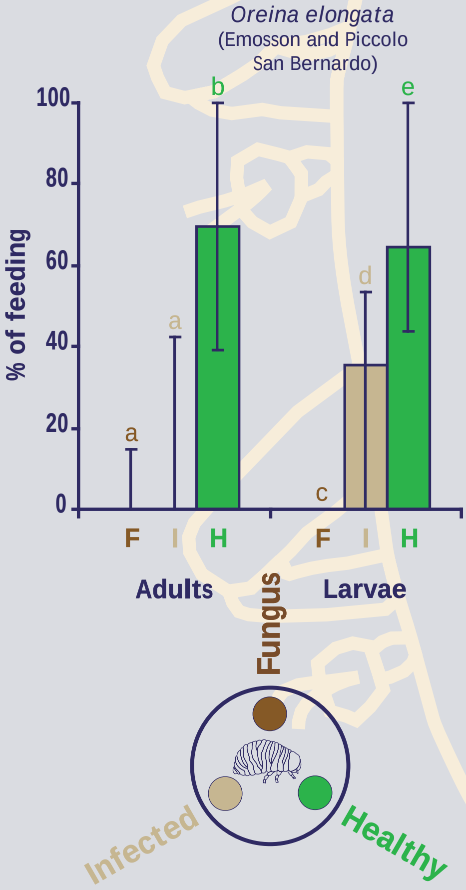
Introduction

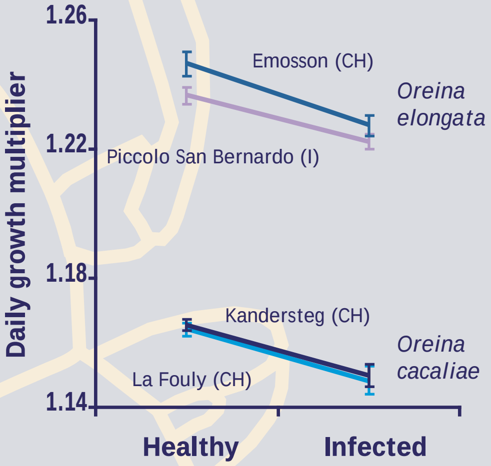
Individually, both pathogenic fungi and herbivorous insects are known to have important effects on plant populations. In turn, plants form the focus for three-way interactions between fungi and insects.

This study tests the indirect effects of a systemic infection of *Adenostyles alliariae* by the rust fungus *Uromyces cacaliae* on the leaf beetles *Oreina elongata* and *Oreina cacaliae*, by examining larval performance, and larval and adult food plant preferences.

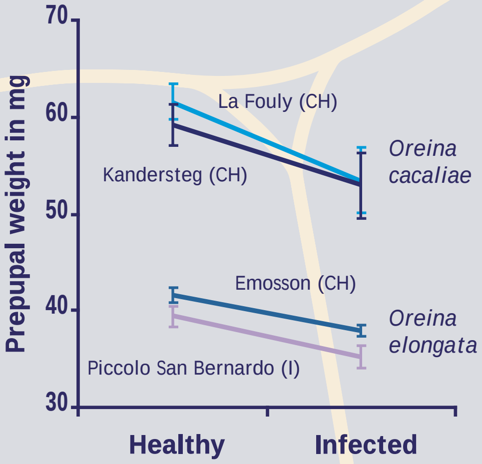


In a three-choice experiment, adults and larvae of both species preferred leaf discs from healthy plants over those containing the rust.

Results



Feeding on infected leaves significantly reduced the growth rate of larvae of both species from all populations.



The prepupal weight of larvae was lower when they had been fed with infected leaves.

Conclusion

Both species avoided infected host plants for adult and larval feeding, and fungal infection negatively affected larval development. Rust infection could therefore influence the distribution and population dynamics of *Oreina* beetles.

