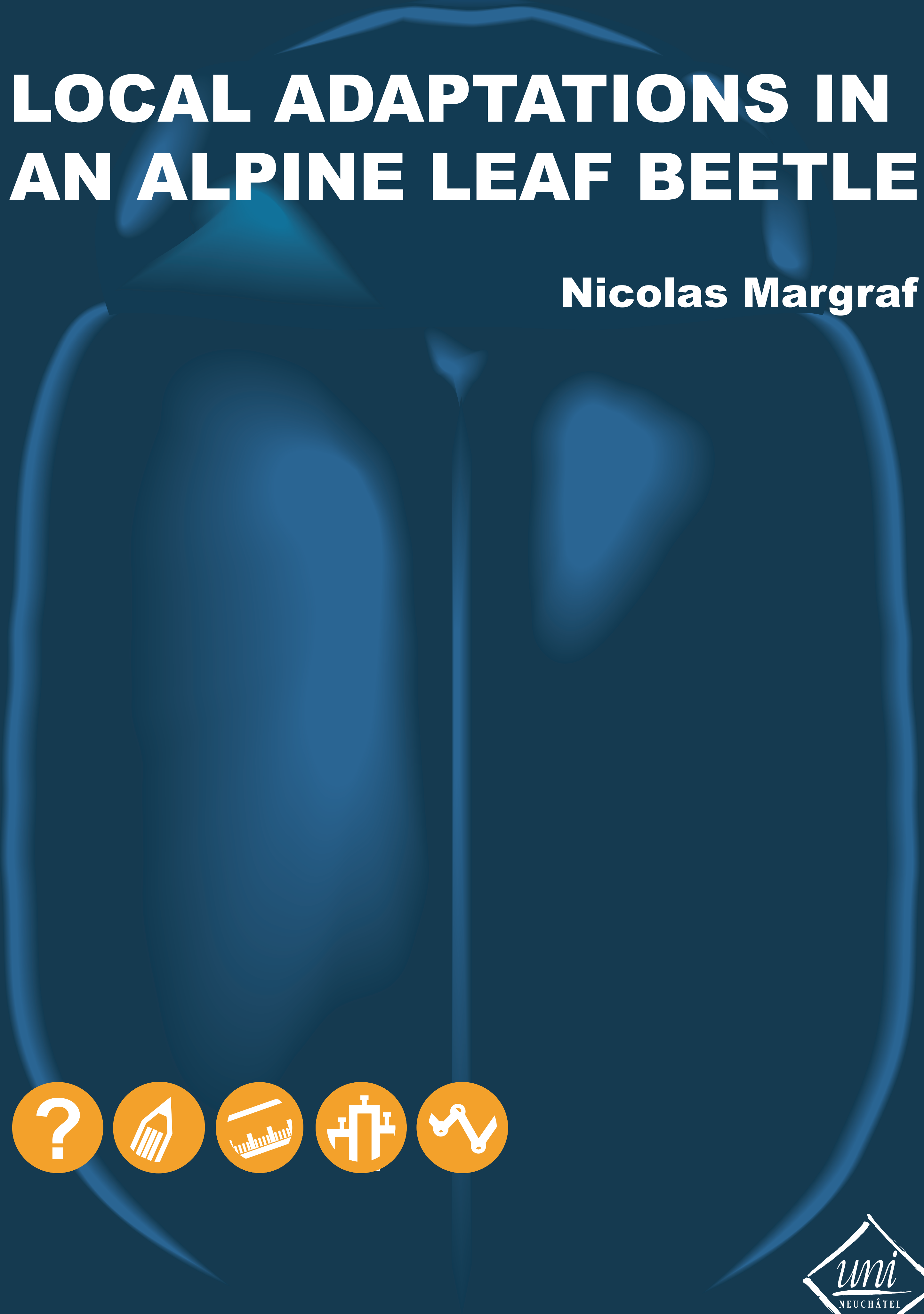

LOCAL ADAPTATIONS IN AN ALPINE LEAF BEETLE

Nicolas Margraf

uni
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IMPRIMATUR POUR LA THESE

Local adaptations in an alpine leaf-beetle

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Neuchâtel, le 25 septembre 2003

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Résumé

L'adaptation locale ainsi que la spécialisation sur un hôte ont souvent été considérés comme les fruits de l'action combinée de trois forces : la sélection naturelle, la dérive génétique et le flux génique. Chez les insectes phytophages, la relation entre l'herbivore et sa plante hôte est si intime que les pressions de sélection entraînant la spécialisation de l'insecte, ne sont souvent envisagées qu'au travers de la plante.

Dans notre étude, nous avons examiné les adaptations locales chez la Chrysomèle alpine *Oreina elongata* dont les populations présentent un sélection variable des quatre plantes hôtes connues chez ce coléoptère. Nous avons testé plusieurs caractères susceptible de révéler des adaptations locales liées à plantes hôtes et qui participent au processus de spécialisation.

Nous n'avons pas mis en évidence de différence entre les populations quant aux performances larvaires, ni aux préférences d'oviposition qui puissent être attribuées à l'utilisation d'un certain type de plante en nature. Cependant, nous avons mis en lumière des adaptations comportementales des populations présentant plusieurs type d'hôtes qui permettent aux larves de ces dernières de bénéficier des avantages liés à l'utilisation de plusieurs hôtes.

Dans une étude génétique comprenant 13 populations et mettant en œuvre des marqueurs microsatellites, nous avons montré la forte structuration génétique des populations d'*O. elongata*, ce qui nous a permis de désigner la dérive génétique comme facteur principal de différentiation génétique entre populations. L'affiliation géographique s'est révélée être bien plus fortement corrélée avec les différences génétiques que l'utilisation d'un type de plante. Les groupes formés par les populations se sont démontrés monophylétique et correspondent aux sous-espèces confirmant ainsi la différentiation morphologique en sous-groupe.

Nous avons pu rejeter l'idée d'une spécialisation liée à la plante hôte chez *O. elongata* et nous avons conclu que les facteurs qui sont susceptibles de conduire cette espèce dans un processus de spéciation sont des forces de sélection indépendantes des plantes hôtes mais qui comprennent un phénomène de dérive génétique souligné par un flux génique faible.

Abstract

Local adaptation and host specialization is often seen as the result of the balance between three major forces: natural selection, genetic drift and gene flow. In phytophagous insects, the relationship between the insect and its host plant is so intimate that the selective pressures leading to local adaptation are often seen as being related to host plant use only.

In this study, we investigate local adaptation in populations of the alpine leaf-beetle *Oreina elongata* that differ in the availability of the four host plant of the species. We tested several traits that are susceptible to adapt according to the host plant locally available and that are known mechanisms of specialization.

We found no differences among populations in larval performance and oviposition preferences that were attributable to differences in host plant use in the field. However, we revealed a behavioural adaptation of a two host population that allows individuals to take advantage of the presence of both plants at this location.

A genetic survey using microsatellite markers on 13 populations of the species showed a highly genetically structured distribution and designated genetic drift as the main factor in population genetic differentiation. Instead of host plant use, regional affiliation turned out to be strongly correlated to genetic differences. Populations perfectly clustered according to their subspecies, therefore confirming previous morphological work.

We can then dismiss the idea of host plant specialization in *O. elongata* and conclude that the factors that may ultimately drive *O. elongata* towards a speciation event are selective forces independent of host plant use but genetic drift supported by very weak gene flow.

MERCI – THANK YOU – GRAZIE – TACK

Prof M. Rahier ● Prof J. McNeil ● Dr F. Bigler ● Prof P. Küpfer ● The Dude ● Le Russ ● Jenny ● Mango-Aline ● Councoun ● Rasmann ● Dal Ness & Tom ● Le Lhoo ● M et Mme Nu ● The Oreina team ● The six-arm olfactometer and its team ● The Bean team ● Les gens du LEAE ● LFB et l'éco-éthol ● Sophie et la Bioche ● Roberto et la Phanéro ● Brigitte et Nat ● Josiane et les gens des livres ● Prof B. Lemaire ● Dr P. Mardulyn ● Y. Borcard ● cMo ● L'Association Internationale du Jardin Alpin de la Chanousia ● Barbara ● Mme Vaser ● E. Noussan ● ECAR ● L'Ordine Mauriziano di Torino ● ECOGENICS GmbH ● Il Museo Civico di Storia Naturale di Verona ● SNF ♥ Camille ♥ Papa et maman

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Conferences:

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"Biology01, Annual meeting of the Swiss Zoological, Botanical and Mycological Societies", Neuchâtel, February 2001, Poster presentation: "Growth rate plasticity in an alpine leaf beetle", N. Margraf, K. Gotthard and M. Rahier.

Publications:

Margraf N., Gotthard K. and Rahier M. (2003) The growth strategy of an alpine beetle : maximization or individual adjustment in relation to seasonal time horizon. Functional Ecology, 17(5): 605-610

Margraf N, Verdon A, Naisbit RE and Rahier M (in prep) Host plant availability and local adaptation in larval performance: a quantitative genetic study in the leaf beetle *Oreina elongata*.

Margraf N and Rahier M (in prep) Host plant specialization or geographic isolation ? Popula-tion genetics of an alpine leaf-beetle.

Gotthard K, Margraf N and Rahier M (in prep) Geographic variation in oviposition choices of a Leaf-Beetle: The relationship between host plant ranking, specificity and motiva-tion.

Gotthard K, Margraf N, Rasmann S and Rahier M (in prep) The evolution of larval foraging behaviour in response to variation in host plant range among populations of a special-ized leaf beetle.

Scriber M. J., Margraf N. and Well T. (2000) Suitability of four families of Florida "bay" species for *Papilio palamedes* and *P. glaucus* (Papilionidae). Journal Lepid. Soc., 54(4): 131-136

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PAPER one 27

Margraf N, Gotthard K, Rahier M (2003) The growth strategy of an alpine beetle: maximization or individual growth adjustment in relation to seasonal time horizons? *Functional Ecology*: 17(4).605-610.

PAPER two 39

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PAPER three 53

Margraf N, Rahier M (in prep) Host plant specialization or geographic isolation ? Population genetics of an alpine leaf-beetle.

PAPER four 69

Gotthard K, Margraf N, Rahier M (submitted) Geographic variation in oviposition choices of a Leaf-Beetle: The relationship between host plant ranking, specificity and motivation.

PAPER five 81

Gotthard K, Margraf N, Rasmann S, Rahier M (in prep) The evolution of larval foraging behaviour in response to variation in host plant range among populations of a specialized leaf beetle.

APPENDIX appendix 99

Margraf N, Gotthard K, Rahier M (poster) Weather conditions influences host-plant choice in an alpine leaf-beetle.

OUTLINE outline

OUTLINE OF THE THESIS

Abstract

Local adaptation and host specialization is often seen as the result of the balance between three major forces: natural selection, genetic drift and gene flow. In phytophagous insects, the relationship between the insect and its host plant is so intimate that the selective pressures leading to local adaptation are often seen as being related to host plant use only.

In this study, we investigate local adaptation in populations of the alpine leaf-beetle *Oreina elongata* that differ in the availability of the four host plant of the species. We tested several traits that are susceptible to adapt according to the host plant locally available and that are known mechanisms of specialization.

We found no differences among populations in larval performance and oviposition preferences that were attributable to differences in host plant use in the field. However, we revealed a behavioural adaptation of a two host population that allows individuals to take advantage of the presence of both plants at this location.

A genetic survey using microsatellite markers on 13 populations of the species showed a highly genetically structured distribution and designated genetic drift as the main factor in population genetic differentiation. Instead of host plant use, regional affiliation turned out to be strongly correlated to genetic differences. Populations perfectly clustered according to their subspecies, therefore confirming previous morphological work.

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Introduction

One of the most striking characteristics of the living world is its extraordinary diversity. This diversity can be understood to include two equally important aspects: diversity of species and diversity of interactions between species, with the two aspects linked through the processes of specialization and coevolution (Darwin 1859; Thompson 1994).

One of the groups that perhaps best demonstrates this extraordinary diversity is the group of insects. Indeed, half of the presumed biodiversity is thought to be due to insects and particularly to phytophagous insects (Bernays and Chapman 1994; Futuyma 1991; Jaenike 1990; Thompson 1994). Assuredly because of their adaptation-rich association with species of plant, herbivore insects have grown to be one of the biggest groups of all organisms. The estimated number of species on the planet varies between authors but the contribution of specialist herbivore insects to the biodiversity makes no doubt. Out of the 50,000 moths collected by Janzen between 1977 and 1987 in the Santa Rosa National Park in Costa Rica, one half of the species were considered as feeding on only one plant species and most of the remainder feed on only a few chemically or taxonomically related hosts (Janzen 1988). This illustrates the importance of specialization for biodiversity, especially in herbivore insects.

However, the process of specialization goes necessarily through the process of adaptations.

ADAPTATION

The association between a specialist insect herbivore and its host plant is intimate. In many cases, the whole life cycle of the herbivore happens on or in the close vicinity of its host plant. The host plant not only represents the places where the insect lives but also its food provider, its shelter against both predator and harsh weather conditions and sometimes its chemical defence supplier. This close association entails adaptation of the insect to its host plant. Through natural selection the host plant shapes numerous life history traits of the herbivore, like oviposition preference of the female, developmental parameters of the larvae, adults feeding preference, etc…

In oligophagous or generalist species, these traits are under sometimes converging sometimes diverging natural selection forces related to the various hosts. These conflicting forces occasionally lead to the formation of host races. Following the pioneering work by Bush (Bush 1969), there is a whole body of evidences that polyphagous herbivores ca form distinct "races" adapted to sympatrically distributed host species. Still, the observed differences between populations may be adaptive, due to selection, or non-adaptive due to genetic drift. This raised the question of whether herbivores are locally adapted to their host plant.

There are three different agents that act together to determine the level of genetic divergence of herbivore populations (Endler 1977; Slatkin 1973; Slatkin 1987). The first is the natural selection which may favour different types in different environments, leading to local adaptation. The second agent, genetic drift, creates genetic differentiation between populations. The third, the counteracting force of gene flow tends to eliminate genetic structure produced by natural selection and prevents random genetic drift. There is then a balance between gene flow and genetic drift on one side and selection on the other that determines the potential for local adaptation.

NATURAL SELECTION

Natural selection is the force that drives adaptation. It operates at the level of genes favouring different combinations in different environments, by the increased fitness of adaptive sets. In other words, individuals that possess a well adapted combination of genes will exhibit an increased fitness while a maladapted set will contribute poorly to the next generation.

Local adaptation can be the result of differing selective regimes imposed by different environments. In theory, spatial variation in the environment and accordingly in selective pressure should promote local adaptation. In this case, the genetic correlation between life history traits across environments should evolve to become negative because adaptation to one environment is often associated with costs of adaptation in the alternate environment. These costs of adaptations are either due to genes that are selected for in one environment and deleterious in the other (antagonistic pleiotropy) or to neutral mutations that accumulate in one environment and are deleterious in the alternative environment (Thompson 1996).

In phytophagous insects, this phenomenon creates a between plant trade-off that prevent individuals from maximizing fitness simultaneously on two or more hosts. This has been hypothesized to explain the high incidence of specialist insect herbivores.

GENETIC DRIFT

Because all populations are finite, alleles at all loci are potentially subject to random genetic drift – but all are not necessarily subject to natural selection. Genetic drift is due to random sampling of alleles during sexual reproduction that generates random fluctuations in their frequency. It assures that in a finite population, at a neutral locus, one allele will ultimately drift and reach fixation – its frequency P will get to one – and that the average time required to reached fixation is shorter, the smaller the population. Because of genetic drift, the frequency of heterozygotes declines and ultimately reaches zero. Hence, unless opposed by gene flow or natural selection, genetic drift reduces genetic variation in a population.

Wright's F-statistics are one of the indirect methods to estimate genetic structure in natural populations and are therefore a powerful tool to detect genetic differentiation due to random genetic drift. F-statistics are a set of tools devised by Wright (Wright 1921; Wright 1969) to partition heterozygote deficiency into within and among population component. Fis measures the heterozygote deficit within populations, Fst among populations (a measure of the Wahlund effect), and Fit the global deficit of heterozygotes. There are several methods to estimate F-statistics that have been debated in the literature for the past 30 years (see review in (Excoffier 2001)).

GENE FLOW

Gene flow among populations is widely considered to be an important factor influencing the evolution of both local adaptation and speciation (Endler 1977; Mayr 1963; Slatkin 1985a). In theory, natural selection can overcome the effects of genetic drift (Fisher 1958; Wright 1931) but moderate gene flow can prevail against local adaptation even if natural selection is strong (Slatkin 1973; Slatkin 1985a). It has been suggested that if gene flow among populations proceeds over short distances, local adaptation should arise at a finer scale than in species with widespread gene flow (Peterson and Denno 1998b; Slatkin 1973). Similarly, factors that diminish gene flow among populations are supposed to favour local adaptation and therefore allow populations to diverge as far as forming separate species (Bush 1975; Futuyma and Mayer 1980; Wiegmann et al. 1993). Various authors have suggested a number of factors that govern the level of gene flow occurring among natural populations. These include geographic distance, dispersal ability, ecological specialization, phenological isolation, habitat patchiness, habitat persistence and population age (see references in (Peterson and Denno 1998a)).

There are direct (e.g. mark-release-recapture studies) and indirect methods to estimate gene flow between populations. One of the indirect method uses Fst from the F-statistics (see below) to assess Nm, the effective number of migrant (Wright 1951). However, the same estimate can be calculated using the rare allele method (Slatkin 1985b).

outline

Material and Methods

STUDY ORGANISM

The genus *Oreina* (Chevrolat) comprises 26 species that are palearctic and herbivorous. They feed exclusively on two plant families: Asteraceae and Apiaceae and are either monophagous or oligophagous (Hsiao and Pasteels 1999). Several species of this family have the ability to sequester plant secondary compounds to use them for their own defence.

Oreina elongata (Suffrian) (Coleoptera: Chrysomelidae) is one of them. This alpine leaf-beetle has an exclusively European distribution that covers the Alps and the Apennines (Appenino-alpine distribution). Adults and larvae of this oligophagous species feed on four hosts belonging to two genera of the Asteraceae family: *Cirsium* and *Adenostyles*.

The three *Adenostyles* species, *Adenostyles alliariae*, *A. glabra* and *A. leucophylla* present hearth shaped leaves that contain pyrrolizidine alkaloids (PAs in the following) which the insect, adult and larvae sequester for their defence. Conversely, *Cirsium spinosissimum* is the only species in *O. elongata*'s diet that does not produce PAs but instead provides mechanical defences due to its spiny and hairy leaves. When *O. elongata* feeds on the latter host, the beetle produces cardenolides as an alternative to PAs (Dobler and Rowell-Rahier 1994). However, this defence strategy is not as efficient as PA sequestration at least when faced to naïve bird predator (Rowell-Rahier et al. 1995).

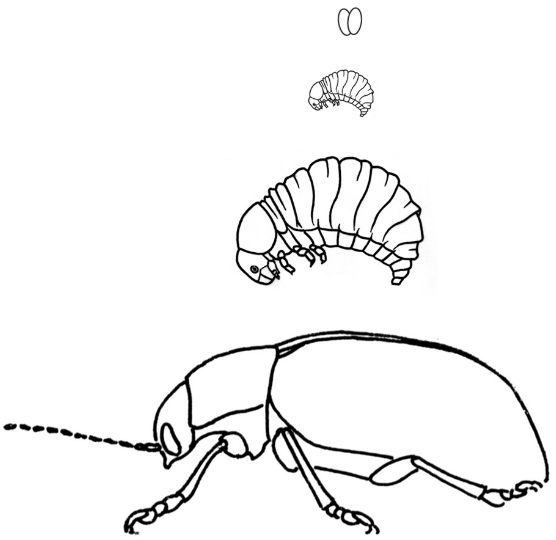

Figure 1. *Oreina elongata adult, larvae and egg*

The high alpine environment has selected *O. elongata* to extend its life cycle over three years. Throughout July of the first year, females lay singly on the host plant up to about 300 eggs from which two weeks later first instar larvae will hatch and start to feed. After around 25 days and three moults, larvae, ready to pupate, will dig down into the soil to overwinter. The next spring (second year) the larvae will pupate in the soil but emerge only in mid August as an adult. At that time it will feed but not mate, and then dig down again to overwinter in the soil, this time as an adult. The third year, the adult will come out in the course of June to reproduce for the first time. There is good evidence that *O. elongata* can reproduce over several consecutive seasons interrupted by overwintering period spent in the soil (Conconi, D., unpublished data)

Because of its high alpine habitat, *O. elongata* exhibits a patchy distribution. So far, 13 populations have been identified that are separated by a minimum of 6 kilometres of unsuitable habitat. The host plant availability differs between populations; most of them only present one host, be it a PA provider or not, but in two sites, *O. elongata* can be found on both an *Adenostyles* species and *C. spinosissimum*.

Despite the possession of flight wings, the dispersal of *O. elongata* is presumably relatively limited and exclusively accomplished by walking. Within a site, host plants are grouped in patches of various sizes and *O. elongata* displays a high fidelity in patch use. Mark-recapture data demonstrated that within one population over a whole season, only about 4 % of the adults left the host plant patch in which they were first captured (Ballabeni et al. 2001a). Moreover, using RAPD markers, a recent genetic survey revealed significant genetic differences between two sub-populations of *O. elongata* separated by not more than 2 km, indicating very low dispersal rate within a site (Hirter 2003).

The species *O. elongata* has been previously split into five or six subspecies depending on the author (Daccordi and Ruffo 1976; Daccordi and Ruffo 1986; Kühnelt 1984). The subspecies were described according to morphological traits and are restricted to one part of the distribution.

EXPERIMENTAL PROCEDURES

The methods used in this thesis can be split into two categories: those concerning laboratory experiments and those used in field experiments. The laboratory methods can themselves be separated into methods involving rearing of beetles, genetic data collection and chemistry. All but the two latter were performed in or around our field station at the col du Petit Saint Bernard, France.