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A paradoxical response by alpine beetles to poor quality food: rust infection as a signal that winter is coming

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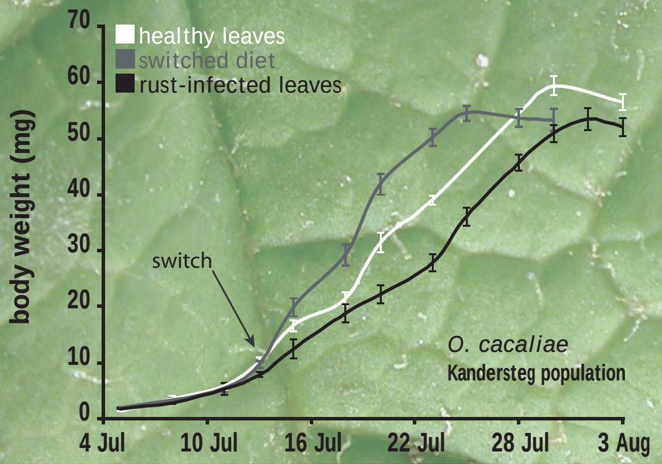
Introduction

Alpine species face extreme time stress during their breeding season, with only a very limited period to complete their life cycle while the habitat is free of snow. The leaf beetles *Oreina elongata* and *O. cacaliae* face a second challenge: competition on their host plant with the rust fungus *Uromyces cacaliae*.

By the end of the summer most plants of *Adenostyles alliariae* are infected, and larvae that feed on such plants show a reduced growth rate, a longer development time, and lower prepupal weight.

Method

Here we test the effect of switching larval diet after one week from healthy to infected leaves.

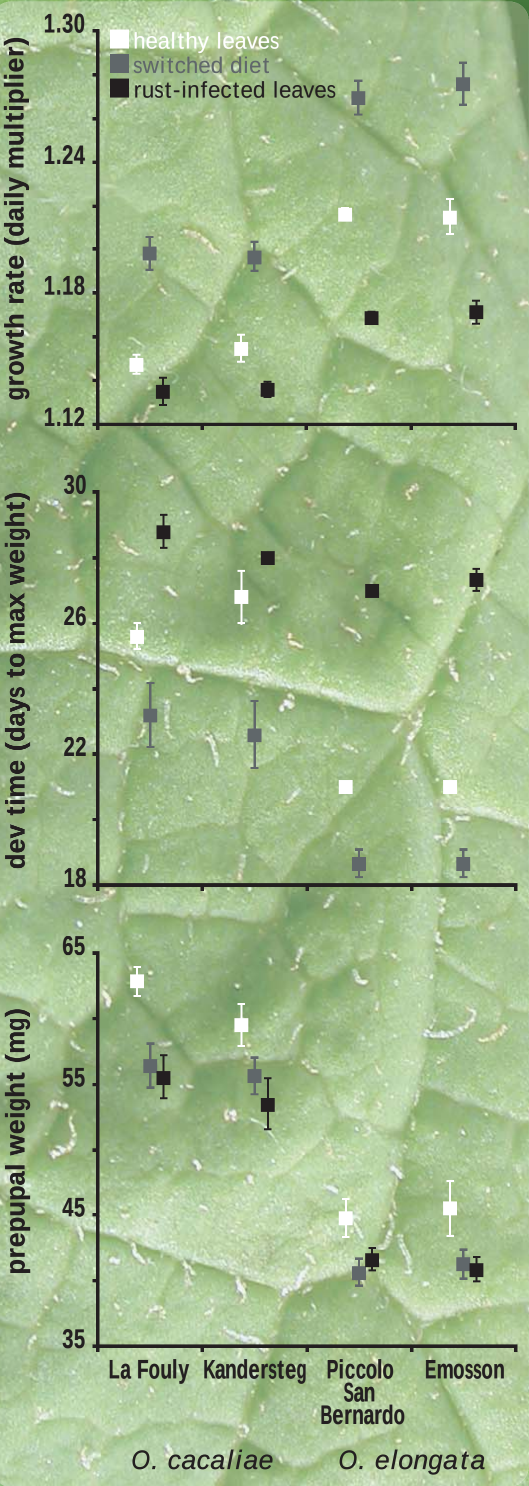


Conclusion

Larvae seem to use the arrival of the rust as a signal that the season is drawing to a close. They accelerate growth and despite a lower body weight, shorten development time to pupate before the winter arrives.

Results

Larvae switched from healthy to rust infected leaves unexpectedly showed an increase in their growth rate and reduced development time, but also a reduced prepupal weight.



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