Beetle rearing was performed individually in round plastic boxes of various sizes that were stored within climate chambers to manipulate temperature and daylength (paper I) or simply stacked randomly in a non-heated laboratory to follow natural temperature and daylength variations (paper II, IV and V). The relevant life history traits were measured on the individual level (larval growth rate, larval development time, prepupal weight, oviposition preference). A number of oviposition experiments were carried out in tents (BugDorm) lined up outside our field station. The plants used for these experiments originated from the col du Petit Saint-Bernard for *Adenostyles alliariae* and *Cirsium spinosissimum* and from the Lautaret and Galibier populations, France for *A. glabra*.

The genetic survey took place in Neuchâtel and was based on microsatellite markers. Microsatellites are regions of the genome that contain a short sequence which is composed of a repeated motif (e.g. poly (CA) or poly (TGG)). These sequences are sometimes referred to as short tandem repeats (STR) and termed as mono-, di-, tri- or tetranucleotides to denote the type of motif being iterated. These regions exhibit a high polymorphism due to allelic length variation, caused by differences in the number of repeats and are therefore the ideal tool to explore genetic variability within and among populations. This technique involves genomic library construction, screening and sequencing for probe development followed by the data collection itself including PCR amplification of the target microsatellite sequence, resolution by electrophoresis on polyacrylamide gels and genotyping. Because we were not experienced and equipped to perform the long and tedious development steps, we hired the company ECOGENIC GmbH, to develop the microsatellite primers.

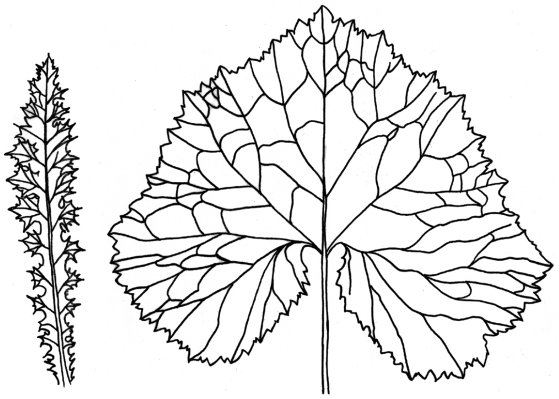

Figure 2. *Typical Cirsium and Adenostyles leaf*

The chemical analysis occurred in our laboratory in Neuchâtel and implied the use of a gas-chromatography device (GC) coupled with a mass spectroscopy detector (MS). This technique allows a purified fraction of a given blend to be split into its elementary components by the transit through a column of gas to finally be detected and determined by comparison to a reference library. This procedure permits the qualification and the quantification of the components of a blend, in our case the PA content of a larva.

Results and discussion

Because of the non-overlapping distributions of a phytophagous insect species and its host plants, the variation in the geographic distribution of host plant may be one of the sources of a herbivore local adaptation and specialization. However, several other explanations have been suggested to account for the general evolutionary drive towards specialization in herbivore insects: insect adaptation to plant chemistry, trade-offs between the physiological performance on different hosts, insect adaptation to the host-specific regime of natural enemies, variation in plant availability, neutral constraints on the processing of information by ovipositing female, genetic constraints on host shifts and the mode of herbivory . In deed, the consensus opinion stresses that there is not a single explanation but several.

In this study, we addressed three potentially important mechanisms.

LARVAL PERFORMANCE

Because of differences in chemistry among plant species and variation in host plant availability between populations, a phytophagous insect may undergo variation in selective pressures that could lead to the development of specialization. The most intuitive traits affected by divergent selective pressures are traits regarding performances and in particular larval performances. In theory, an insect should exhibit higher performances on its native host and reduced fitness on the alternative plant.

Our results do not confirm the prediction of a higher larval performance on the host plant *O. elongata* typically encounters in the field (Paper I and II). We did find, however, differences in growth rate between populations as well as differences in growth strategy. In both experiments, the *Cirsium*-only populations, i.e Mattmark and Bosco Gurin, displayed a different growth strategy including a higher growth rate overall, a shorter developmental time and a smaller prepupal weight, as compared with populations encountering *Adenostyles* in the field (Lautaret, Nice and Petit Saint-Bernard). This has been hypothesized to be the result of the less efficient protection of cardenolides that are the only chemical defence solution offered in absence of PAs containing plants. Larvae would increase growth rate and shorten development time to reduce chances of being killed by a predator that is not deterred by the poor chemical protection. Still, increasing growth rate requires more feeding and therefore more time spent unsheltered and at a risk of predation. Simpler explanations such as differences in climate or in predation rate, do not depend on host plant availability. More experiments are needed to determine the cause of the differences in larval strategy between northern population and more southern ones, but the interesting element is that these differences have also been found in morphological studies (Daccordi and Ruffo 1976; Daccordi and Ruffo 1986; Kühnelt 1984) as well as in a genetic survey (Paper III).

Despite the relatively short period favourable for development in high alpine habitat and their reproduction strategy, i.e. income breeders, *O. elongata* larvae were shown not to grow at their maximal rate and displayed the ability adjust their growth rate in relation to time stress (Paper I). The general finding that *O. elongata* larvae exhibit a flexible growth indicates that this feature may be more common that previously thought.

OVIPOSITION

Several lines of evidence suggest that the oviposition behavior of females may be more important than larval performance in driving the evolution of host specialization (Futuyma 1983; Jaenike 1990; Janz et al. 2001; Thomson 1988). An evolutionary logic predicts that oviposition behaviour should be favoured by natural selection to lay eggs on the host on which the offspring fitness is maximized (Thomson 1988; Yamaga and Ohgushi 1999). Our results do not support a simple preference – performance association. In both no choice and multiple choice experiments, *Cirsium spinosissimum* is the host preferred for oviposition by all populations indicating that the oviposition preference for *C. spinosissimum* is an ancestral trait in *O. elongata*. The differences between populations seems mainly to be in the general motivation to oviposit; the *Adenostyles* population (Lautaret) being selected for a reduced choosiness due to the absence of the preferred host (i.e. *C. spinosissimum*).

The absence of an association between oviposition preference and larval performance is likely to result from the higher protection provided to eggs and larvae by *C. spinosissimum* (Ballabeni et al. 2001b), paper V and poster). In *O. elongata*, we are faced with a preference – protection association rather than the traditional preference – performance association.

LARVAL BEHAVIOUR

It has been repeatedly suggested that for an insect with several alternative host plant a single plant species may not provide the most beneficial conditions in all parts of the life-cycle (Janz 2002; Reavey and Lawton 1991; Scheirs and De Bruyn 2002; Scheirs et al. 2000). In this case, natural selection may favour a dynamic use of the various host plants in different life stages.

We found this pattern in *O. elongata* (Paper V). The two host population exhibited a behavioural local adaptation being generally more active, more likely to leave the host they had been feeding on earlier and showing a significantly lower feeding preference. Despite the lower larval developmental rates of larvae feeding on a mixed diet composed of both *C. spinosissimum* and *A. alliariae* compared to *A. alliariae* only diet (Ballabeni and Rahier 2000), this result has been explained by differences in plant specific mortality risk. If *A. alliariae* provides the highest larval performance, *C. spinosissimum* has been shown to better protect larvae as well as eggs (Ballabeni et al. 2001b), Paper V). In the two host populations, practically all larvae hatch on the host preferred for oviposition and seem then to commute between both hosts, benefiting from the PAs provided by *A. alliariae* and of some protection by *C. spinosissimum*.

GENETIC

Because genes are the support of heredity and because local adaptations have to be genetically-based to spread, it is of great interest to investigate the population genetic of phytophagous insects susceptible to local adaptation and specialization. The relationship between host plant use and genetic distance between populations has to be positive to indicate host race formation. On the other hand, geographical distances may explain part of the genetic variation (Isolation by distance) or populations may cluster according to the general region they come from.

We found a highly structured pattern of genetic differentiation among 13 populations of *O. elongata*. This was coupled with significant isolation by distance among populations and a very low number of migrant, suggesting a genetic drift. The genetic differentiation between populations appeared to be weakly correlated with host plant availability but instead highly explained by the region of origin of the population. Within the five populations tested in paper II, the genetic differences turned out to be related to larval performance differences, in other words population that were more different in larval performance, appeared more genetically different. Additionally, the cluster tree based on genetic distance grouped the 13 populations according to the subspecies described by (Daccordi and Ruffo 1976) on morphological traits. The overall pattern of genetic structure in *O. elongata* therefore seems to indicate that populations undergo genetic drift perhaps ultimately resulting in speciation.

Conclusions

In *Oreina elongata*, we found no evidence of any mechanism susceptible to lead to specialization. The tested populations appeared to be different in several life history traits but these differences do not relate to host plant availability in the field. The only exception concerns a behavioural adaptation to the presence of the two hosts in the Petit Saint-Bernard population. It is not surprising that the only measurable adaptation turns out to be behavioural since behavioural traits are known to evolve more rapidly than other life history traits. This finding seems to indicate that natural selection needs more time to give rise to adaptation to host plant use. On the other hand, natural selective pressure associated with the host plant might be relieved by more important selective pressures related with life at high altitude. The genetic analysis indicated genetic drift as the main factor responsible for genetic variation among populations. It looks as if out of the three agents that determine genetic divergencebetween population, genetic drift is the most important in *O. elongata*. Natural selection associated with the host plant does not appear to be sufficiently strong and divergent to lead to any pattern of specialization, while the counteracting force of gene flow does not seem high enough to prevent genetic drift. Other experiments are needed to determine the cause of the divergence in life-history traits among subspecies, but this variations seems to match both morphological and genetic differentiation. It seems that in *O. elongata*, we are faced with the start of a speciation event, driven by an unknown selective pressure, sustained by genetic drift and supported by low gene flow.

outline

Acknowledgements

I thank M. Rahier, C. Truong, K. Gotthard and R.E. Naisbit for helpful discussion of the results.

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PAPER one

THE GROWTH STRATEGY OF AN ALPINE BEETLE: MAXIMIZATION OR INDIVIDUAL GROWTH ADJUSTMENT IN RELATION TO SEASONAL TIME HORIZONS?

Nicolas Margraf, Karl Gotthard and Martine Rahier

Abstract

Life history theory typically predicts that juvenile growth rate should be maximized and consequently juvenile period minimized. However, in several examples it has been shown that insect larvae do not always grow as fast as they can and this has been explained by costs of high growth rate, typically higher juvenile mortality rate. Hence, some insect larvae have the ability to adaptively adjust growth rate to catch up if development is delayed.

We tested for the presence of such ability in the alpine beetle *Oreina elongata*. In this species, the favourable period for development is relatively short and of unpredictable length, and individuals are chemically defended against predation; factors that could affect the balance between the benefits and costs of high growth rate.

Here we show that when time stressed *O. elongata* larvae were able to increase growth rate, accelerate development and reach the normal final weight.

Hence, individual growth rate adjustment was present in a situation where its adaptive value appeared to be relatively weak, which supports the notion that flexible growth strategies are a common phenomenon in temperate insects.

Keywords: Phenotypic plasticity, life history, photoperiod, seasonality, growth rate

Introduction

A short juvenile period is generally believed to be advantageous mainly because this reduces the time window for juvenile mortality (Roff 1992; Stearns 1992). It is also frequently observed that a large adult size increases fitness (Roff 1992; Stearns 1992). Therefore, life-history models typically predict that development time should be minimized and concurrently that juvenile growth rate should be maximized. However, theoretical and empirical investigations suggest that under a range of conditions it may be favourable for an individual to grow more slowly although this leads to a longer juvenile period or a smaller adult size (Abrams et al. 1996). The reasons for this are that high growth rates are often associated with fitness costs and there will be a balance between the benefits and costs of a high growth rate (Arendt 1997; Gotthard 2000). Hence, for any organism there may be an optimal growth rate that is not necessarily the maximum. The optimal balance between the costs and benefits is likely to be influenced by environmental factors such as predation rate and time stress (Nylin, Gotthard & Wiklund 1996; Gotthard 2000). In temperate regions, where part of the year is unsuitable for growth and development, most insects have to reach a certain size or developmental stage before the onset of winter in order to survive. Hence, temperate insects typically face time horizons that limit their development, and variation in climatic variables such as temperature and photoperiod are often used as cues to estimate the remaining time available (Nylin & Gotthard 1998). Time is an important resource for developing juveniles and there should be strong selection for its efficient use. In a seasonally changing environment, it may then be beneficial for an individual to be able to adjust its growth rate in response to the amount of time left for development as well as other environmental factors that affect the balance between the costs and the benefits of fast growth (Abrams et al. 1996).

Several temperate butterfly species have been shown to have plastic growth that allows individuals to grow more slowly or to speed up development in relation to seasonal time stress (Nylin, Wickman & Wiklund 1989; Leimar 1996; Gotthard 1998; Gotthard, Nylin & Wiklund 1999). Individuals experiencing "end of the favourable season" conditions are able to increase growth rate, speed up development and finish at the normal size rather than at a smaller size, which might otherwise result in lower adult fitness.

In this study, we wanted to test if the predictions of a plastic growth strategy hold for Oreina elongata Suffrian (Coleoptera: Chrysomelidae), a beetle with life-history characteristics that are distinctly different from the butterflies mentioned above. This species lives at high altitudes in the Alps (alt. 2000 - 2200 m). The snow-free period is about three months in summer and the growth season for the host plants of O. elongata is even shorter, which leaves little time for growth and development within a single season. Moreover, weather conditions at such high altitudes are highly unpredictable and even in the middle of summer, temperatures can drop below zero and snowfall can occur (pers. obs.). Hence, growing larvae constantly face a high risk of being interrupted in their development and this risk cannot be readily predicted from cues of seasonal change (e.g. photoperiod or temperature). These factors are probably the main reasons why O. elongata have a two-year juvenile period including two winter diapauses, which guarantees sufficient time for development. Furthermore, O. elongata larvae are to some degree chemically defended due either to sequestration of plant defensive compounds or to endogenously produced compounds (Dobler & Rowell-Rahier 1994a). An increased predation risk is one of the major costs of both a higher growth rate and a long juvenile development and it seems likely that the chemical defence may reduce the effect of these costs and that it will affect the optimal growth rate. Despite specific studies on potential parasitoids of O. elongata, there has not been a single observation of parasitoid emergence from eggs or larvae, suggesting that this is a minor mortality factor. Finally, in contrast to the butterflies, O. elongata is more of an income breeder (Stearns 1992; Jonsson 1997). Resources for reproduction are accumulated not only throughout the juvenile period (as in capital breeders) but also during the adult stage. Since adults are long-lived an individual may reproduce during several consecutive summers (D. Conconi, unpublished data). This may imply a less direct impact of size at maturity on adult fitness. It seems likely that the short and unpredictable season increases the benefit of growth maximisation while the chemical defence lowers the cost of fast growth and together they suggest that a flexible growth strategy would be less advantageous for O. elongata than for most other temperate insects. The effect of income breeding is less obvious but it does suggest that variation in size at maturity has weaker fitness consequences and that selection for an ability to reduce such variation through a plastic growth strategy may be weak.

The conditions outlined above suggest that selection for a flexible growth strategy should be relatively weak in O. elongata larvae and that they should typically maximize growth rate to be able to finish larval growth during the first summer (first winter diapause is in the prepupal stage), independently of what time horizons they are experiencing (Abrams et al. 1996). Since daylength is a more accurate predictor of date than temperature (Nylin & Gotthard 1998), we assumed it would be the most important cue used by the beetle larvae to gauge the actual time left for development. These predictions were tested in a laboratory experiment where daylength conditions were systematically manipulated. To provide a general picture of the seasonal growth strategy we also included several populations and host plants in the experimental setup.

Material and Methods

STUDY ORGANISM

Oreina elongata is an alpine species found in the Alps and Apennines. Both larvae and adults feed on the leaves of three host-plants from different tribes of the Asteraceae: Adenostyles alliariae (Gouan) Kerner, A. glabra (Miller) DC. (both Senecioneae) and Cirsium *spinosissimum* (L.) Scop. (Cardueae). Adenostyles alliariae and A. glabra contain pyrrolizidine alkaloids (hereafter abbreviated to PAs) that O. elongata larvae and adults can sequester for their own defence (Dobler & Rowell-Rahier 1994b). Cirsium *spinosissimum* lacks PAs and beetles feeding on this species synthesize cardenolides endogenously (Dobler & Rowell-Rahier 1994b). Some populations of O. elongata encounter only one of the three host-plants whereas in other sites both A. alliariae and C. *spinosissimum* are present and are used by the beetles. Earlier studies indicate that O. elongata females from all populations have a strong oviposition preference for C. *spinosissimum* (Paper IV).

The juvenile period of O. elongata extends over two years. Eggs are laid during the earliest part of the short alpine summer season (July) and hatch after two to three weeks, after which the larvae grow through four instars before digging down in the soil to overwinter and pupate. Surviving adults emerge in the second part of the next summer, feed and again dig down into the soil for a second winter diapause. The second year, reproductive adults emerge early in the season (mid-June), mate and oviposit. Adults can survive and reproduce up to three consecutive seasons (D. Conconi, unpublished data).

STUDY POPULATIONS

In the first week of July 2000 we collected beetles from three populations in the Alps: from Petit Saint-Bernard (PSB) on the border between France and Italy (45°40'20.6" N, 6°52'39.4" E, WGS84, altitude 2188 m) where insects can be found on both A. alliariae and C. *spinosissimum* in mixed patches (beetles collected in equal numbers from both plants); from Mattmark (MTT) in the Swiss Alps (46°1'56.4" N, 7°57'25.9" E, WGS84, altitude 2200 m) where beetles feed only on C. *spinosissimum* (the only plant available at MTT); from Lautaret (LAU) in the Vanoise region of France (45°0'39.0" N, 6°22'19.3" E, WGS84, altitude 2058 m) where O. elongata is present only on A. glabra (the only plant available at LAU).

EXPERIMENTAL DESIGN

We performed a larval growth experiment using a full factorial design with three factors: population (three levels), host-plant (two levels) and daylength treatment (two levels). We reared 15 larvae in each of the 12 combinations. Field collected females from each population were given A. alliariae and C. *spinosissimum* for oviposition in plastic jars. Eggs were collected daily and placed in transparent plastic Petri-dishes. To provide the necessary humidity, the bottom of each dish was filled with a layer of moistened plaster covered with filter paper. Sixty newly hatched larvae from each population were weighed, and placed individually in Petri-dishes of the type described above, in incubators set at constant temperature cycles and one of two daylength treatments. The incubators were programmed to follow the natural decrease in photoperiod and the first treatment was chosen to mimic the first part of the summer (start 5th of July) and the second to mimic the end of the summer season (start 14th of August). The temperature cycle was identical for both daylength treatments: 17 °C for 16 hours and 7 °C for 8 hours, more or less corresponding to the day:night cycles. Larvae were randomly assigned to one of the two hosts plants (A. alliariae or C. *spinosissimum*) and every third day, larvae were weighed and new food was provided in excess until they reached the fourth instar (~ 25 mg). At that time they were transferred to larger plastic boxes where they were provided with a 2 cm layer of soil for pupation. Their food

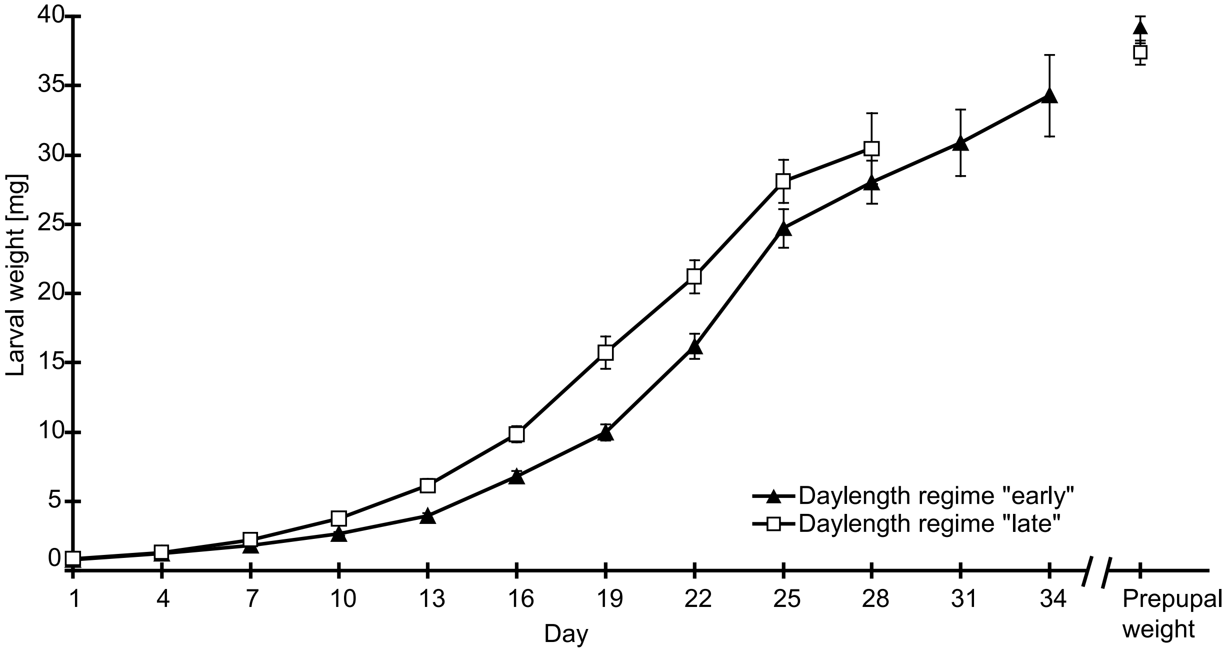


Figure 1. *Mean growth trajectory and mean prepupal weight of Orein1a elongata larvae under two daylength regimes, mimicking early and late light conditions.*

was renewed and the soil moistened every third day. The boxes were placed in an unheated room where the temperature fluctuated between 7 and 17 °C (values that fall within the range of daily temperature fluctuations in the field). The day of disappearance of the larvae into the soil was recorded and on 14 September all prepupae were dug out and weighed.

STATISTICAL ANALYSES

After log-transformation of the weight data, regression lines were fitted to the individual growth trajectories (Fig 1). The slope of each line was used as a measure of the growth rate of each individual larva. Development time was calculated as the period from the beginning of the experiment to the day the larvae disappeared into the soil. The effects of daylength, diet and population and their interactions on growth rate, development time and prepupal weight were tested with analyses of variance (ANOVA). Analyses were carried out using the Splus statistical package with type III sums of squares and all effects were considered as fixed factors. Because the three-way interactions were non-significant in all analyses, we removed them and recalculated the ANOVAs.

Results

Growth rate differed significantly between daylength treatments and populations (Table 1). Except for the Mattmark beetles reared on A. alliariae, all populations increased growth rate when reared in end of the season light conditions (Fig 2). However, Mattmark larvae generally grew faster than larvae from the two other populations. There was a significant interaction between plant and daylength treatment, which indicates that depending on the host plant on which they were reared, Oreina elongata larvae responded differently to time stress.

As for growth rate, development time was significantly affected by population and daylength treatment (Table 2). Larvae had a shorter development time under late season light conditions, with the exception of larvae from Mattmark reared on A. alliariae (Fig 2). There was again a significant interaction between plant and daylength regime but also between population and daylength treatment. In other words, instead of shortening their development time under late season conditions as did the other populations, Mattmark larvae reared on A. alliariae increased their developmental period.

Prepupal weight was unaffected by daylength treatment and host plant. Only population had a significant effect on prepupal weight (Table 3). Mattmark prepupae were lighter than other populations, Petit Saint-Bernard prepupae were intermediate, and Lautaret larvae ended with the highest prepupal weight, independent of light conditions or host plant (Fig 2).

Table 1. *ANOVA for relative growth rate*

	SS	DF	F	P value
Population	0.0010	2	10.53	0.0001
Host-plant	0.0000	1	0.24	0.6288
Daylength treatment	0.0009	1	19.36	<.0001
Population X Host-plant	0.0002	2	2.44	0.0920
Population X Daylength treatment	0.0001	2	1.00	0.3703
Host-plant X Daylength treatment	0.0003	1	7.09	0.0090
Residuals	0.0049	101		

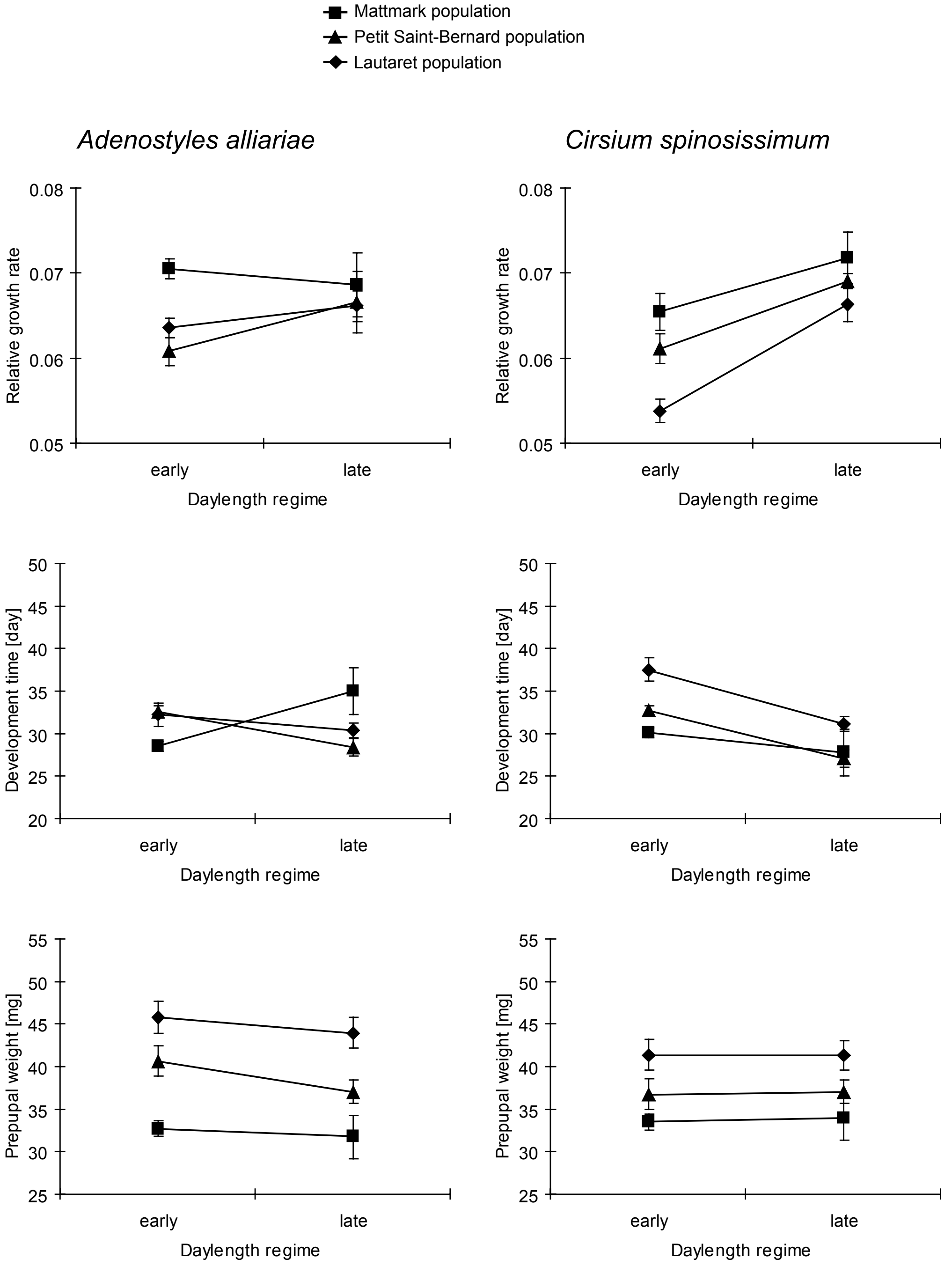
Table 2. *ANOVA for development time*

	SS	DF	F	P value
Population	131.6630	2	3.22	0.0441
Host-plant	11.5130	1	0.56	0.4547
Daylength treatment	140.6620	1	6.88	0.0101
Population X Host-plant	177.7220	2	4.35	0.0155
Population X Daylength treatment	263.2560	2	6.44	0.0023
Host-plant X Daylength treatment	153.8170	1	7.52	0.0072
Residuals	2044.3210	100		

Table 3. *ANOVA for prepupal weight*

	SS	DF	F	P value
Population	1611.4280	2	37.37	<.0001
Host-plant	54.0080	1	2.51	0.1174
Daylength treatment	17.5290	1	0.81	0.3699
Population X Host-plant	120.9570	2	2.81	0.0664
Population X Daylength treatment	3.7910	2	0.09	0.9159
Host-plant X Daylength treatment	26.7850	1	1.24	0.2683
Residuals	1746.3030	81		

Figure 2. *Interaction plots of the three fitness parameters: relative growth rate, development time and prepupal weight, showing mean and standard error, split by host-plant, daylength regime and populations*



Discussion

Our results indicate that larvae of Oreina elongata from all the tested populations are able to adjust their growth rate in response to seasonal time stress. However, this ability varies, depending on which host plant the beetles are feeding. On Cirsium *spinosissimum*, all populations increased their growth rate when experiencing the photoperiod typical of late in the season. On Adenostyles alliariae the pattern was the same, with the exception of beetles from Mattmark. Development time followed the inverse pattern; an increase in larval growth rate led to a shorter development time. As a consequence, prepupal weight was not affected by daylength regime, varying only between populations.

It seems that beetles from Mattmark have a different life history compared to the other populations, with a higher larval growth rate and smaller prepupal weight. This confirms previous results (Ballabeni et al. 2003) suggesting that local selection on the life cycle may be different in Mattmark compared to the two other populations. Moreover, potential explanations for the relatively limited degree of local adaptation to variation in host plant use have also been examined elsewhere (Ballabeni et al. 2003). The focus of the present experiment was plasticity in relation to seasonal cues and the Mattmark population feeding on A. alliariae provided the only observation against the general pattern of increasing growth and decreasing development time in late season conditions (Fig. 2). The main difference between the populations was that the Mattmark larvae grew very fast in the early season conditions whereas larvae of the two other populations displayed reduced growth rates. Indeed, it seems as if the Mattmark larvae are maximising growth independently of seasonal cues, which would lend some support to our original expectations. However, in light of the rest of the results it seems more likely that this is a consequence of the Mattmark population never encountering Adenostyles plants in the field and that feeding on this novel host plant somehow disrupts the seasonal adjustment of growth (the other two populations do use Adenostyles in the field). In all treatments where larvae are feeding on native hosts the trend is that larvae grow and develop faster in late season conditions (Fig. 2).

In contrast to the expectations outlined in the Introduction, it appears that selection is favouring an ability to adjust growth in response to time stress in a chemically defended beetle that is restricted to an alpine habitat where the length of the favourable season is highly unpredictable. This ability may allow larvae to catch up after weather-induced delays in developmental schedules, a pattern found in a range of ecothermic animals (Gotthard 2001). The results of the late season treatment suggest that for O. elongata larvae the costs of being small at the time of diapause exceed the costs of growing fast. Larvae that finish at a reduced size may, for example, suffer from higher winter mortality in addition to any costs of smaller adult size. On the other hand, the reduction in growth of larvae in the early treatment suggests that the benefit of an even larger size is typically lower than the cost of maximizing growth rate. These results indicate that both size at diapause and larval growth rate in O. elongata are under stabilizing selection and that a plastic growth strategy rather than growth rate maximization seems to be beneficial in this insect.

The combination of a short and unpredictable period available for larval growth, chemically protected larvae and an income breeding strategy suggest to us that O. elongata larvae should be likely to experience selection for growth rate maximization and it is of interest to examine why this expectation was not supported. It seems very likely that the two-year juvenile period of O. elongata is an adaptation for coping with the short alpine season and it may thus reduce the effect of time limitation on juvenile development even if larval growth has to completed in the first summer. Hence, the time constraints on larval development may be less severe than we expected. There is also considerable variation among individuals in the start of larval growth as oviposition goes on for at least one month, and therefore it may be beneficial to adjust growth in relation to seasonal time horizons. However, this potential benefit depends on the presence of costs of high growth rates, which we assumed to be relatively low because of the chemical defence. Field studies, performed subsequent to this experiment, do suggest that despite their chemical defence predation rates of O. elongata larvae are substantial (Paper V). Predators that have been observed predating on O. elongata larvae include the commonly occurring harvestman Mitopus morio and sawflies of the family Tenthredinidae (Hymenoptera), and it is quite likely that fast growth and increased feeding effort may be associated with a higher risk of detection by these predators. Increased growth may also occur at the expense of investment in defence.

Finally, we expected that the income breeding of O. elongata was likely to weaken the correlation between size at metamorphosis and adult fitness. However, in O. elongata the size at metamorphosis also coincides with the size at winter diapause and it is possible that below a certain size there is indeed a tight correlation between weight and over-wintering survival. Unfortunately there are no data on the relationship between winter mortality and the size at winter diapause in this species but increased winter survival with increased size at diapause has been found in other insects (Gotthard et al. 1999). Such a situation could help to explain why larvae increase growth rate if they have fallen behind their developmental schedule. On the other hand, the finding that larvae reduce their growth in early season conditions suggests that becoming a very large over-wintering larva or reaching a large adult size does not pay off in terms of total fitness. This pattern might be expected from an income breeding strategy in that above a given threshold size, determined by the chance of surviving winter diapause, the relationship between final size and fitness shows a strong diminishing returns pattern. Even so it is fascinating that in the time constrained growth conditions of O. elongata there seems to be room for fine-tuning of growth by means of a plastic developmental strategy.

In conclusion, this study shows that in an insect species whose life cycle characteristics and habitat suggest a very high degree of time limitation of the larval period, larvae do not grow at their maximal rate unless they experience late season conditions. Hence, individual growth rate adjustment was present in a situation where many of the theoretical prerequisites for it to be adaptive appeared to be relatively weak. Therefore juvenile growth rate adjustment in relation to time horizons in insects may be a more common phenomenon than previously thought.

Acknowledgements

We thank K. Besomi and Y. Borcard for assistance with the experiments and E. Do Linh San and R. Naisbit for helpful comment on the manuscript. The Ordine Mauriziano (Torino, Italy) and the Association Internationale du Jardin Alpin de la Chanousia very kindly provided logistic support at the field site. This work was supported by the Swiss National Science Foundation grant number 31-64’864.01.

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PAPER two

HOST PLANT AVAILABILITY AND LOCAL ADAPTATION IN LARVAL PERFORMANCE: A QUANTITATIVE GENETIC STUDY IN THE LEAF-BEETLE, *OREINA ELONGATA*

Nicolas Margraf, Aline Verdon, Russell E. Naisbit and Martine Rahier

Abstract

In phytophagous insects the association between female oviposition preference and larval performance and among plant trade-offs in performance have been hypothesised to be two of the main forces driving host specialization. However empirical studies rarely uncover these two correlations.

In this study, we examine the larval performance of five populations of the leaf-beetle *Oreina elongata*, on their respective host-plants in a quantitative genetic experiment.

We show that in this alpine species, there is no negative genetic correlation between three life history traits on the three main host plants. Additionally, larval performance did not appear to be associated with maternal oviposition preference, for females instead lay their eggs on the host that offers better protection.

Introduction

Theory suggests that in the absence of genetic constraints, a heterogeneous environment should lead to the evolution of ecological generalists because niche breadth should evolve to match the amount of environmental variation (Kassen 2002). On the other hand, there is strong evidence that spatial variation in the environment facilitates the maintenance of diversity by promoting local adaptation (Jaenike 1990; Mopper 1996). Which of the two outcomes will hold depends on the relative spatial scales of dispersal and environmental heterogeneity. Local adaptation can occur when dispersal among different patches of the environment is low relative to the strength of selection favouring different types (Slatkin 1987; Barton and Whitlock 1997; Lenormand 2002). Theory then predicts that the genetic correlation in fitness across environments should evolve to become negativefor two reasons: firstly, the direct responses to selection simply tend to be larger than the correlated responses; and secondly, adaptation to one type of environment is associated with a cost of adaptation in alternative conditions (Kassen 2002). These costs of adaptation arise either directly due to genes that are selected for in one environment but deleterious in the other or indirectly via neutral mutations that accumulate in one environment and are deleterious elsewhere (Kawecki et al. 1997).
In phytophagous insects, the existence of between plant trade-offs for larval performance, due to antagonistic pleiotropic effects of alleles of host use, has been hypothesized to explain the high incidence of specialist insect herbivores (Jaenike 1990). However, most quantitative genetic studies of insect performance on different host plants failed to show this negative genetic correlation. Instead, studies typically report either no correlation, a positive correlation, or mixed results (Hare and Kennedy 1986; James et al. 1988; Rausher 1984; Via 1984). Several hypotheses have been put forward to explain this, invoking the masking effect of other factors.

Mutation-selection balance hypotheses assume that factors such as recurrent mutation can generate variation in general vigour that may obscure the antagonistic pleiotropic effects of genes even once a population reaches genetic equilibrium (Charlesworth 1990; Fry 1993; Houle 1991).

The equilibrium trade-off hypothesis suggests that the effects of genes showing negative pleiotropy will be evident only once selection has fixed the alleles that increase performance on one host and on both hosts. Only after that point will the measurable genetic variation consist of those genes that increase performance on one host and decrease it on the other (Joshi and Thompson 1995; Thompson 1996).

In the coadapted gene hypothesis, populations that have been under selection for a relatively long period of time on one host will have been purged of genotypes with low performance on that particular host. Therefore, when a population first encounters a novel host, it will only reveal genotypes ranging from those performing well on both hosts to those that perform well on the normal host but poorly on the novel host (Thompson 1994; Thompson 1996)

It is also possible that trade-offs are sufficient to be evolutionarily significant, but weak enough to be difficult to detect statistically (Rausher 1988). In any case, strict trade-offs may not be necessary to drive specialisation (Fry 1996).

A second correlation, between maternal oviposition preference and larval performance, would also favour specialisation and facilitate sympatric speciation (Hawthorne and Via 2001; Via 1990).

In this study we test for these correlations in the alpine leaf beetle *Oreina elongata* (Coleoptera: Chrysomelidae), taking advantage of variation in host plant availability across the range of the species.

Oreina elongata is specialized on host plants in two genera in the family Asteraceae. *Adenostyles alliariae* and *A. glabra* have large heart-shaped leaves and produce pyrrolizidine alkaloids (PAs) that the insects, both adults and larvae, can sequester for their own defence (Dobler and Rowell-Rahier 1994). *Cirsium spinosissimum* has highly dentate and spiny leaves, which provide some protection to eggs (Ballabeni et al. 2001a) and shelter against severe weather conditions for larvae (Appendix 1). Beetles feeding on *C. spinosissimum* rely on endogenous production of cardenolides for their protection (Dobler and Rowell-Rahier 1994), although the PAs from *Adenostyles* protect *O. elongata* more efficiently against naïve predators (Rowell-Rahier et al. 1995). The beetles and their hosts are patchily distributed at high altitude (1500-2200 m asl) throughout the Alps and the Apennines and host plant availability varies between sites: several populations have only a single host while others encounter up to three. Mark-recapture studies have shown that *O. elongata* has a very low rate of dispersal (Conconi, unpublished data) and the species has never been known to fly (pers. obs). This genetic isolation, coupled with the heterogeneity in host plant availability, suggests that local adaptation is likely to evolve.

Materials and methods

STUDY POPULATIONS

Beetles were collected during the last week of June 2002 from five populations: Mattmark (MTT) in the Swiss Alps where only *C. spinosissimum* is present; the col du Lautaret (LAU) in the Vanoise region of France and L'Authion (NCE) around Nice in France where the beetles live on *A. glabra* exclusively; Bosco Gurin (BOG) on the southern side of the swiss alps where the beetles are found on *C. spinosissimum* with just a small patch of *A. alliariae* nearby; and the col du Petit Saint-Bernard (PSB) on the border between France and Italy where both *C. spinosissimum* and *A. alliariae* are found in mixed patches.

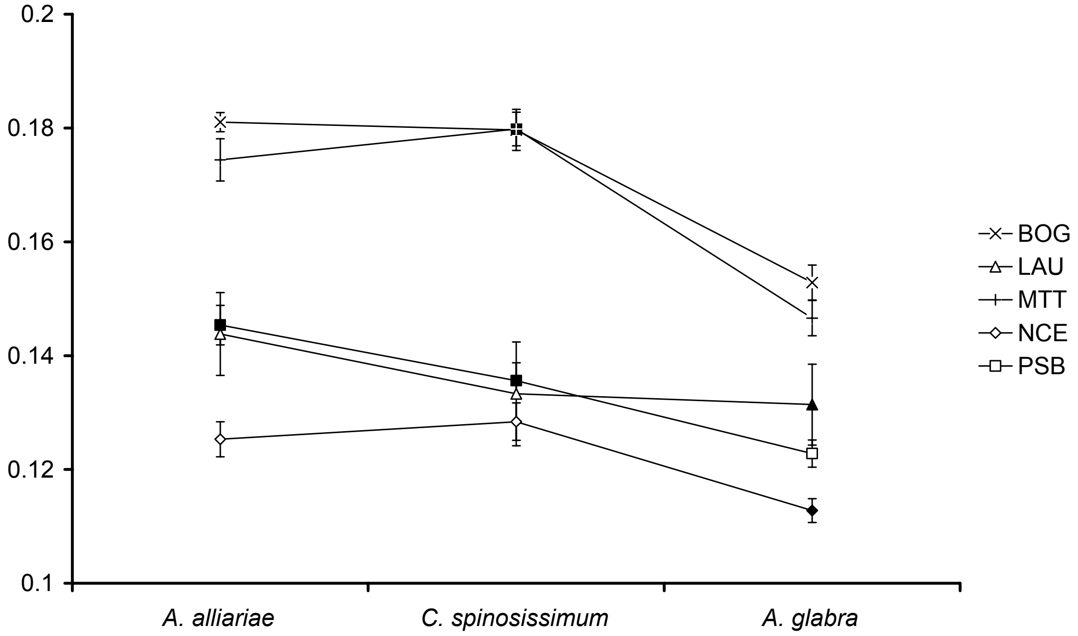

Fig 1 *Larval growth rate of the five populations on the three host plants. Filled symbols represent the host plants of each population in the field*

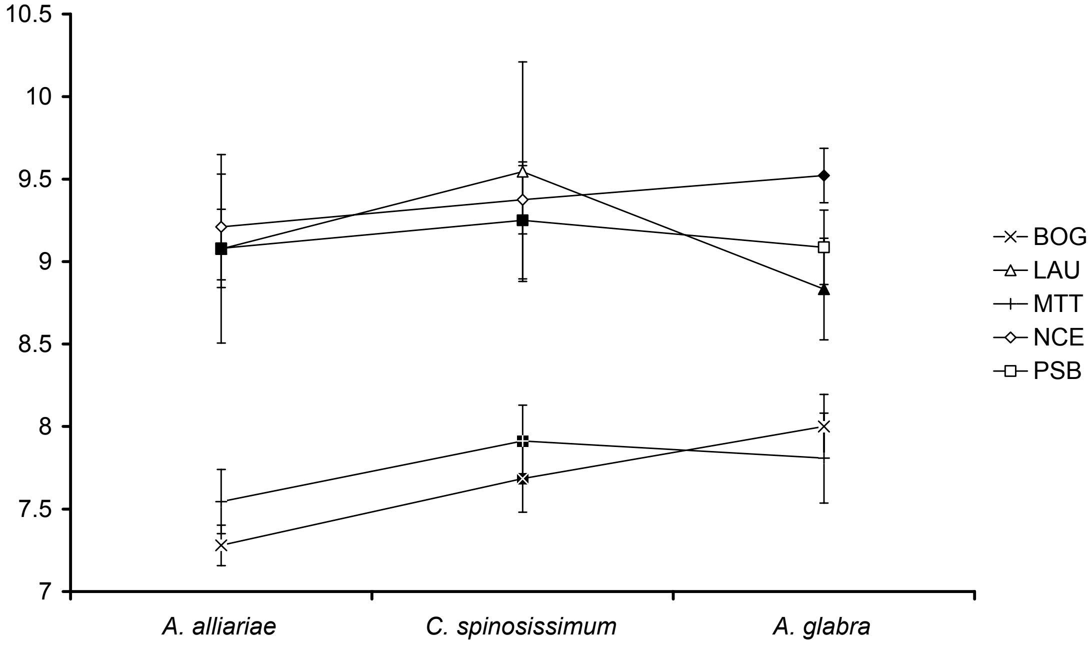

Fig 2 *Larval development time of the five populations on the three host plants. Filled symbols represent the host plants of each population in the field*

EXPERIMENTAL DESIGN

All experiments were conducted at the col du Petit Saint Bernard. Females were kept together in plastic boxes and provided with the three host plants simultaneously to reduce any potential effects of learning in the field sites. They were then tested for oviposition preference in a sequential no-choice experiment. Females were placed individually in plastic yogurt cups with a moistened filter paper at the base and provided with a cutting of each host plant in a randomly assigned order. After each round of 48 hours, the plants were changed and the eggs counted and removed from the plant. They were placed according to family in Petri dishes with a moistened chalk bottom covered with a filter paper to maintain humidity.

Seven families of 12 larvae from each population were then used to compare larval performance on different host plants. Larvae were assigned to one of the three feeding treatments (*A. alliariae*, *A. glabra* & *C. spinosissimum*), weighed and transferred to individual Petri dishes as described above where they were provided with a cutting of their treatment host plant. Every third day, the larvae were weighed, their food renewed and the chalk moistened. When they reached a weight of 30 mg, larvae were transferred to a round plastic box containing soil for them to dig into and pupate and a cutting of their treatment host plant. On 23 September, larvae were dug out and weighed to obtain their prepupal weight.

DATA ANALYSIS

After log-transformation of the weight data, regression lines were fitted to the individual growth trajectories. The slope of each line represents the natural log of the exponential growth factor and was used as a measure of the growth rate of each larva. Development time was calculated as the period from the beginning of the experiment to the day the larvae reached 30 mg.

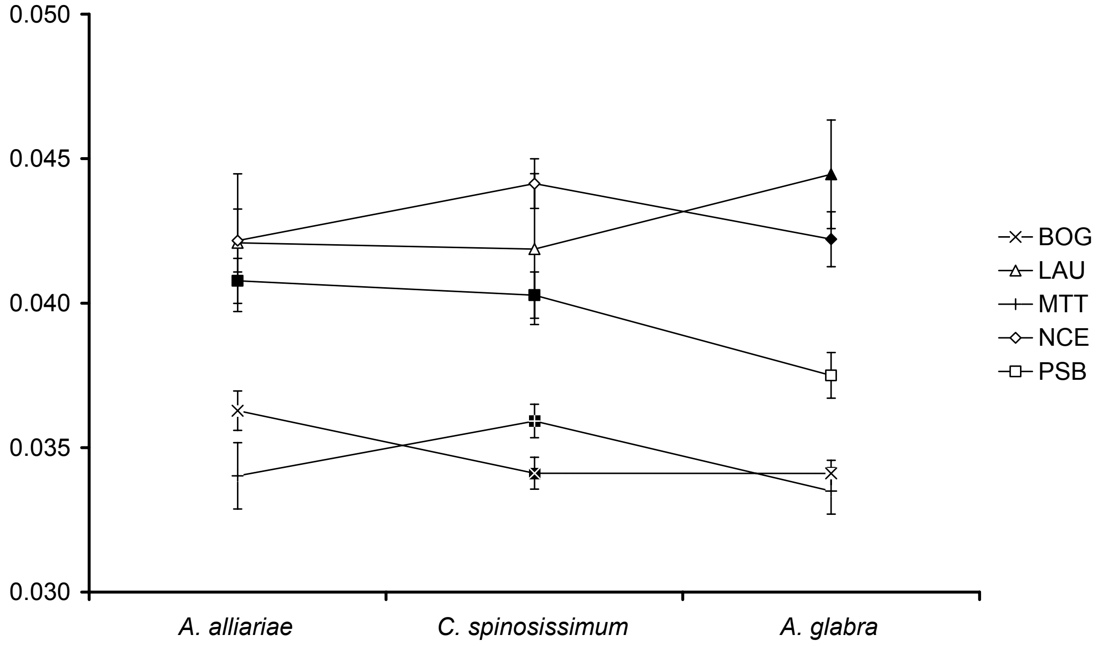

Fig 3 *Larval prepupal weight of the five populations on the three host plants. Filled symbols represent the host plants of each population in the field*

Table 1. *ANOVAs for performance traits. The family effect and the family-host interaction were nested within population*

Source	SS	DF	F	P value
Response	**growth rate**			
Population	0.1024	4	121.62	<.0001
Family[Population]	0.0054	26	0.81	0.7159
Host-plant	0.0146	2	29.23	<.0001
Population X Host-plant	0.0061	8	3.00	0.0062
Family X Host-plant[Population]	0.0135	52	1.17	0.2276
Error	0.0420	189		
Response	**development time**			
Population	155.2880	4	54.21	<.0001
Family[Population]	16.9853	26	0.42	0.9906
Host-plant	0.7485	2	0.25	0.7759
Population X Host-plant	9.7069	8	0.80	0.6038
Family X Host-plant[Population]	80.9761	52	1.27	0.1287
Error	232.2500	189		
Response	**prepupal weight**			
Population	0.0028	4	24.58	<.0001
Family[Population]	0.0008	25	1.63	0.0678
Host-plant	0.0001	2	1.64	0.2004
Population X Host-plant	0.0003	8	1.84	0.0870
Family X Host-plant[Population]	0.0009	50	1.35	0.0792
Error	0.0025	180		

The effects of diet, family and population and their interactions on growth rate, development time and prepupal weight were tested with mixed model ANOVAs. Host-plant, population and their interaction were considered as fixed factors whereas family and the family-host interaction were considered random effects. Family was nested within population. Analyses were carried out using the JMP statistical package with type III sums of squares (SAS 1989).

To estimate the maternal preference – larval performance correlation, we first corrected performance measures for variation in general vigour. We estimated the family performance on each host by substracting the mean overall performance of a family from the mean performance of that family on each host. For oviposition preference we had noted a significant effect of the order of presentation of the host plant on the proportion of eggs laid by a female on a given plant. Therefore, we corrected the number of eggs laid on each plant by a female by subtracting the mean proportion of eggs laid by all females of this population during that round. We then regressed preference against performance separately for each combination of host plant and performance trait (Via 1986).

We estimated broad sense heritabilities of performance traits on each host in each population according to (Roff 1997) using the standard formulae based on a one-way ANOVAs. We tested the heritabilities against zero with one sample t-tests based on standard errors calculated using the formulae given by (Roff 1997). The significance thresholds were corrected for multiple testing using sequential Bonferroni adjustments (Rice 1989).

Genetic correlations between each pair of hosts, for each performance trait in each beetle population were calculated through Pearson product-moment correlation of family means (Roff 1997). The significance thresholds were corrected using sequential Bonferroni adjustments (Rice 1989).

Results

GROWTH RATE

Populations differed significantly in their growth rate (Table 1). Host plant also had a significant effect and this varied between populations. Overall, beetles from Mattmark and Bosco Gurin grew faster than those from the three other populations (Figure 1). Neither family nor its interaction with host-plant were statistically significant (Table 1). After Bonferroni adjustment, broad sense heritabilities of growth rate were not significantly different from zero (Table 2).

DEVELOPMENT TIME

Development time was only affected by population (Table 1). As a result of the differences in growth rate, larvae from Lautaret, Petit Saint-Bernard and Nice took longer to reach 30 mg (Figure 2).

Heritabilities of development time were never significant (Table 2).

Table 2. *Broad sense heritabilities of larval performance traits for each host and beetle population. P values for differences from zero are given in parentheses. Gaps are because of low survival of larvae from Lautaret reared on A. glabra.*

	A. alliariae	***C. spinosissimum***	***A. glabra***
Response	**growth rate**		
Bosco Gurin	0.111 (0.793)	0.040 (0.944)	-0.211 (0.628)
Lautaret	-0.962 (0.013)	0.028 (0.979)	
Mattmark	0.349 (0.508)	-0.166 (0.665)	0.248 (0.642)
Nice	-0.151 (0.773)	-0.332 (0.276)	-0.510 (0.058)
P^t S^t-Bernard	0.198 (0.658)	0.668 (0.254)	0.794 (0.145)
Response	**development time**		
Bosco Gurin	0.120 (0.778)	-0.558 (0.175)	0.256 (0.634)
Lautaret	-0.624 (0.213)	0.110 (0.918)	
Mattmark	0.378 (0.478)	0.032 (0.943)	0.248 (0.642)
Nice	-0.160 (0.759)	0.415 (0.410)	0.128 (0.782)
P^t S^t-Bernard	0.535 (0.291)	0.012 (0.982)	0.122 (0.791)
Response	**prepupal weight**		
Bosco Gurin	0.279 (0.59)	0.073 (0.897)	-0.653 (0.027)
Lautaret	-0.468 (0.481)	-0.278 (0.840)	
Mattmark	0.488 (0.384)	0.939 (0.084)	0.618 (0.277)
Nice	0.325 (0.588)	0.483 (0.360)	-0.011 (0.981)
P^t S^t-Bernard	1.072 (0.043)	0.025 (0.961)	0.373 (0.466)

Table 3. *R^2 and P values of regressions of the mean performance traits of families on the three hosts against the proportion of eggs laid by the mother on the given plant.*

	Adenostyles alliariae		*Cirsium spinosissimum*		*Adenostyles glabra*	
	R^2	P value	R^2	P value	R^2	P value
Prepupal weight	0.016	0.485	0.033	0.320	0.102	0.075
Growth rate	0.003	0.776	0.040	0.274	0.000	0.975
Development time	0.051	0.229	0.049	0.230	0.006	0.679

PREPUPAL WEIGHT

Population was the only factor that had a significant effect on prepupal weight (Table 1). Despite their higher growth rate, larvae from Mattmark and Bosco Gurin reached a smaller weight before pupation (Figure 3).

After Bonferroni adjustment, heritabilities of prepupal weight were never significantly different from zero (Table 2).

Preference-performance correlations

The correlations between maternal oviposition preference and larval performance for each combination of host plant and performance trait were never statistically significant (Table 3).

GENETIC CORRELATIONS

After Bonferroni adjustment, the genetic correlations of larval performance traits between hosts were significant and positive in only two cases (Table 4).

Discussion

Local adaptation predicts that beetles experiencing one host plant for a relatively long period of time should exhibit a higher growth rate on this host. Our study does not confirm this prediction. Although larvae from different populations grew at different rates on the different host plants, there was little correlation between the optimal plant for each population and that which it encounters in the wild. Bosco Gurin and Mattmark were distinct in all life history traits and in general most populations performed worst on *A. glabra*. A similar conclusion can be drawn from a previous study, using fewer populations and host plants, which found significant but very slight host-plant by population interactions (Ballabeni et al. 2003). The degree of local adaptation to host-plant availability in larval performance traits therefore appears to be relatively limited in *Oreina elongata*.

Maternal oviposition preference of individual females was uncorrelated with the three measures of performance in their larvae. All populations also show a strong overall preference for *C. spinosissimum*, despite most showing equal or better performance on *A. alliariae*. In theory, everything else being equal, phytophagous insects should exhibit a positive association between oviposition preference and larval performance (Futuyma and Peterson 1985). Nevertheless, despite better larval growth on one host, other selective forces can drive a population to develop an oviposition preference for another host. This common but counterintuitive result has been explained by several factors, including evolutionary lag, larval host choice, and enemy-free space ([Thomson, 1988 #119]. In the case of *O. elongata* the lack of correlation probably results from plant specific mortality risks and the ability of larvae to move between hosts. Previous studies in the two host population of Petit Saint Bernard have shown that both eggs and larvae have higher survival on *C. spinosissimum* than on *A. alliariae* (Ballabeni et al. 2001b), Paper V). Moreover, under semi-natural conditions, larvae were shown to be better protected against harsh weather conditions (i.e. hard rain and strong wind) on *C. spinosissimum* (Appendix). Larvae move between hosts and so have a mixed diet, and while this has little effect on growth rate, it does allow them to sequester PAs from *A. alliariae* (Ballabeni et al. 2001a; Ballabeni and Rahier 2000). A combination of female oviposition preference and larval movement would therefore allow offspring to benefit from both mechanical and chemical defence.

Populations differed strongly in their growth rate and in particular those populations that experience *C. spinosissimum* as their main host seemed to have a different life history. Bosco Gurin and Mattmark showed a higher growth rate, a shorter developmental period and a lower prepupal weight whichever plant they fed on. These differences in life history might be a response to climatic variation, if the two sites provide a shorter favourable season for growth. They may also result from differences in the availability of defensive compounds. Populations that live only on *C. spinosissimum* do not have PAs available for sequestration in their host plant and have to rely on endogenously produced cardenolides. More studies are needed to compare the costs and benefits of the two defensive strategies, but larvae that do not encounter sequestrable PAs might speed up development to avoid prolonged exposure to natural enemies. *A. glabra* which also produces PAs provided the lowest growth rate in all populations, even those in which it is the only natural host. This may perhaps relate to the presence of additional toxins, sesquiterpenes, in the leaves of that species, or to other differences in chemical composition (Hägele and Rowell-Rahier 1999).

Table 4. *Genetic correlations of each performance trait between each pair of hosts, estimated by Pearson's product moment coefficient. AC stands for Adenostyles alliariae X Cirsium spinosissimum, AG for A. alliariae X A. glabra and CG for C. spinosissimum X A. glabra. P-values for differences from zero are given in parentheses. After sequential Bonferroni adjustments for multiple comparisons, we accept only the correlations in bold type as significant (α = .05)*

	AC	**AG**	**CG**
Response	**growth rate**		
Bosco Gurin	0.249 (0.591)	-0.022 (0.963)	0.834 (0.02)
Lautaret	-0.759 (0.732)	0.377 (0.729)	-0.497 (0.01)
Mattmark	-0.119 (0.800)	0.128 (0.784)	0.490 (0.265)
Nice	-0.285 (0.535)	-0.244 (0.598)	-0.164 (0.726)
P^{t} S^{t}-Bernard	-0.412 (0.358)	-0.463 (0.296)	0.063 (0.893)
Response	**development time**		
Bosco Gurin	-0.438 (0.326)	-0.48 (0.276)	0.114 (0.807)
Lautaret	**0.629 (0.003)**	-0.127 (0.811)	
Mattmark	0.384 (0.395)	-0.240 (0.604)	-0.751 (0.052)
Nice	-0.284 (0.538)	-0.331 (0.469)	0.158 (0.735)
P^{t} S^{t}-Bernard	-0.616 (0.141)	-0.700 (0.080)	0.413 (0.357)
Response	**prepupal weight**		
Bosco Gurin	0.106 (0.821)	0.835 (0.019)	0.121 (0.795)
Lautaret	-0.434 (0.098)		0.995 (0.329)
Mattmark	0.055 (0.906)	0.251 (0.587)	-0.299 (0.515)
Nice	**0.927 (0.003)**	0.142 (0.761)	0.179 (0.702)
P^{t} S^{t}-Bernard	0.091 (0.847)	0.820 (0.024)	-0.440 (0.323)

The heritabilities of larval performance traits in the tested populations were not significantly different from zero. There was no support for the prediction that a history of selection on native hosts would have produced lower heritabilities for performance traits than those on alternative hosts. Our results do confirm the general finding of low heritabilities of life history traits (Price and Schluter 1991).

We found no evidence for trade-offs in larval performance across hosts. As many of the genetic correlations between hosts were positive as were negative, and the only two significant correlations were both positive. Similarly at the population level, larvae from Mattmark and Bosco Gurin which performed best on one host were also superior on the two other plant species that they do not encounter in the field. As mentioned in the previous sections, there are several explanations to account for the lack of negative genetic correlation between performance traits among environments. We have no empirical evidence that would help us determine which factors have prevented this negative genetic correlation from arising in the *O. elongata* system.

In conclusion, there is little evidence for negative genetic correlations or the start of host plant specialization among populations of *Oreina elongata*. This is despite variation in host plant availability and very low gene flow between these isolated populations, the prerequisites for local adaptation and specialization (Margraf 2003). Larval performance is characterised by stasis among populations, except in the response of the two populations lacking *C. spinosissimum*. It seems as if larval physiology is of minor importance in driving host specialization in *O. elongata*, but that instead protection against natural enemies and sequestrable plant defensive compounds are of larger impact. Rather than the traditional preference – performance association, it looks as if in the *Oreina elongata* system we are instead faced with a preference – protection association.

Acknowledgements

We thank G. Aerni and M. Tschan for assistance with the experiments. We are very grateful to K. Gotthard and people from the LEAE laboratory for helpful discussion of the manuscript. Logistic support at Petit Saint Bernard was kindly provided by L. Vaser, P. Küpfer and the Ordine Mauriziano, Torino, Italy. This work was supported by the Swiss National Science Foundation (Grant number - 31-64’864.01)

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PAPER three

HOST PLANT SPECIALIZATION OR GEOGRAPHIC ISOLATION ? POPULATION GENETICS OF AN ALPINE LEAF-BEETLE

Nicolas Margraf and Martine Rahier

Abstract

The variations in host plant availability in the population of Oreina elongata, as well as the low dispersal ability of the species have been hypothesized to favour host plant specialization in this species. We used six microsatellite loci to examine genetic divergence in 13 populations from across the range of the species. Genetic variability was found to be high among populations but relatively low within populations, and the estimate of Fst of 0.382 suggests extremely low dispersal. A phylogenetic tree derived from a genetic distance matrix clustered O. elongata populations according to geographic region, and showed almost perfect monophyly of the four subspecies considered. Host plant in the field appeared to explain poorly the diversification of the species. Larval performance data correlates with genetic differentiation in a subset of five of the populations.

Introduction

The balance between gene flow and spatial variation in selection pressure has been thought to be one of the agents that determine the degree of adaptation of separated populations to their local environment.[Peterson, 1998 #219; Slatkin, 1973 #224; Slatkin, 1987 #454] Gene flow within a species largely determines the extent to which populations evolve independently [Slatkin, 1985 #452]and has therefore often been perceived as the homogenising factor that prevents local adaptation. However, (Wright 1982) in his “shifting balance theory” suggested that gene flow could also play a creative role in the process of local adaptation. Well-adapted combinations of genes that would then emerge in one local populations by the combined action of natural selection and selective drift would spread to other local population through gene flow.

In phytophagous insects, host plant specialization on different host plants has been advocated to be a major factor in generating their extraordinary diversification..[Bernays, 1994 #268; Futuyma, 1991 #267; Jaenike, 1990 #261; Thompson, 1994 #465] However, recent evidence suggest that specificity is often a local phenomenon that may or may not spread throughout the range of an herbivorous species.[Fox, 1981 #226; Leebens-Mack, 1998 #45; Thompson, 1994 #465] In this study examines the genetic population structure of an alpine leaf-beetle, *Oreina elongata* (Coleoptera: Chrysomelidae) and relate it to geographical isolation and host plant use.

Probably because of its high alpine habitat, *O. elongata* displays a very patchy distribution throughout its appenino-alpine range. Both adults and larvae feed on four different plant species from two genera of the Asteraceae family: *Adenostyles alliariae*, *A. glabra*, *A. leucophylla* and *Cirsium spinosissimum*. The two genera of host plant differ in their characteristics: *C. spinosissimum* is known to better protect eggs and larvae against predation (Paper V) and severe weather conditions (Appendix) probably through its hairy, highly dentate and spiny leaves. On the other hand, the heart-shaped leaves of the Adenostyles species contain pyrolizidine alkaloids (PAs) that adult and larvae sequester for their own defence (Hartmann *et al.*, 1997). Beetles feeding on *C. spinosissimum* rely on an endogenous production of cardenolides for their protection (Dobler, Rowell-Rahier, 1994), which seems to be an inferior chemical defense compared to the PAs from Adenostyles that protect *O. elongata* more

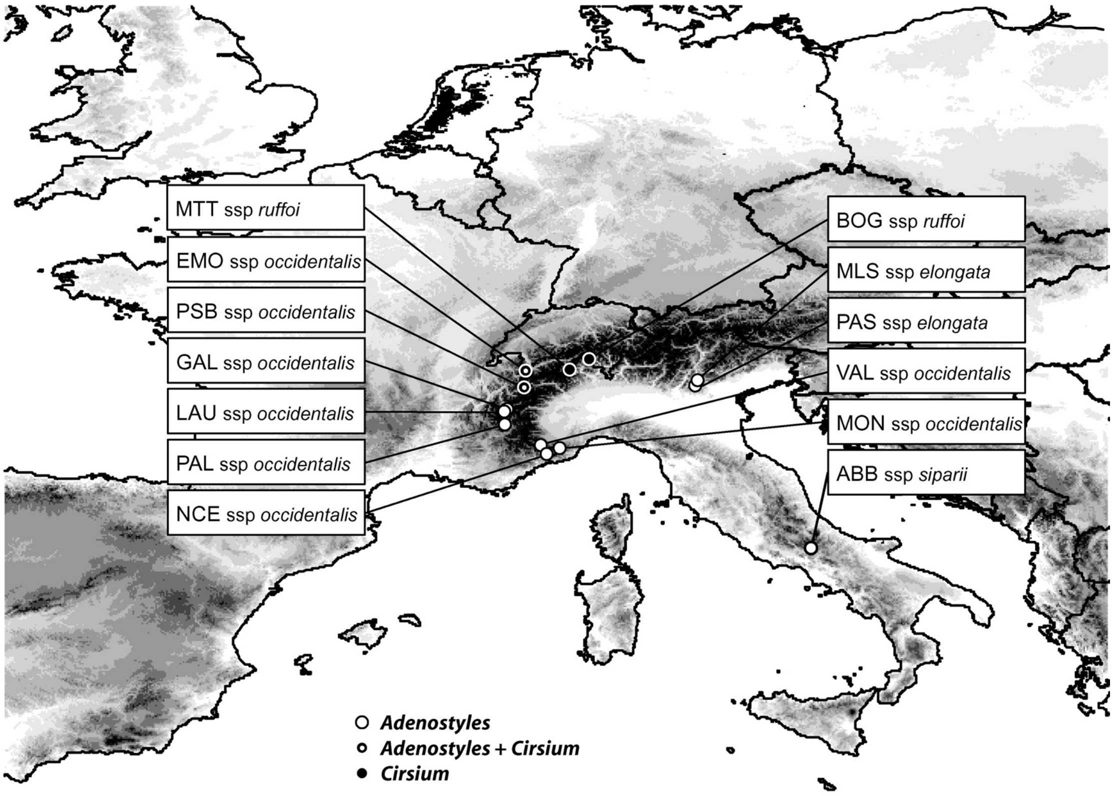

Figure 1. *Sampling locations of Oreina elongata populations throughout the appenino-alpine distribution of the species*

efficiently against naïve bird predators (Rowell-Rahier *et al.*, 1995).

The differences in host plant availability between populations, the very patchy distribution coupled with a very low dispersal rate (the adults seems not to fly and mark-recapture studies have revealed a maximal dispersal distance of 80 m over two years (Conconi, unpublished data)) and the total absence of flying behaviour despite their effective flight muscles, suggests that local adaptation and genetic differentiation is likely to evolve in *O. elongata*.

Recent evidence suggests there has been little adaptation of *O. elongata* populations to the host plant availability at least in females oviposition preferences; *C. spinosissimum* is always the host preferred to lay eggs (Verdon, A., Margraf, N., Naisbit, R.E., Rahier, M., in prep). Larval performance does vary between populations but in general the superior host is not that which is encountered in nature (Paper II).

In this study, we investigated the population genetic differentiation of *O. elongata* throughout its European range using microsatellites markers. We wanted to see if genetic variation between populations was present and if it could be explained by geographic isolation or/and differences in host plant availability in the field. Moreover, we aimed to compare the genetic similarities with the ecological similarities for the five populations that we had previously tested for larval performance.

Material and Methods

During the summer 2001, we collected individuals from 13 populations of *O. elongata* throughout the Alps and the Appenines (fig. 1). The individuals were starved overnight to avoid contamination by plant material contained in the gut, preserved in pure ethanol and stored at -80°C as quickly as possible.

DNA was extracted using the PUREGENE kit (Gentra) from the head, thorax and legs only, to ensure that no plant material contained in the gut could contaminate the samples.

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repeat Motif	Ta (°C)	Size-range (bp)	Accession N°
CAA3	CCGAAGCTTCCACTTGAGAC	CAGGGTACTTTGTCCCGAAC	$(GT)_{12}$	58	142-176	AY380123
CAA5	AGGCAACAACGATGTCTCG	GGTGCTGGCTGTAGGTTCAC	$(GT)_{15}$	60	115-141	AY380124
CAB1	ATCCGGTGTGAAAGACTTCG	CATGCTCCTCTGAAGGAACC	$(CA)_8n(CA)_{11}$	58	176-196	AY380129
CAB6	GCAACAGTTCATGGCAGAATC	CCATTGCGAGGTCTGTCC	$(GT)_{13}$	58	96-114	AY380125
CAO7	AGATGCCGGTGACACAATG	TTGGTCATTAGGGCTCCATC	$(CA)_{12}$	62	106-126	AY380131
TGG6	CACTCGATGCTGATGCAGAC	ACGCTCGAAAAGATCACCTG	$(CCA)_6$	58	162-168	AY380130

Table 1. *Microsatellite loci for Oreina elongata with their accession number in GenBank*

An enriched library was made by ECOGENIC GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer et al. 1999) and enriched by magnetic beads selection with biotin-labelled (CA)13 and (GA)13 oligonucleotide repeats (Gautschi et al. 2000a; Gautschi et al. 2000b). Out of the 1536 recombinant colonies screened, 519 gave a positive signal after hybridization. Plasmids from 104 positive clones were sequenced and primers were designed for 6 microsatellite inserts (Table 1) in which polymorphism has been tested.

To assay variation among individuals, polymerase chain reactions (PCR) were performed in a 5 µl volume containing about 1.5 ng of template DNA, 1.25 µM of each primer and 2.5 µl of HotstarTaq master mix (Qiagen). Amplification were proceeded using the following hotstart protocol: A first step of 15 min of prolonged denaturation at 95 °C, followed by 25-30 cycles, each consisting of 30 s denaturation at 95°C, 30 s annealing at the specific annealing temperature and 30 s extension at 72°C. The last cycle was followed by an extra 8 min at 72°C to complete extension. PCR products were mixed with a stop solution and denatured at 95°C for 1 min, before electrophoresis on a 6% denaturing polyacrylamide gel Sequagel *XR* (National Diagnostics) running on a Li-Cor sequencing system. Isolated bands were scored according to their size, using SAGA IR^2software, version 2.1.2.

For each population and locus, we calculated the mean and expected heterozygosity and tested for linkage disequilibrium using ARLEQUIN (Schneider et al. 2000). We assessed the degree of departure from Hardy-Weinberg equilibrium using exact tests given by GENEPOP (Raymond and Rousset 1995). We calculated genetic differentiation among populations and level of inbreeding within population using Fstat version 2.9.3 (Goudet 1995; Goudet 2001). The program uses the procedure of (Weir and Cockerham 1984) for multilocus estimates and weights by sample size within populations. The coefficients estimated by this procedure are related to the Wright's F-statistics of (Wright 1921; Wright 1969) as $F = F_{IT}$, $\Theta = F_{ST}$ and $f = F_{IS}$. The 95 % confidence intervals (CI) around F-statistics were estimated by bootstrapping.

We estimated genetic distances between individuals using Nei's genetic distance using the R4 package (Casgrain and Legendre 1997). To assess the influence of different factors on genetic differentiation in *O. elongata*, we calculated pairwise similarities between individuals for different factors using asymmetrical Jaccard's coefficient as it does not take double zeroes into account.

$I_j = \dfrac{a}{a+b+c}$ where a is the number of shared characteristics between the two individuals and b and c the number of characteristics present only in individual 1 and exclusively in individual 2 respectively.

We used host plant availability, individual affiliation to population and to regional groups (north, east, west and south of the distribution) and geographic distances between populations to calculate resemblance matrices. We transformed pairwise similarities into pairwise distances using the formula distance = 1 – similarity. We then performed Mantel test (999 permutations) using the progiciel R to assess the correlation coefficient between pairwise genetic distance and geographic distance matrix as well as distance matrices of host plant availability and population affiliation.

We tested for isolation by distance using linear distance between populations and estimated the number of effective migrant (Nm) using private alleles method with the program GENEPOP (Raymond and Rousset 1995)

We built dendrograms using the progiciel R for a better capture of how population cluster together. We used pairwise Nei's genetic distance calculated on number of copies of alleles in each population to build the tree with the UPGMA method.

Finally we calculated from a previous data set (Paper II), mean larval performance (i.e. larval growth rate, developmental time and prepupal weight) distances between five populations (MTT, BOG, LAU, PSB and NCE) using the Euclidean distance on standardized data. We transformed these distances into similarities using the formula similarity = 1- distance and compared this matrix to the Nei's genetic distance matrix for those same five populations using a Mantel test (999 permutations). We clustered these populations according to genetic and performance distances in two trees using the UPGMA method

Table 2. *Microsatellite loci of Oreina elongata with their observed heterozygosity (Ho), the P value of the test for a departure from HWE with a deficit of heterozygotes (HW), F which is related to F_{IT}, Θ which is related to F_{ST} and f which is related to F_{IS}*

Locus	No. of alleles	H_O	HW	F	Θ	*f*
CAA3	15	0.241	0.000	0.733	0.288	0.624
CAA5	14	0.307	0.000	0.656	0.370	0.454
CAB1	9	0.081	0.000	0.753	0.455	0.547
CAB6	9	0.269	0.000	0.770	0.581	0.451
CAO7	10	0.620	0.770	0.142	0.250	-0.144
TGG6	3	0.100	0.668	0.466	0.483	-0.032

Table 3. *Locations, sample sizes, unbiased heterozygosity, observed heterozygosity and mean number of allele per locus for the 13 populations of Oreina elongata. Numbers in parentheses are standard deviations*

Population	Sample size	Exp Hz	Obs Hz	No Alleles
ABB	24	0.28 (0.12)	0.11 (0.03)	2.83 (1.47)
BOG	24	0.37 (0.18)	0.31 (0.05)	3.00 (1.41)
EMO	24	0.32 (0.13)	0.19 (0.03)	3.67 (3.08)
GAL	21	0.33 (0.12)	0.12 (0.03)	3.00 (1.67)
LAU	24	0.21 (0.11)	0.09 (0.02)	2.33 (1.51)
MLS	12	0.45 (0.12)	0.18 (0.05)	3.20 (1.3)
MON	24	0.58 (0.07)	0.23 (0.04)	4.83 (2.99)
MTT	24	0.50 (0.19)	0.26 (0.05)	4.00 (2.35)
NCE	24	0.36 (0.15)	0.12 (0.03)	3.17 (2.14)
PAL	21	0.23 (0.13)	0.08 (0.02)	2.50 (2.26)
PAS	35	0.53 (0.14)	0.20 (0.04)	4.20 (2.59)
PSB	24	0.35 (0.12)	0.06 (0.02)	2.83 (1.94)
VAL	24	0.38 (0.08)	0.15 (0.03)	3.00 (1.55)

Results

All six microsatellites loci were variable and consistently scorable in *Oreina elongata*. The number of alleles per locus ranged from 3 to 15 for the six microsatellites loci. Exact tests for departure from Hardy-Weinberg equilibrium (HWE) indicated a significant deficit of heterozygotes for four out of six loci (CAA3, CAA5, CAB1 AND CAB6) and overall through the multilocus test (Table 1). This may suggest the presence of null alleles at these loci. We found no linkage disequilibrium between the loci.

Hierarchical analysis revealed an overall high level of inbreeding within populations ($f = 0.336$) with 95% *CI* just over zero. The f values differed between loci, being slightly negative for CAO7 and TGG6 but higher than 0.4 for the four other loci (Table 1). On the other hand, Θ-values turned out to be relatively consistent, ranging from 0.250 for CAO7 to 0.581 for CAB6. The overall Θ-value revealed a high genetic differentiation among populations ($\Theta = 0.382$) with 95% *CI* well above zero. There was no difference between males and females in Fst nor in Fis values.

The Isolation By Distance (IDB) test performed by GENEPOP (slope = 0.173, intercept = -0.264, P value = 0.016) confirmed the Mantel correlation between pairwise genetic distance and population geographic distance (r = -0.463, P value < 0.001), showing isolation by distance between the sampled populations of *O. elongata*. The estimated number of migrant appeared consistent between the private allele method (Nm = 0.215) and the Wright's method (Nm = 0.249).

Mantel correlation between estimates of pairwise genetic distance and population affiliation matrix was positive (Mantel r = 0.409) and significantly different from zero (P value = 0.001) confirming Fst results of population differentiation. Additionally, the distance matrix of host plant availability was significantly correlated with the pairwise genetic distance matrix (Mantel r = 0.238, P value < .001) indicating an relationship between genetics and hostplant availability in *O. elongata*. The division of populations into four regional groups also appeared to be strong with a Mantel r of 0.634 (P value < .001). To dismiss the opportunity of a regionalization of host plant availability, we performed a partial Mantel test between genetic distance matrix and host plant availability distance matrix controlling with the regional affiliation distance matrix, which correlation turned out to be weaker than without correction (Mantel r = 0.173) but still significant (P value = 0.001). Finally, we found a high Mantel correlation between the distance based on mean larval performance of the five populations and their genetic distances (Mantel r = 0.811) although it did not attain statistical significance (p = 0.072) (Fig 3).

Table 4. *Mantel correlations between Nei's genetic distances and four variables. N = 305 individuals*

	Mantel r	P value
Geographic distance	-0.462	0.001
Population affiliation	0.351	0.001
Geographical regions	0.366	0.001
Host plant availability	0.238	0.001
Host plant availability controlling for regionalization	0.160	0.001

Discussion

The level of genetic differentiation between the different populations of our study (Θ = 0.382) is relatively high compared to those reported for other chrysomelid species [Knoll, 1996 #233] The high level of genetic differentiation among populations of *Oreina elongata* can be attributed to the extreme patchiness of their distribution. This is partly due to their restriction to high altitude: at such altitudes, populations are more likely to be isolated from one another by non suitable habitat [Knoll, 1996 #233] Still our result for genetic differentiation is even higher than expected and this can be explained by the extremely low dispersal rate of *O. elongata*. Despite several years of intense study, *O. elongata* adults have never been seen to fly even though they possess flight wings. Moreover, mark-recapture studies reveal a dispersal range of maximum 80 m. (Conconi, unpublished data). A recent genetic analysis using RAPD markers has shown that within the Petit Saint Bernard population, *O. elongata* collected on patches approximately 1 km apart showed a significant genetic differentiation [Hirter, 2003 #336] Considering the very low dispersal rate and subsequent gene flow as well as extreme patchiness of the habitat, it is not surprising to find a high value of Θ in *O. elongata*.

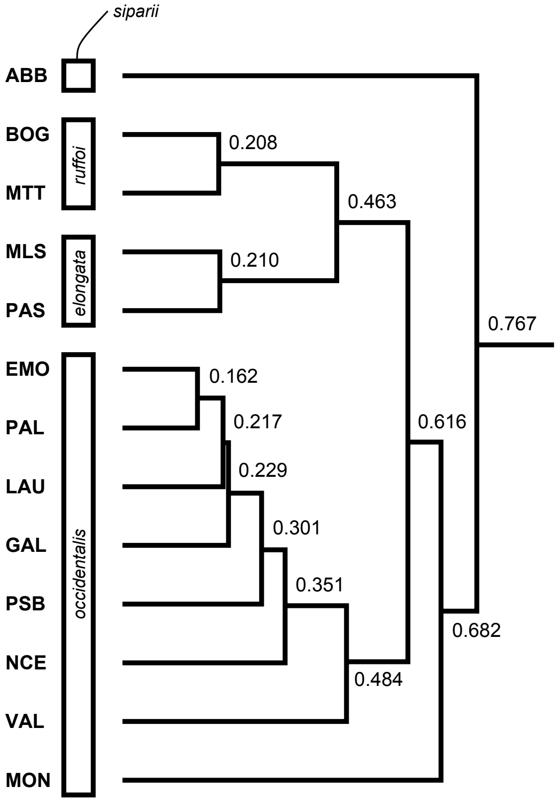

Figure 2. *UPGMA dendrogram based on Nei's genetic distances from microsatellite allele frequencies*

On the other hand, several explanations could account for the high inbreeding coefficient found within population. A Wahlund effect can be dismissed because we collected beetles from a limited area. Within each site, null alleles may be the reason for an overestimation of homozygotes and consequently an underestimation of heterozygotes leading to a high inbreeding coefficient within each population. However, despite the suspicion of the presence of this artefact in our study, we think that it is probably not the only reason for the high inbreeding coefficient.

Strong inbreeding may have caused the observed deficit in heterozygotes. In several other organisms, the pattern of a high level of genetic differentiation among populations and a high inbreeding coefficient has been found.

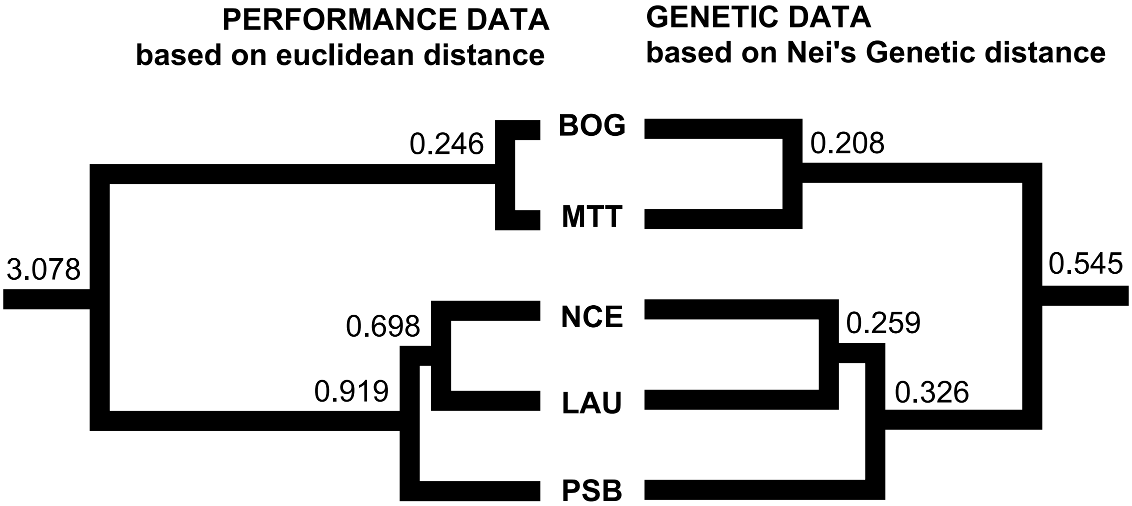

Figure 3. *Comparison between the UPGMA dendrogram obtained using Nei's genetic distance from microsatellite allele frequencies in the five tested populations and the UPGMA dendrogram obtained using Euclidean distance on the mean larval performance data (Paper II)*

This indicates that *O. elongata* populations are not at the equilibrium between immigration rate and genetic drift as confirmed by the weak pattern of IDB. The slope of the IBD was relatively low as expected for a "sedentary species" (Peterson and Denno 1998) suggesting that the relationship between populations is determined more by historical factors than by effect of distance. Additionally, the low estimated number of migrant found with the two methods confirms the observation of very low dispersal capacity of *O. elongata*.

The threshold value of 0.5 migrants per generation is not reached in our beetles, predicting separate genetic evolution of the populations under genetic drift (Slatkin 1985). This result explains both the high value of Θ and the high inbreeding coefficient: due to the very low immigration rate, populations evolve as entities developing genetic differences that will not spread over the whole distribution of the species.

However, there are other explanations to account for the genetic differentiation among populations of *O. elongata*. One of them has been outlined by the Mantel correlation between estimates of pairwise genetic distances and pairwise plant availability distances, which was positive, indicating an effect of host plant availability on genetic distance among populations even when corrected for regionalization of host plant availability. Populations of *O. elongata* experience various mixtures of host plants in the field and presumably divergent local selection pressures. Adaptive genetic structuring has been found in other herbivorous insects (Mopper 1996). Nevertheless, in *O. elongata*, past and recent studies have failed to show local adaptation in life history traits. Tests for local adaptation in oviposition preference and larval performance have produced unvarying results: *O. elongata* females prefer to lay their eggs on *C. spinosissimum* whereas overall *A. alliariae* or *C. spinosissimum* allow the best larval performance, whatever plant the population experiences in the field. This might correspond to the relatively low correlation between host plant availability and genetics, suggesting that we are not confronted to host races. Nevertheless, a recent study in larval performance of five populations differing in their host plant availability has been able to confirm a tendency of beetles living on *C. spinosissimum* in the northern part of the distribution to exhibit a different life history strategy (Paper II). Larvae from MTT and BOG showed a higher larval growth rate, a shorter larval developmental time and a smaller prepupal weight. We performed a cluster analysis on the performance data for the five populations using

Euclidean distances and the UPGMA method to be compared with the cluster based on the genetic distances of these same five populations. The two trees did split the population in similar groups: populations living in the Swiss Alps on *C. spinosissimum* (MTT and BOG) differed from beetles from the more occidental part of the distribution with accessibility to Adenostyles plants (Fig 3). Moreover, even though marginally significant, the Mantel correlation between the performance similarity matrix and the genetic distance matrix appeared to be relatively high (Mantel r = 0.811), revealing that populations that are more similar genetically are also more similar in term of larval performance. The differences in life history traits found in previous studies are therefore also evident at the genetic level. More than differences in host plant availability, shared region of origin seems to be a good predictor of genetic similarity. The Mantel correlation between regional affiliation and genetic differences as well as the general pattern of the overall cluster analysis separate *O. elongata* into four geographic groups. These groups correspond to the subspecies previously described on the basis morphology.[Daccordi, 1986 #40; Daccordi, 1976 #60; Kühnelt, 1984 #234] The first group includes all the western part of the distribution (NCE, MON, VAL, PAL, LAU, GAL, PSB, EMO) forming the *occidentalis* subspecies, the southern population of the Abbruzes (ABB) is the only representing population of the *siparii* subspecies, the eastern group unites PAS and MLS populations as the *elongata* subspecies and finally the two *C. spinosissimum* only populations, MTT and BOG, forming the *ruffoi* subspecies.

In conclusion, we have been able to document the genetic divergence of populations of the alpine leaf-beetle, *O. elongata* showing that the underlying process does not seem to be related to host plant availability in the field but instead related to a common history that led to subspecies formation. Our results seem to indicate that we are faced with a speciation phenomenon that appears to be due to the low dispersal ability of the species that triggers a genetic drift rather than divergent selective pressures associated with variation in host plant use.

Acknowledgments

We thank B. Gautschi, S. Marc-Martin, A. Aebi, N. Alvarez and R. Guadagnolo for technical assistance and C. Truong, K. Gotthard and P. Mardulyn for helpful discussion on the manuscript. This work was supported by the Swiss National Science Foundation (Grant number - 31-64’864.01)

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PAPER four

GEOGRAPHIC VARIATION IN OVIPOSITION CHOICES OF A LEAF BEETLE: THE RELATIONSHIP BETWEEN HOST PLANT RANKING, SPECIFICITY AND MOTIVATION

Karl Gotthard, Nicolas Margraf and Martine Rahier

Abstract

The degree of adaptation of herbivorous insects to their local flora is an important component of the evolutionary processes that lead to host plant specialization in insects. In this study we investigate geographic variation in the oviposition preference of the leaf beetle, Oreina elongata, in relation to the pattern of host plant specialization in the field. We focus on the mechanisms of host choice and ask whether potential differences among populations are due to variation in host plant ranking and/or host plant specificity. We performed a combination of simultaneous choice and sequential no-choice experiments with two of the major host plants of the beetle. The results suggests that spatial variation in host plant specialization have resulted in differences between populations in some aspects of the oviposition choice of O. elongata, while other aspects seem unaffected. We found no variation in host plant ranking among populations as estimated in simultaneous choice tests. In contrast, the sequential no-choice test indicate that host plant specificity was lower in a population that never encounter the highest ranked plant in the field. This finding was in line with expectations and we discuss our results in relation to the commonly used hierarchical threshold model. The results suggest that the most likely mechanism for the differences in specificity is variation among populations in the general motivation to oviposit rather than quantitative differences in relative preference for the two hosts. We stress that it is essential which of these mechanisms is most important since it will effect the probability of evolutionary change in host plant ranking.

Introduction

The spatial structure of populations and its ecological and evolutionary consequences are central issues in theoretical ecology and evolution (Endler, 1977; Thompson, 1994; Hanski & Gilpin, 1997; Mopper & Strauss, 1998). In the field of insect-host plant biology there has been a great interest in the spatial patterns of host plant use (Thompson, 1994; Mopper, 1998).This is probably due to the long-standing interest for the evolutionary processes that lead to host plant specialization (Futuyma & Moreno, 1988; Jaenike, 1990; Futuyma, 1991; Thompson, 1994; Bernays, 1998), and the realization that the spatial dynamics of insect-plant interactions is often a central component of these processes (Thompson, 1994). In this study we investigate variation in oviposition preference among populations of the beetle, *Oreina elongata* Suffrian (Coleoptera: Chrysomelidae), in relation to differences in host plant specialization in the field. We focus on the mechanisms of oviposition host choice and ask whether potential differences are due to variation in host plant ranking and/or host plant specificity, and relate our results to a commonly used model for host plant choice (Courtney, *et al.*, 1989).

Several lines of evidence suggest that the oviposition behavior of females may be more important than larval performance in driving the evolution of host specialization, and the selection pressures that may influence oviposition preferences of herbivorous insects have been studied in some detail (Futuyma, 1983; Thompson, 1988a; Jaenike, 1990; Janz, *et al.*, 2001). One important part of these studies have been to describe and understand the decision process that leads an individual female to either accept or reject a given host for oviposition (e.g. Wiklund, 1981; Singer*, et al.*, 1992). The results suggest that the oviposition decision depends on the hierarchical ranking of the potential hosts according to their general suitability and the instantaneous motivational status of the ovipositing female. This view has been formalized in the hierarchical threshold model of host choice (Courtney*, et al.*, 1989), which applies to the typical situation where potential hosts are encountered in sequence. In this model the ranking of host plants is a consequence of variation in insect preferences for the different plants, which is determined by the positive stimuli that the insect receives from the plant (in contrast to Courtney*, et al.* (1989), we prefer to use the term preference rather than acceptability to describe the insect trait, see Singer (2000)). The rank hierarchy is thought to be genetically fixed and invariable during the life of an individual female. However, whether or not a given female accepts a potential host will depend on her current motivational status to oviposit, which may be influenced by factors such as egg load, age or immediate host plant density and will vary throughout female life. The motivational status of a female will constitute the threshold criterion for host acceptance/rejection and it will determine how far down in her host plant hierarchy she will accept hosts at any given time.

The model and its empirical foundations (Wiklund, 1981; Singer, 1983; Thompson, 1988a, b; Courtney*, et al.*, 1989) suggest that two insect females could rank two host plant in the same order but still differ in host plant specificity; i.e. the likelihood that the lower ranked plant will be accepted at encounter. Specificity is influenced by two factors in the model: the quantitative difference in preference between the hosts, and rate of change/average level of motivational status (the threshold criterion). Large differences in preference between hosts and/or a slowly increasing motivational status will lead to high specificity. The level of preference as well as rate of change in motivational status are likely to be under genetic control and may respond to natural selection. Indeed, several workers have suggested that oviposition specificity should be less evolutionary conservative than host plant ranking (e.g. Courtney*, et al.*, 1989; Janz, 1998).

We investigated host plant choices of ovipositing *O. elongata* females from three different populations that vary in host plant specialization in the field. Two of the populations have only one host plant each available (although different from each other) and they only experience selection for utilizing their respective host plant. The third population has available, and use, two host plants and experience selection for using both hosts (Ballabeni*, et al.*, 2001a). Earlier studies indicate strong genetic differentiation among these populations in several developmental traits (age and size at pupation, larval growth rate) but only a relatively limited degree of local adaptation in larval performance on different host plant species (larvae of single-host populations grow slightly faster on their native host genus while the difference is smaller in the two-host population, Ballabeni, *et al.*, 2003). We expected that the differences in host plant availability and utilization among populations should have led to divergent evolution of oviposition preferences that should be manifested in some of the components of the hierarchical threshold model. These expectations were tested in experiments where two hosts species were presented simultaneously to females as well as in experiments where these two hosts were presented singly in sequence. We used these two setups to estimate rank order of the two hosts (simultaneous choice trials) independently of host plant specificity (sequential no-choice trials). Despite the fact that these methods have been used extensively for investigating oviposition choices of insects they have rarely been used together in the same insect species.

Materials and Methods

STUDY ORGANISMS

Oreina elongata is found exclusively in alpine areas at altitudes between 1600 m - 2300 m and its geographical distribution extends throughout the Alps and further south in the Apennines. Due to its alpine habitat the distribution of *O. elongata* populations is highly heterogeneous and this is also true within sites where the occurrence of beetles is patchy. Field observations and mark-recapture studies strongly indicate that the dispersal rates of *O. elongata* are very low (Conconi, D. unpublished). The beetles have flight wings but despite several years of intense studies both in the field and in the laboratory there are no recordings of them flying (personal observations, Conconi, D. unpublished). Hence, it seems likely that the beetles disperse mainly by walking.

The life cycle of *O. elongata* is adapted to the high alpine environment and includes a two year juvenile period as well as the potential for several consecutive reproductive seasons once the adult stage is reached (personal observations, Conconi, D. unpublished). The host plant utilization (oviposition and adult and larval feeding) of *O. elongata* is specialized to three different plant species in the Asteraceae: *Adenostyles alliariae* (Gouan) and *A. glabra* (L.) as well as the thistle *Cirsium spinosissimum* (L.). Both in terms of morphology and chemistry the *Adenostyles*-species are distinctly different from *C. spinosissimum*. Plants in the genus *Adenostyles* produce pyrrolizidine alkaloids (PAs in the following) and both adults and larvae of *O. elongata* (and several other *Oreina* species) sequester PAs and use them as a chemical defense against natural enemies (Rowell-Rahier*, et al.*, 1991, 1995; Dobler & Rowell-Rahier, 1994; Pasteels*, et al.*, 1995). The third host, *C. spinosissimum*, does not provide the beetles with any sequesterable defensive compounds. In addition to the chemical differences between the host plants there are substantial morphological differences: the *Adenostyles* species have large, heart-shaped and relatively smooth leaves while the leaves of *C. spinosissimum* are strongly dentate, hairy and spiny, which may provide mechanical protection against predators for all life stages of the beetle.

The availability of host plants varies geographically in the Alps and there are populations of *O. elongata* that only has *Adenostyles*-species available in their habitats and others that only have *C. spinosissimum* available, while others again have both an *Adenostyles*-species and *C. spinosissimum* present in their habitat. The three populations that were used in this study represent these three categories of host plant availability: the population at Col du Lautaret (France, altitude 2058 m) has no *C. spinosissimum* in its habitat and feeds almost exclusively on *A. glabra*, whereas the population at the Mattmark dam (Switzerland, altitude 2200 m) only has *C. spinosissimum* available and is exclusively found on this plant, finally the population at Col du Petit Saint Bernard (France, altitude 2188 m) has both *A. alliariae* and *C. spinosissimum* in its habitat and all life stages of *O. elongata* can be found on both plants (in the following they will be referred to as the *Adenostyles*, *Cirsium* and two-host populations, respectively). However, females of this two-host population seems to prefer to oviposit on *C. spinosissimum*, which may be due to a higher egg survival on this plant compared to *A. alliariae* (Ballabeni*, et al.*, 2001a, b). The shortest distance between any of these populations is at least 200 km and they are separated by high altitude mountain ranges (3000 – 4000 m a s), making dispersal between them very unlikely.

EXPERIMENTAL DESIGN– SIMULTANEOUS CHOICE

During the first week of July 2000 we collected at least 100 adult beetles from each of the three populations above and brought them to a field station close to the two-host population where the host choice experiment was carried out. Prior to the experiments all populations were given simultaneous access to leaves of *A. alliariae* and *C. spinosissimum* in population boxes, which should reduce any potential effects of learning in the field sites. The oviposition experiment was performed outdoors in cages made of mesh and plastic (60 x 60 x 60 cm BugDorm-2, MegaView Science Education Services Co., Taiwan) that allowed the natural temperature, wind and rain to affect the ovipositing females. The cages were placed in three rows on flat ground next to the field station and each row consisted of 10 cages. In every cage a single female was given the choice between *A. alliariae* and *C. spinosissimum* that were grown in pots. The plants were placed in the cages so that they had leaf contact and each female was randomly placed on either of the two plants at the start of the trial. Within each row of cages the females were randomized with respect to population. Hence, we tested 30 females (10 per population) in each trial that lasted for three days and we performed four such trials. In total we tested 40 individuals per population and each female was only used once. The potted host plants were reused in the different trials but were randomized over the cages before each trial. In the end of each trial we counted the number of eggs that each female had laid on the two hosts as well as any eggs laid elsewhere in the cage.

EXPERIMENTAL DESIGN– SEQUENTIAL NO-CHOICE

During less than one week in the end of June 2001 (28.06 – 04.07) we collected at least 150 adults at each of the three populations and brought them to the field station. Prior to the experiments all populations were given simultaneous access to leaves of *A. alliariae* and *C. spinosissimum* in population boxes. We used 60 females from each population in the oviposition experiments. These experiments were designed as "no-choice trials" where each female only had access to one host plant species at the time (*A. alliariae* or *C. spinosissimum*). For logistic reasons the experiment was performed in the laboratory of the field station where females were put singly in round plastic cups (height 10 cm and width 5 cm) together with one leaf of host plant. At the start of the experiment females from each population were randomly assigned to one of the two host plant treatments (*A. alliariae* or *C. spinosissimum*) and were left during 48 h. After this first part of the experiment all females were switched to the alternative host plant and were given the next 48 h to oviposit on this other plant exclusively. At the end of each 48 h trial we counted the number of eggs that each female had laid on the host plant and elsewhere in the plastic cup. The plastic cups were randomized in a portable shelf system that was placed in one of the windows of the laboratory in order to provide more natural light conditions. The shelves were rotated three times per day to avoid any position effects. The laboratory were left unheated during the experiments which led to a daily variation in temperature that was similar to outdoor conditions. During the experiment we measured the temperature in the laboratory three times per day (the range of temperatures were 15°C – 18.5°C).

STATISTICAL TREATMENT

Throughout the analysis, values for individual females were used as independent observations and we used standard parametric statistical methods. All analyses were performed with StatView 5.0. All proportions were Arcsine-root transformed prior to analysis ($X' = \text{Arcsine}(\sqrt{X})$), while egg numbers were Box-Cox transformed when it improved normality of the data ($X' = ((X+0.1)^{\lambda} - 1)/\lambda$, to avoid problems with zero-observations when estimating λ we always performed transformations on $X+0.1$, see Sokal & Rohlf, 1995). The values of λ were in each case found by maximum likelihood (Sokal & Rohlf 1995) and are presented along with the respective analysis.

In the simultaneous choice experiment we first analyzed if the three populations differed in the total numbers of eggs laid and also if there were any effects of trial on oviposition rate. In this ANOVA we included all females that survived (i.e. also females that did not oviposit during the trials). In a next step we tested if there was a difference in host plant preference among populations and if it mattered which plant the female was placed on at the start of the trial (start-plant). In this analysis we used the proportion of eggs that each female laid on *C. spinosissimum* as our measure of host plant preference (i.e. only females that did oviposit during the trials were included). We also tested if this measure of host plant preference deviated significantly from 0.5 in any of the populations, which would mean that they on average showed a preference for one of the two hosts presented. Prior to testing we inspected the forms of the distributions of preference values carefully to check that there were no obvious differences among populations.

The two time periods of the no-choice experiment were analyzed separately and in each case we tested the effect of population and host plant treatment on female oviposition preferences. Throughout the experiment an unexpectedly large proportion of eggs were deposited on the plastic boxes where they were kept during the experiment. Because of this we calculated the proportion of the total amount of eggs that each female deposited on the plant it had available and performed the analysis on that measure. We also performed an analysis using for the total number egg laid during each of the time periods. To correct for multiple testing we performed sequential Bonferroni-adjustments to keep the table-wide level of α at 0.05 (Rice, 1989). Finally, we used the oviposition on the plants during both time periods to calculate the proportion of eggs that each individual female laid on *C. spinosissimum* during the whole experiment. This data was used to test the effects of population on host plant preference while controlling for any effects of the sequence in which the plant species were presented to the females.

Results

HOST PLANT RANKING IN SIMULTANEOUS CHOICE EXPERIMENT

Ten of the120 females that were used in the experiment died during the trials (1, 3 and 6 from the three populations, respectively) and of the surviving females between 24 and 27 females per population did lay eggs. When including all surviving females there was a significant effect of trial starting date on the number of eggs laid but there was no significant difference between populations (ANOVA on Box-Cox transformed total egg number ($\lambda = 0.132$): Start date: $F_{3,\,98} = 6.88$, $P < 0.001$; Population $F_{2,\,98} = 2.22$, $P = 0.11$; Interaction $F_{6,\,98} = 1.05$, $P = 0.40$). This is due to a general seasonal increase in the number of females that terminated oviposition in all three populations.

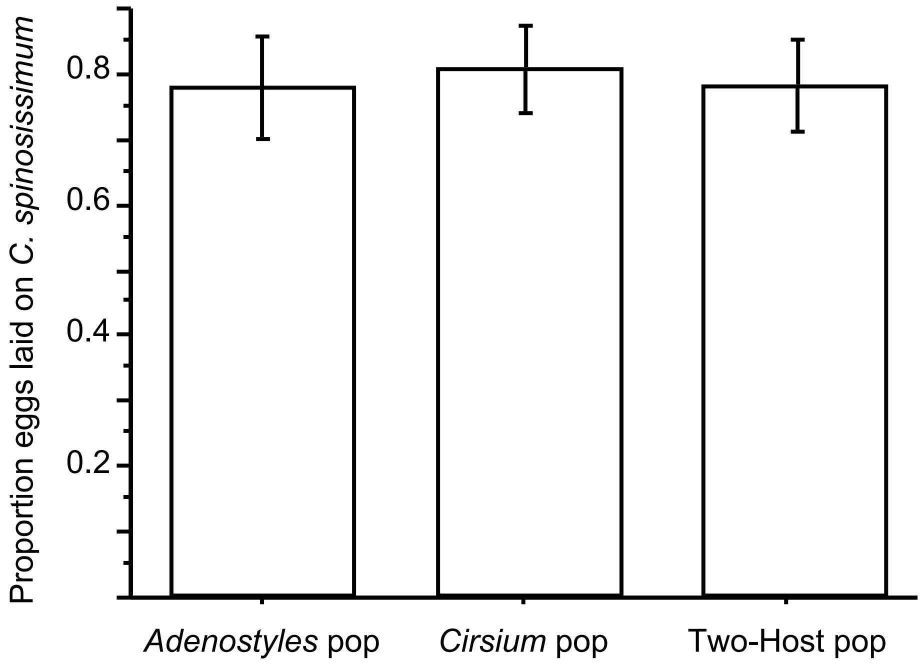

Figure 1. *Results from the simultaneous choice experiment showing the mean proportion of eggs (± 1 SE) that each population laid on C. spinosissimum. Only females that did oviposit are included*

Neither population nor start-plant had significant effects on host plant preference (Fig. 1, ANOVA on arcsine-root transformed values: $F_{population} = 0.14$, df = 2, $P_{population} = 0.87$, $F_{startplant} = 1.13$, df = 1, $P_{start-plant} = 0.29$, $F_{interaction} = 0.77$, df = 2, $P_{interaction} = 0.47$, Error df = 71 in all cases). However, all three populations significantly preferred *C. spinosissimum* to *A. alliariae* (t-test for difference from 0.785 which is the arcsine-root transformed value of 0.5 ; *Adenostyles*-population: t = 3.72, df = 23, P = 0.0011; *Cirsium*-population: t = 4.52, df = 26, P = 0.0001; two-host population: t = 3.83, df = 25, P = 0.0008). There were no apparent differences between populations in the form of the preference distributions. Arguably the measure of host plant preference used here (proportion of eggs laid on a certain plant species) is better estimated in females that lay many eggs. Therefore we performed a new set of identical analyses where we only included females that laid more than 5 eggs during their trials. However, also in these analyses the effects of population and start plant were non-significant, while all populations significantly preferred *C. spinosissimum* to *A. alliariae*. Since the re-analysis did not change any of the conclusions from the analysis of the whole dataset it is not presented in detail.

HOST PLANT SPECIFICITY IN NO-CHOICE EXPERIMENT

In total 17 out of the 180 adults used in this experiment did not oviposit during any of the time periods and they were excluded from the data. Most of these individuals were probably males that were included in the experiment because of mistakes in the sexing procedure. When excluding these individuals the total number of eggs laid per female during both experimental periods ranged from 11 to 59.

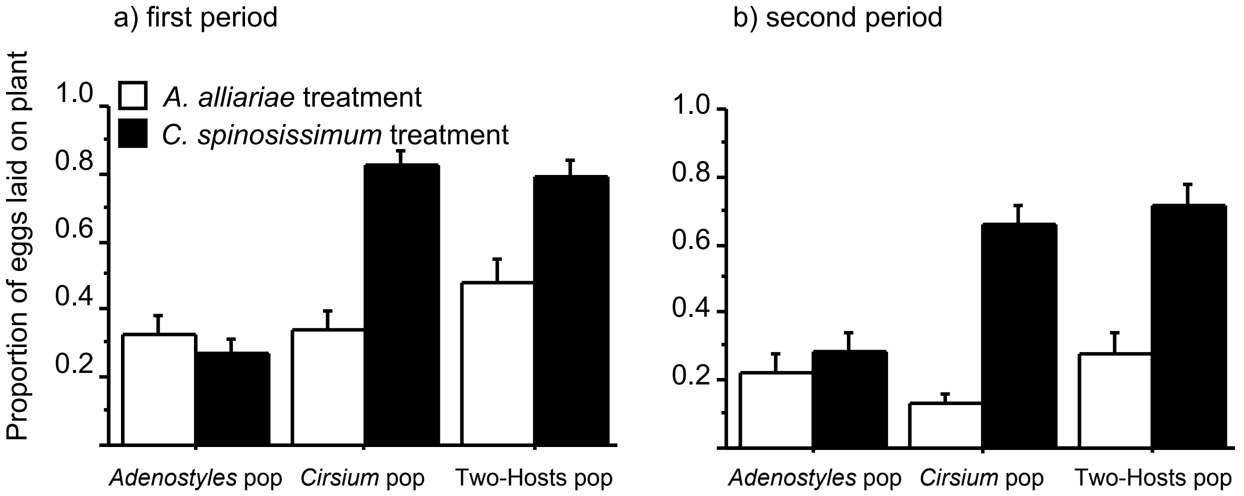

Figure 2. *Results from the sequential choice experiment showing the mean number of eggs (± 1 SE) laid on the plant (a-b) separated by populations and host plant treatment. The left graph show results of the first time period of the experiment while the right graph show oviposition taking place in the second period after the host switch (each period lasted for 48 h)*

During both time periods of the experiment (before and after the plant switch) there were significant effects of population and plant as well as of the interaction between these factors on the proportion of eggs laid on the plants (Fig. 2, ANOVA on arcsine-root transformed values for the first period: Population, $F_{2, 157} = 18.69$, $P < 0.0001$; Plant, $F_{1, 157} = 33.47$, $P < 0.0001$; Interaction $F_{2, 157} = 12.19$, $P < 0.0001$; for the second period: Population, $F_{2, 157} = 10.64$, $P < 0.0001$; Plant, $F_{1, 157} = 67.13$, $P <0.0001$; Interaction $F_{2, 157} = 10.04$, $P <0.0001$). The consistent significance for the interaction term indicates that the *Adenostyles*-population behaves differently compared with the other populations. In both time periods females from the *Cirsium*- and two-host populations laid more eggs on *C. spinosissimum* than on *A. alliariae*, while for the *Adenostyles*-population there was no such difference between host plant treatments. All populations laid significant amounts of eggs on the plastic box (Fig 2) but during both time periods the pattern of oviposition on the box was largely a mirror image of oviposition on plants. When *C. spinosissimum* was available in the box the females from the *Cirsium*- and the two-host populations laid a significantly lower proportion of their eggs on the plastic compared to the situation when they only had *A. alliariae* available, whereas females from the *Adenostyles*-population deposited a majority of their eggs on the plastic independent of host plant treatment (Fig 2).

When all eggs (plant + box) were summed up there was a significant difference between populations in that the *Cirsium*-population laid slightly fewer eggs compared with the other populations (mean ± 1SE egg number for the first and second period for the respective population; *Adenostyles*: 20.7 ± 0.8, 21.2 ± 0.7, *Cirsium*: 16.3 ± 0.8, 15.4 ± 0.6, two-host: 21.6 ± 0.8, 19.4 ± 0.7). However, there was no significant effect of host plant treatment (ANOVA for the first period: Population, $F_{2, 157} = 14.35$, $P < 0.0001$; Plant, $F_{1, 157} = 0.24$, $P = 0.63$; Interaction $F_{2, 157} = 4.34$, $P = 0.015$ (should be lower than 0.013 to be significant at a table-wide α of 0.05) ; for the second period: Population, $F_{2, 157} = 18.92$, $P < 0.0001$; Plant, $F_{1, 157} = 0.83$, $P = 0.37$; Interaction $F_{2, 157} = 0.32$, $P = 0.73$).

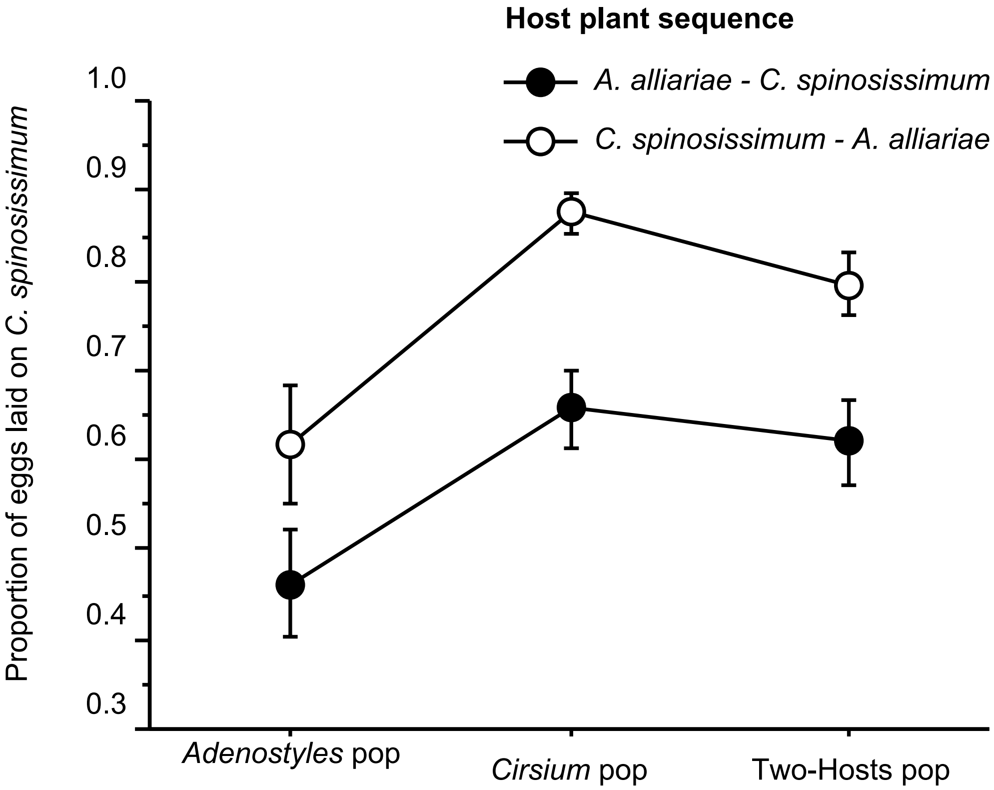

Figure 3. *Results from the sequential choice experiment showing the mean proportion of eggs (± 1 SE) that each population laid on C. spinosissimum during both time periods of the experiment. Result for each host plant sequences is shown separately*

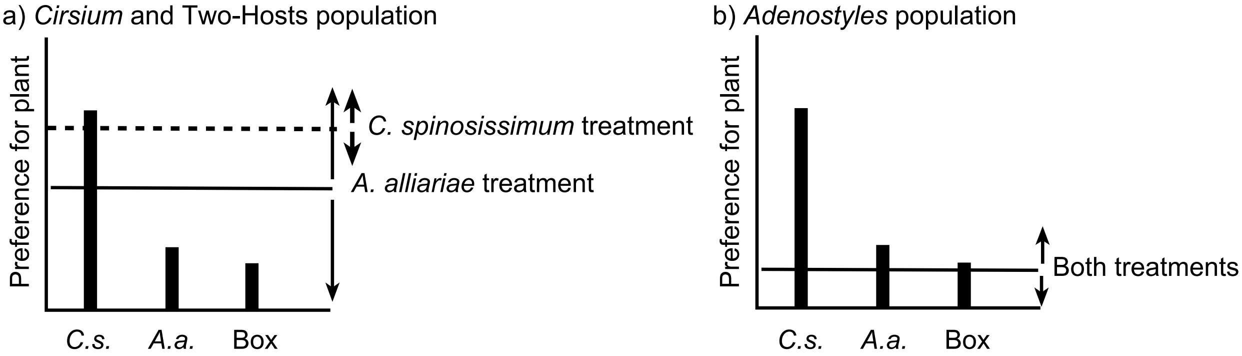

Figure 4. *Interpretation of the results in relation to the hierarchy threshold model when the difference between populations is due to variation in motivation to oviposit. The left column shows the Adenostyles population while the right column shows the Cirsium and two-host populations. Plant species are abbreviated on the X-axis (C.s – C. spinosissmum, A.a – A. alliariae). The horizontal lines in each graph represents examples of acceptance thresholds and when needed the thresholds are separated by host plant treatment (C. spinosissimum – thick, dashed; A. alliariae – thin, full). Note that the thresholds represent the motivation of a female at a given instance and they may move both down (when searching for a host) and up (after oviposition) during the experiment. The arrows to the right of each graph show, for each host plant treatment, the region within which the motivational threshold typically moved during the experiments. The upper limit of the range within which each threshold-line moved is determined by the general motivational level/speed of increase in motivation during the search for oviposition sites, whereas the lower limit is determined by host plant treatment. For example, a female with any of the motivational thresholds depicted in (a) would only accept C. spinosissimum for oviposition, but if this plant is not available the threshold will descend in the graph until she starts accepting other oviposition sites at encounter. When she eventually oviposits, the threshold may move up again and she will perhaps again reject the lower ranked plants for some time. In contrast, the female in (b) would at this point accept all the oviposition sites depicted and due to her genetically high level of motivation her threshold would only rise very little after oviposition and then start to descend again*

The proportion of eggs laid on *C. spinosissimum* by each female during the whole experiment is a measure of the relative preference for the two hosts and it was strongly affected both by population and the sequence in which the plants were presented to the beetles (Fig. 3, ANOVA on arcsine-root transformed proportions: Population, $F_{2,156} = 11.10$, $P < 0.0001$; Start plant, $F_{1,156} = 22.79$, $P < 0.0001$; Interaction $F_{2,156} = 0.20$, $P = 0.82$). The post-hoc test revealed that there were no significant differences between the *Cirsium*- and the two-host populations while both these population showed significantly higher preference for *C. spinosissimum* than did the *Adenostyles*-population (Scheffe's test for C-TH: $P = 0.42$, A-C: $P < 0.0001$, A-TH: $P= 0.0043$). The effect of plant sequence was due to a stronger preference for *C. spinosissimum* in all populations when this plant was presented first (Fig. 3).

Discussion

The results suggest that some aspects of the oviposition preference of *O. elongata* varies among these populations while other aspects seems to be unaffected by the differences in natural host plant use. We found no difference in host plant ranking among populations when it was estimated in simultaneous choice tests. All populations significantly preferred *C. spinosissimum* to *A. alliariae* (Fig. 1), which raises questions concerning the *Adenostyles* population that never encounters *C. spinosissimum* in its habitat. The pattern of oviposition in the no-choice experiment confirmed the preference for *C. spinosissimum* over *A. alliaria*e in the *Cirsium* and the two-host populations, while oviposition of the *Adenostyles* population was unaffected by host plant treatment when the two hosts were presented in sequence (Fig. 2). This result suggests that the *Adenostyles* population is less specific in its choice between these two hosts compared to the other populations.

The analysis of total number of eggs laid suggests that in the present experimental conditions the oviposition rate of *O. elongata* females was largely independent of which host plant was available. Matching of egg maturation in relation to availability of preferred hosts has been documented in some insect groups including the Coleoptera (Hopkins & Ekbom, 1999; Papaj, 2000). However, *O. elongata* is adapted to high alpine environments with short summers and highly variable weather conditions, which can be expected to select for an ability to take advantage of a period of favorable temperature conditions to mature and lay eggs. The experimental conditions used here is likely to correspond to such an advantageous period since it represents an upper part of the natural temperature distribution. Potential host plants will always be present during the oviposition period and it is unlikely that selection should favor an ability to restrain the rate of egg maturation or oviposition in *O. elongata*.

In the no-choice experiment females from all three populations deposited unexpectedly large numbers of eggs on the plastic boxes rather than on the plants (Fig 2). To what extent females oviposit on other objects than the host plants in the field has not been studied in detail but observations during several years in two-host population suggests that it is rare at this site. It seems likely that the oviposition off the plants here is mostly a consequence of the limited space of the containers in combination with high rates of egg maturation/oviposition. Nevertheless, the consistent significant effect of the interaction between population and host plant treatment on the patterns of oviposition shows that oviposition decisions are influenced by which host plant is available, and that the particular form of this influence of host plant varies among the three populations (Fig 2).

When females from the *Cirsium* and two-host populations are put in a situation where they experience a high rate of egg maturation but do not find their preferred host (*C. spinosissimum*) they are forced to oviposit on a lower ranked host (*A. alliariae*) or on the box (Fig 2). Females of these two populations will always find *C. spinosissimum* during the ovipositing period. In fact, results from field studies of the two-host population strongly suggest that females actively move from *A. alliariae* (where they spend most of their time) to *C. spinosissimum* to oviposit (Ballabeni*, et al.*, 2001a; Gotthard, K,. Rasmann, S., Magraf, N., Rahier, M. submitted). The frequent oviposition on the box when only *A. alliariae* is present may reflect this behavior.

In contrast, the oviposition pattern of the *Adenostyles* population did not differ between host plant treatments (Fig. 2), suggesting that host plant specificity, with regard to the two hosts tested, is lower in the *Adenostyles* population than in the other two populations. This lower specificity was in line with expectations since these females will never encounter the higher ranked *C. spinosissimum* in the field and selection should work against individuals that continuously reject suitable *Adenostyles* plants. However, we expected that natural selection in the *Adenostyles* population should have acted to increase the acceptance rate of *Adenostyles* plants (rather than lowering the preference for *C. spinosissimum*) simply because acceptance/rejection of *Adenostyles*-plants is the only trait that is expressed in this population. The result of the no-choice experiment does not support this expectation since the population difference is not due to variation in the *A. alliariae* – treatment and there is no indication that the preference for *A. alliaria*e is higher in the *Adenostyles* population compared to the other two populations. The *Adenostyles* population is instead less likely to oviposit on *C. spinosissimum* when this is the only plant available (Fig. 2).

One possibility is that females from the *Adenostyles* population would have reacted differently if they had been offered their native host species (*Adenostyles glabra*) but additional experiments with the same populations refute this explanation (Verdon, A., Magraf, N. unpublished). Hence, the *Adenostyles* population combines a high preference for *C. spinosissimum* (Fig. 1) with a low specificity for the choice between this plant species and the *Adenostyles* plants (Fig. 3) and the difference is not due to quantitative variation in the preference for *A. alliariae*. An alternative explanation that is perhaps better supported by the results suggests that females of the *Adenostyles* population have a generally higher motivation to oviposit than females from the other two populations (see Fig 4 for a graphical representation). It implies that in the beneficial temperature conditions of the no-choice experiment the motivational threshold of the *Adenostyles* population females constantly moved in a range of very low acceptance values leading to the equal acceptance rates of both plants and frequent acceptance of the plastic box (Fig. 4b).

It is of interest to speculate on how the difference in host specificity among these three populations may have evolved. It seems likely that a high preference for *C. spinosissimum* is an ancestral trait in *O. elongata*. The total absence of this highest ranked host in the *Adenostyles* population may, however, led to selection for reduced "choosiness" by an increase in the general level of motivation to oviposit. In the natural situation beetles in the *Adenostyles* population spend practically all their time on, or very close by *A. glabra*, and even with a very high motivational level, eggs will practically always end up on this plant. Indeed, Courtney *et al.* (1989) proposed that differences in host specificity should typically evolve by genetic modifications of the motivational level rather than by changes in preferences for different host plants. However, there is no *a priori* reason to believe that these two evolutionary mechanisms are mutually exclusive and oviposition choices may evolve by a combination of changes in relative preferences and changes in motivational level (Singer*, et al.*, 1992). In relation to the present results it is interesting to note that in a population of the butterfly *Euphydrias editha* where the highest ranked host plant was naturally absent, females seemed to have a higher general motivation to oviposit (Singer*, et al.*, 1992).

Studies of spatial variation in oviposition preferences of phytophagous insects in relation to variation in host plant availability have produced results ranging from essentially no genetic differentiation among populations at a wide spatial scale to a high degree of local specialization on a small spatial scale (Singer*, et al.*, 1993, 1994; Thompson, 1988b,1994; Wheling & Thompson, 1997; Thomas & Singer 1998; Peterson & Denno, 1998; Pappers*, et al.*, 2002). Since it appears that *O. elongata* never flies it is highly improbable that there is gene flow among the populations investigated here. Indeed, there are genetic differences among these populations in several developmental traits such as larval growth rate and body size (Ballabeni *et al.*, 2003). From the present study we can conclude that there are also differences between populations in host specificity that correspond with variation in local selection pressures. These differences seem mainly to be due to variation between populations in the general motivation to oviposit rather than to quantitative differences in relative preference for the two hosts. For most studies of the evolution of insect host utilization it is essential which of these mechanisms is most important, because changes in relative preference may ultimately lead to evolutionary change in host plant ranking, while changes in motivational level will not necessarily affect ranking at all.

Acknowledgements

We thank Mathieu Rapp and Katya Besomi for their fieldwork and Urs Schaffner for valuable discussion. Barbara Barisani, Philippe Kuepfer, Ephyse Noussan, Lucie Vaser, the Association Internationale du Jardin Alpin de la Chanousia, and the Ordine Mauriziano, Torino, Italy, all provided valuable logistic support at the field site. This study was financed by grant 3146850.96 from the Swiss National Science Foundation.

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PAPER five

THE EVOLUTION OF LARVAL FORAGING BEHAVIOUR IN RESPONSE TO VARIATION IN HOST PLANT RANGE AMONG POPULATIONS OF A SPECIALIZED LEAF BEETLE

Karl Gotthard, Nicolas Margraf, Sergio Rasmann and Martine Rahier

Abstract

1. The evolutionary causes of variation in host specialization among phytophagous insects are still not well understood and identifying them is a central task in insect-host plant biology.
2. Here we examine host utilization of the chrysomelid beetle Oreina elongata that shows inter-population variation in the degree of specialization. We focus on what selection pressures may favour the use of two different host plants in one population as opposed to specialization onto one of them as is seen in other populations.
3. We report on a field experiment that tests if larval mortality rates differ between host plant species in a way that favours the use of two hosts, and a laboratory experiment where we test if the more heterogeneous host situation in the two-host population has selected for a different larval feeding strategy than in two single-host populations.
4. The results suggest that the combination of differences in larval mortality and growth performance among host plant species forms a selective regime that favours the use of two hosts, and that individual larvae move between host plant species in the field.
5. In line with these propositions we also found that the degree of exploratory feeding behaviour was higher in larvae from the two-host population than in single host populations.
6. We conclude that in this two-host situation a single plant species does not provide the most beneficial conditions in all parts of O. elongata life cycle and individual insects may obtain different plant-specific benefits by moving between host species.
7. The results support the notion that to understand patterns of host plant use in insects it is often vital to consider a range of host related selection pressures whose relative importance varies between life stages of the insect.

Introduction

A high degree of host specialization is perhaps the most general ecological pattern identified in studies of insect-host plant interactions and it is thought to be one of the major explanations for the remarkable diversity of herbivorous insects (Bernays & Chapman, 1994; Futuyma, 1991; Jaenike, 1990; Thompson, 1994). Nevertheless, there are enough generalist species present to show that specialization is not the only evolutionary possibility and evidence is mounting that the drive towards specialization is not irreversible (Bernays & Minkenberg, 1997; Janz, Nyblom & Nylin, 2001. Hence, there is a need to identify evolutionary processes that can explain the predominance of specialists as well as the exceptions of polyphagy [Futuyma, 1988 #2562; Janz & Nylin, 1998; Janz & Thompson, 2002). Most attempts in this direction have concentrated on finding benefits of host specialization and this has led to the recognition of a number of important evolutionary mechanisms (Bernays, 1998; Bernays & Bright, 2001; Bernays & Graham, 1988; Futuyma, 1983, 1991; Futuyma & Moreno, 1988; Jaenike, 1990; Janz & Nylin, 1997; Thompson, 1988; Thompson, 1994). Thus, it seems unlikely that a single evolutionary process causes the general drive towards specialization in plant feeding insects. Instead, it appears that host range evolution is a more dynamic process than previously thought and that it is necessary to consider how a wide combination of selection pressures may determine host plant range in any given species (Janz, 2002; Scheirs & De Bruyn, 2002; Thompson, 1988).

It has been repeatedly suggested that for an insect with several alternative host plants a single plant species may not provide the most beneficial conditions in all parts of the life cycle (Janz, 2002; Reavey & Lawton, 1991; Scheirs & De Bruyn, 2002; Scheirs, De Bruyn & Verhagen, 2000). For example, the best host plant for the larva may not be the best site for the egg or for adult feeding. Such situations are interesting since they can lead to variable and elaborate host utilization strategies that may allow studies of general aspects of host range evolution. Depending on the exact ecological circumstances one may find that individual fitness is maximized by a generalist host utilization strategy, or by specialization to a certain host plant that is suboptimal for some or all life stages of the insect. Alternatively, selection may favour a dynamic use of different host plants in different life stages, which would necessitate that individuals move between host plant species. In the present study we investigate host plant utilization of a specialized chrysomelid beetle, *Oreina elongata* Suffrian, that in some locations uses two different host plants in different parts of the life cycle, while in other locations only one host species is available and is used throughout the life cycle. This relatively simple insect-plant system allows us to study a situation where selection on the actual host range varies geographically and we can investigate which selection pressures may lead to the use of more than one host plant when they are available to the beetles.

Oreina elongata is a high-alpine species (altitude 1600 m - 2300 m) with a patchy distribution throughout the Alps and further south in the Apennines. Both adults and larvae feed exclusively on three species of alpine plants in the Asteraceae: *Adenostyles alliariae* (Guoan), *Adenostyles glabra* (L.) (Senecioneae) and the thistle *Cirsium spinosissimum* (L.) (Cardueae). The two *Adenostyles* species are chemically and morphologically similar and both contain pyrrolizidine alkaloids (hereafter abbreviated PA:s) that are sequestered and used as a chemical defence by both adults and larvae. The third host species, *C. spinosissimum*, is distinctly different from the other two and individuals that feed only on this plant contain no PA:s (Dobler & Rowell-Rahier, 1994; Pasteels *et al.*, 1996).

The two-host population of *O. elongata* studied here use both *A. alliariae* and *C. spinosissimum* and at this location the beetles are exclusively found where both plants grow close to each other. Adult beetles in the mixed host plant stands spends significantly more time on *A. alliariae*, and after the period of oviposition there are very few beetles on *C. spinosissimum* (Gotthard, K., Rasmann, S., Rahier, M. unpublished). In contrast to the apparent adult preference for *A. alliariae* practically all eggs in the field are found on *C. spinosissimum* (Ballabeni *et al.*, 2001a), which is due to a strong oviposition preference for this plant (Paper IV). However, after the eggs hatch the number of larvae that are found feeding on *A. alliariae* in the field increases with time (Ballabeni *et al.*, 2001a). The number of final (4th) instar larvae on the two hosts plants is not significantly different (Ballabeni *et al.*, 2001a) and if anything, it is easier to find 3rd – and 4th instar larvae feeding on *A. alliariae* than on *C. spinosissimum* (personal observation). Field surveys have also shown that most eggs are found on *C. spinosissimum* individuals that have leaf contact with *A. alliariae* plants (Ballabeni *et al.*, 2001a), which facilitates larval movement between plants. Hence, it seems that a vast majority of larvae in this two-host population start their growth on one plant species (*C. spinosissimum*) but that a large proportion of larvae move to nearby plants of the alternative host species (*A. alliariae*) during the growth period. This host utilization pattern may be adaptive since egg survival is significantly higher on *C. spinosissimum* in the field (Ballabeni, Wlodarczyk & Rahier, 2001b) whereas larval growth rate is higher on *A. alliariae* (Ballabeni & Rahier, 2000b).

Indeed, these results have led to the adaptive hypothesis that *O. elongata* females oviposit on the plant that gives the eggs best protection but that larvae continuously move to the plant that support the highest growth and contains PA:s that can be sequestered and used as a chemical defence. Here we further investigate this hypothesis by exploring other potentially important selection pressures acting on larvae in this two-host situation.

First, we investigated if larval mortality rate is higher on *C. spinosissimum* than *on A. alliariae* in the field, which could constitute an additional selection pressure favouring larval migration from *C. spinosissimum* to *A. alliariae*. This expectation was based on the notion that larvae can only sequester PA:s while feeding on *A. alliariae*.

Second, we tested whether larval movement rates were higher and larval feeding preferences less specialized in the two-host population compared to single-host populations. This would suggest that larvae from a two-host situation have a stronger tendency to explore their host plant environment and that larval movement between plants may be a local adaptation in response to a heterogeneous host plant composition. With respect to the situation in the two-host population we also wanted to test if earlier experience with the different host species would affect larval feeding behaviour.

Third, in the light of the results of the two first studies we wanted to evaluate to what degree larvae in the field typically stay on *A. alliariae* once they have reached this plant, or if they continue to move between and use both host species throughout their development. If so, individual larvae may move back to *C. spinosissimum* (where practically all larvae start development) after having fed and sequestered PA:s on *A. alliariae*. This was investigated by comparing the amount of PA:s in larvae collected on *C. spinosissimum* in the field with the PA-content of larvae reared in the laboratory on a *C. spinosissimum*-only diet (free of PA:s). Hence, we were using the presence of PA:s as an indicator of earlier feeding on *A. alliariae*.

Materials and Methods

STUDY ORGANISM AND POPULATIONS

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

FIELD EXPERIMENT ON LARVAL DISAPPEARANCE RATES IN THE TWO-HOST POPULATION

The aim of this field experiment was to estimate potential differences in host-specific mortality due to natural enemies if larvae use a single host plant. Early in the season of 2001 we collected approximately 30 mating pairs of *O. elongata* from the two-host population. The beetles were kept together in one cage (30cm x 30cm x 30 cm) in the field station at the site where they were given constant access to both host plants and were allowed to oviposit freely. Eggs were collected daily and at hatching larvae were transferred to plastic boxes where either *C. spinosissimum* or *A. alliariae* were provided as larval food until they were released in the field.

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

We estimated disappearance rates from the plants by counting all remaining larvae twice a day during four days. In the Glue treatment we also counted the number of larvae that got stuck in the glue. The experiment was performed within the host plant patch where the adults had originally been collected and larvae were put out in the field at the rate at which they reached the desired instars (second or third). Consequently, the experiment stretched over approximately three weeks covering a significant part of the larval growth season. The numbers of plant pairs (one plant of each host species) in the treatments were: 15 in the Control treatment, 10 in the Enclosed treatment and 5 in the Glue treatment (leading to a total number of larvae in each treatment of 300, 200, and 100, respectively). Logistic problems led to a smaller sample size in the Glue treatment than planned. The larger sample in the Control treatment was chosen because we expected the measurement error to be larger in this unmanipulated treatment.

LABORATORY EXPERIMENTS ON LARVAL EXPLORATORY BEHAVIOUR

During two field seasons (2000, 2001) we conducted experiments to test for differences in larval exploratory behaviour among the three described populations and if earlier host experience affect subsequent larval feeding behaviour. All larvae originated from field-collected females that were handled in the same laboratory conditions as described for the field experiment above (50 females per population). At hatching larvae were randomly assigned to either a *C. spinosissimum* - only or an *A. alliariae* - only diet on which they were reared until they had reached at least the second larval instar. The experiments were started by weighing the larvae (second or third instar) and then placing them individually in round plastic boxes (D =100 mm, H = 50 mm) where one leaf of *C. spinosissimum* and *A. alliariae* were available. In both experiments all larvae were started simultaneously and were put on the plant species that they had been reared on previously (the start plant).

We monitored movements and host plant choices over three days by observing the positions of all larvae two (experiment in 2000) or three (experiment in 2001) times per day. To estimate larval feeding preferences we counted the number of feeding marks on both host plants during the three-day period. In the 2001-experiment we used image analysis to calculate the leaf areas that had been consumed (NIH Image, version 1.62) and estimated the relationship between leaf area and dry weight of both plant species using leaf pieces of known areas (equation for *C. spinosissimum*: Dry weight (mg) = 0.029 * leaf area (mm^2), N = 80, $r^2 = 0.85$; for *A. alliariae*: Dry weight (mg) = 0.028 * leaf area (mm^2), N = 79, $r^2 = 0.89$, there was no significant difference between plant species and none of the intercepts were significantly different from 0). These equations allowed us to estimate larval consumption of each host plant. Further analysis showed a strong positive correlation between the proportion of feeding marks that a larva made on the start plant (the measure of feeding preferences used in 2000) and the proportion of plant material actually consumed of that plant (linear regression:$F_{1,\ 170} = 805.5$, $P < 0.0001$, $r = 0.91$). Since both measures seemed to estimate the same underlying feeding preference we used feeding marks as our estimate of feeding for both years (this simplified comparison between years).

In the 2001 experiment we marked five equally sized compartments in the plastic boxes in order to estimate larval movements in the box at a finer scale. In 2000 we only registered movements between plants or between the box and the plants (i.e. the plastic box was treated as one compartment). With these data we calculated the number of position changes made by each larva, the proportion of the time that each larva spent on the different host plant species, and the relative consumption of the two hosts by each larva. We then investigated the effect of population origin and start plant on these variables.

PA CONTENT OF LARVAE IN THE TWO-HOST POPULATION

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

Late in the season (August 30, 2001) we collected 10 *O. elongata* larvae from *C. spinosissimum* plants in the two-host population. These larvae were starved for 24 h (to allow plant material in the gut to be excreted) and then weighed and frozen at - 80 °C. The equivalent treatment was given to 12 larvae from the same population that had been reared their whole life in the laboratory on a *C. spinosissimum*-only diet. All larvae were either third or fourth instar when they were killed. We extracted the total fraction of PA:s of all 22 larvae individually using a standard protocol (Pasteels et al. 1995). We analyzed the presence and quantity of PA:s in each larva by gas chromatography – mass spectrometry (GC-MS, Agilent 5973) using split/splitless injection (injector temperature 250°C), aliquots of 2 µl were injected onto a HP1-MS 30m x 0.25mm x 0.25µm column. Initial oven temperature was set at 40°C for 3 min and thereafter increased 15°C/min to 300°C, where it was held for 10 min. The MSD was run under the following conditions: transfer line 230°C, source 230°C, quadrupole150°C, ionization potential 70 eV, and a scan range 0-400 amu. Heliotrine was used as internal standard. The identity of PA:s was confirmed by comparing the spectra from the samples with the NIST reference library.

STATISTICAL TREATMENT

We estimated the larval disappearance rates from plants in the field with the remaining number of larvae after four days. We performed a Wilcoxon signed rank test to assess the difference in disappearance rate on the two hosts. To compare treatments overall, we first performed a Kruskall-Wallis test and then compared the treatments pairwise using a Mann-Whitney test correcting the significant threshold with sequential Bonferroni adjustment [Rice, 1989 #870].

For both years of experiment on larval feeding behaviour we first performed a MANOVA where the effects of population, start plant (both fixed factors) and their interaction on the three response variables was analyzed (no. of moves, no. observations on start plant, proportion of feeding on start plant). We then performed the corresponding univariate ANOVA:s on each response variable to investigate how each response variable was affected by the two factors. Larval weight was initially included as a covariate in the MANOVA:s but since it had no significant effect it was excluded from the models. To meet model assumptions all proportions were Arcsine-root transformed ($X' = \text{Arcsine}(\sqrt{X})$) prior to analysis, whereas the number of position changes and observation on the start plant were Box-Cox transformed ($X' = ((X+0.1)^{\lambda} - 1)/\lambda$). The value of λ was in each case found by maximum likelihood (Sokal & Rohlf, 1995). In the analysis of such data we give the value of λ that was used in the transformation. The untransformed data are displayed in all graphs and all statistical analyses were performed with SPSS 10.0.7a for Macintosh.

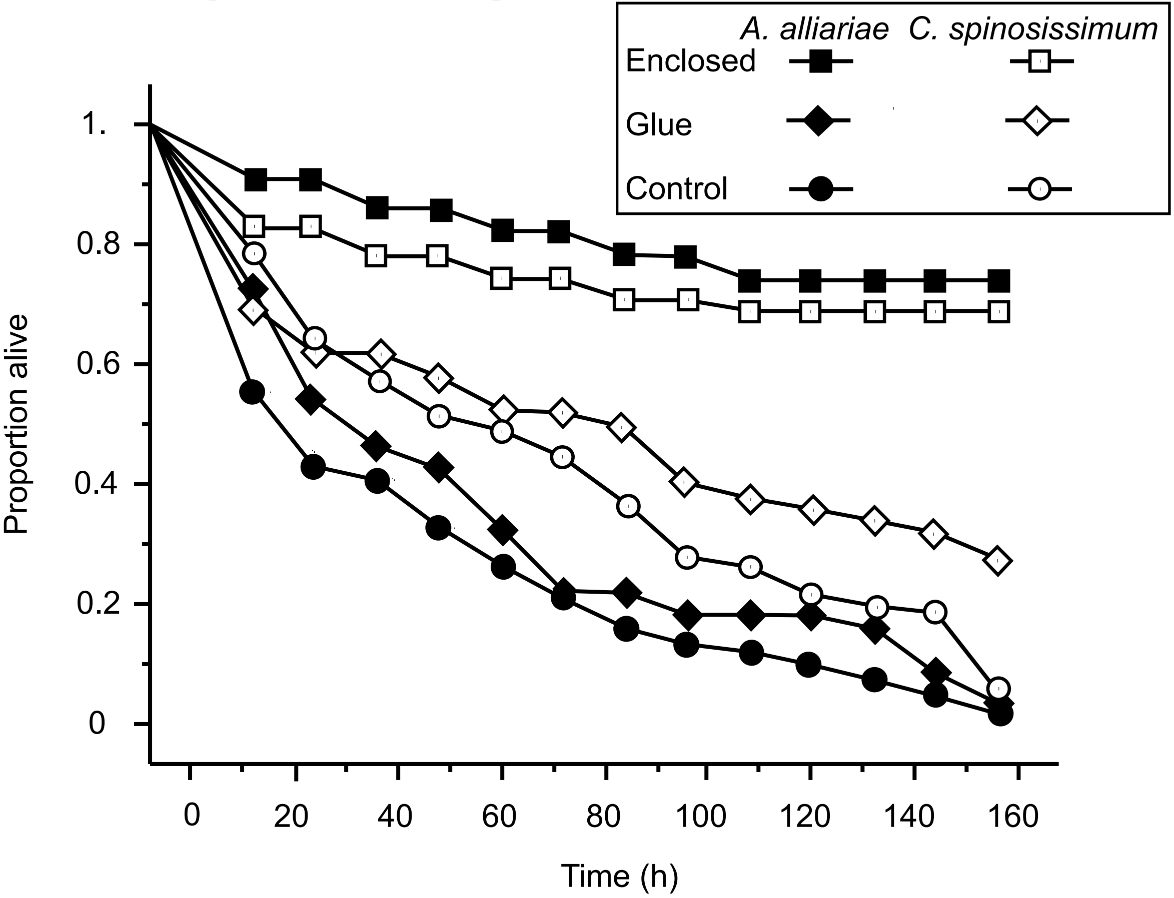

Figure 1. *Results from the field experiment on larval disappearance rates in the two-host population showing survival functions separated by three experimental treatments and plant species*

Results

RATE OF LARVAL DISAPPEARANCE FROM THE PLANTS IN THE TWO-HOST POPULATION

Of the total of 600 larvae that were put out in the field 347 disappeared from the plants during the four days. We found a significant difference between the two hosts (Z = -2.391, Pvalue = 0.017). Unexpectedly, *C. spinosissimum* appeared to better protect the larvae compared to *A. alliariae*. The overall Kruskal Wallis test showed a significant difference between the three treatments (χ^2 = 24.609, df = 2, Pvalue < 0.001). This was explained by the pair wise comparisons of the treatments (Mann-Whitney tests) which revealed significant differences between enclosed vs. control plants and between glue vs. control plants but no difference between glue vs. enclosed plants (Table 1). The two manipulated treatments (Glue and enclosed) seem to group together by being safer for larvae while the control plants hosted a significantly inferior number of larvae after the four days.

LARVAL EXPLORATORY BEHAVIOUR AND FEEDING PREFERENCES

In the 2000 experiment we found significant effects of population and start plant in the multivariate analysis indicating variation in the general feeding strategy (Table 2). The following univariate analyses of the two movement estimates indicate that all populations were more likely to move and leave their start plant when it was A. alliariae (Table 2, Figs 2a,c). The population effect on these variables seems mainly to be due to the two-host population being more likely to move in general as well as being more likely to leave the start plant (Bonferroni/Dunn post-hoc for no. moves : PTH-C = 0.0097, in the two other cases; P > 0.17; for no. obs. on start plant: : PTH-A = 0.024, PTH-C = 0.022, PA-C = 0.96; the P-value should be lower than 0.0167 to be significant at the 0.05-level in all post-hoc tests).

The relative amount of feeding on the two plant species was estimated as the proportion of feeding marks that each larva made on its start plant species. There was a significant effect of the interaction between population and plant species on this measure and it seems to be due to the two-host population behaving differently than the other two (Table 2, Fig. 2e). The two-host population fed on average more on *A. alliariae* than on *C. spinosissimum* when this was their start plant, whereas the single-host populations showed the opposite pattern with a stronger preference for *C. spinosissimum* (Fig. 2e).

Table 1. *Pair wise comparisons between treatments; result of Mann-Whitney test on number of remaining larvae on the plant of the 10 larvae initially placed after 4 days.*

	Mann-Whitney U	P value
Enclosed - Glue	28	0.001
Enclosed - Control	64.5	< 0.001
Glue - Control	108	0.198

Table 2. *Results of a) MANOVA and b) univariate ANOVA:s for the analysis of larval exploratory behavior and feeding preference in the 2000-experiment. The number of moves and the number of observations on the start plant were Box-Cox transformed before analysis ($\lambda_{moves} = 0.436$, $\lambda_{omplant} = 0.385$), while the proportion of feeding marks on the start plant was Arcsine-square root transformed.*

a) MANOVA	Wilk's λ	DF	F	P value
Population	0.84	6, 214	3.37	0.0034
Start plant	0.80	3, 108	9.10	< .0001
Population x Start plant	0.93	6, 214	1.33	0.2400

b) Univariate ANOVAs	MS	DF	F	P value
Response **No.moves**				
Population	3.88	2	3.26	0.0420
Start plant	16.45	1	13.85	0.0003
Population x Start plant	1.00	2	0.85	0.4300
Error	1.18	109		
Response **No observation on start plant**				
Population	6.69	2	3.29	0.0410
Start plant	30.9	1	15.20	0.0002
Population x Start plant	2.44	2	1.20	0.3100
Error	2.03	109		
Response **Proportion feeding marks on start plant**				
Population	0.18	2	0.50	0.6100
Start plant	0.88	1	2.45	0.1200
Population x Start plant	1.13	2	3.16	0.0460
Error	0.36	109		

In line with the first year results the multivariate analysis of the 2001 experiment showed strong significant effects of both population and start plant, but this time also the effect of the interaction was significant (Table 3). The marking of boxes and the increased frequency of observations allowed us to estimate movement levels in more detail and the univariate analyses showed again that all populations were more likely to move and leave the start plant when it was *A. alliariae* (Table 3, Figs 2b,d). As in the previous experiment it was larvae from the two-host population that showed the highest level of movement and that were most likely to leave the start plants (Figs 2b,d; Bonferroni/Dunn post-hoc for no. moves: $P < 0.005$ in all three comparisons; for no. obs. on start plant: $P_{TH\text{-}A} = 0.0002$, $P_{TH\text{-}C} = 0.0054$, $P_{A\text{-}C} = 0.37$; the P-value should be lower than 0.0167 to be significant at the 0.05-level in all post-hoc tests).

The relative feeding on the two host plants showed a similar pattern as the year before with the interaction between population and start plant being significant (Table 3). The difference between plant treatments was smallest in the two-host population also in this experiment although all populations fed more on *C. spinosissimum* when this was the start plant (Fig. 2f).

Table 3. *Results of a) MANOVA and b) univariate ANOVA:s for the analysis of larval exploratory behavior and feeding preference in the 2001-experiment. The number of moves and the number of observations on the start plant were Box-Cox transformed before analysis ($\lambda_{moves} = 0.584$, $\lambda_{omplant} = 0.549$), while the proportion of feeding on the start plant was Arcsine-square root transformed.*

a) MANOVA	Wilk's λ	DF	F	P value
Population	0.71	6, 336	10.41	< .0001
Start plant	0.58	3, 168	41.45	< .0001
Population x Start plant	0.86	6, 336	4.46	0.0002

b) Univariate ANOVAs	MS	DF	F	P value
Response **No.moves**				
Population	50.83	2	31.42	< .001
Start plant	61.72	1	38.15	< .001
Population x Start plant	3.54	2	2.19	0.120
Error	1.62	170		
Response **No observation on start plant**				
Population	11.37	2	7.75	0.001
Start plant	160.97	1	109.66	< .0001
Population x Start plant	1.52	2	1.03	0.360
Error	1.47	170		
Response **Proportion feeding marks on start plant**				
Population	1.08	2	11.97	< .0001
Start plant	11.24	1	124.72	< .001
Population x Start plant	0.32	2	3.56	0.031
Error	0.09	170		

PA-CONTENT OF LARVAE IN THE FIELD AND IN THE LABORATORY

We could identify three different PA:s in our samples: Seneciphylline, Senecionine and Plathyphylline. The two latter compounds were always present in very small quantities and therefore we only included Seneciphylline in the quantitative analysis. All larvae contained PA:s although five of the laboratory reared larvae contained only unquantifiable traces. Therefore, we first analyzed the results assuming that these five individuals contained no PA:s and then re-analyzed the data assuming that they had the same concentration as the larva with the lowest quantifiable concentration (= 0.009 µg/mg). The analyses gave qualitatively identical results and statistics of the second analysis is given in parenthesis.

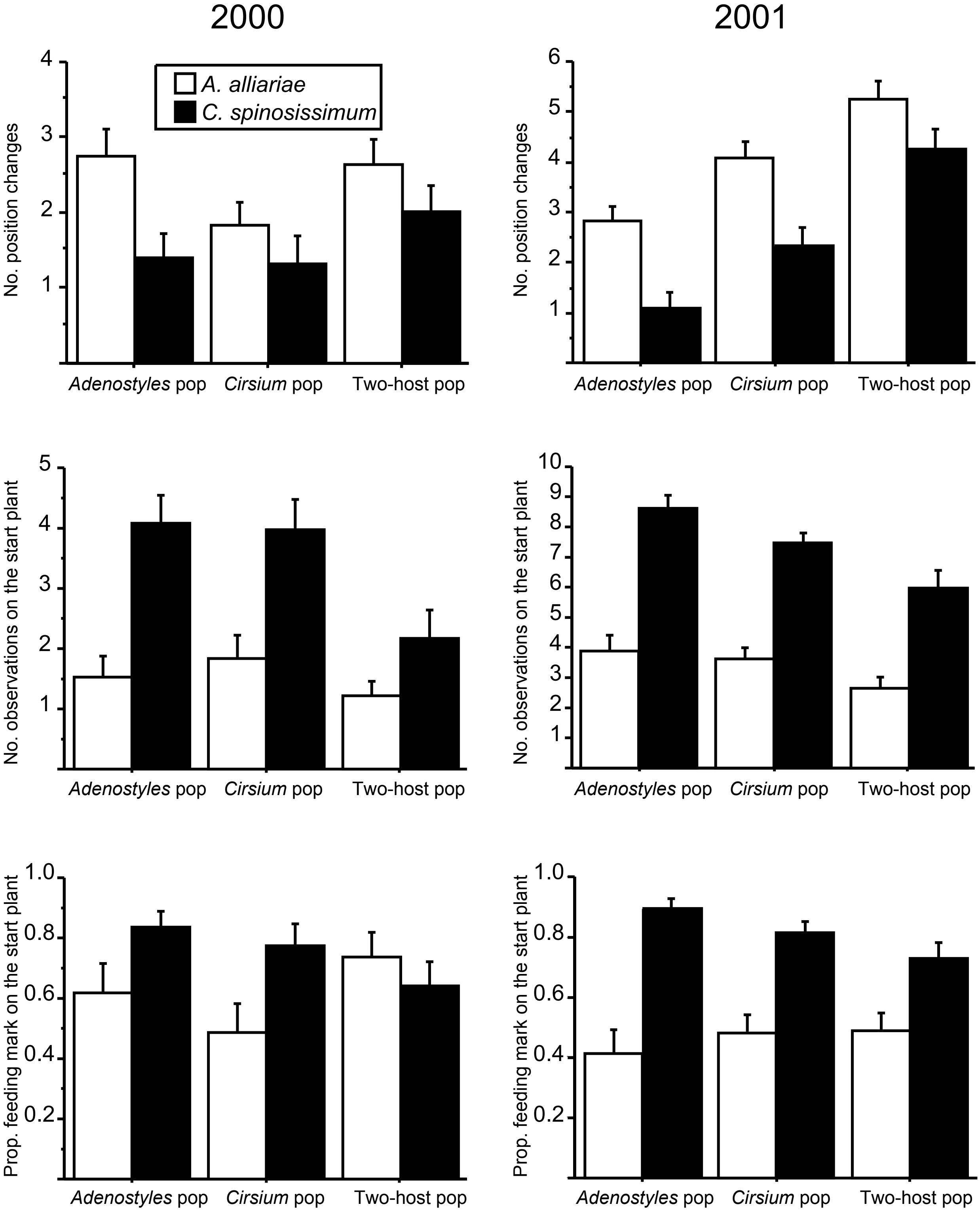

Figure 2. *Results from the two experiments on larval behavior and feeding preferences. The left column show results from the 2000 experiment (a, c, e) while the 2001-experiment is displayed in the right column (b, d, f). The total number of observations per individual larva was 7 in 2000 and 10 in 2001. Means ± 1 SE is given for the number of position changes (a, b), number of observations on the start plant (c, d), and proportion feeding performed on the start plant (e, f). The data is split by population and start plant (the plant species that each larva was put on at the start of the experiment)*

Despite the small sample we found that the concentration of PA:s in the field-collected larvae was significantly higher than in the laboratory-reared larvae (Fig. 3, ANOVA,: $F_{1,20}$ = 5.86 (5.59), P = 0.025 (0.028)). There was no significant difference in average weight between groups (mean ± 1SE for field = 21.1 ± 3.2 mg , for laboratory = 26.8 ± 2.3 mg, ANOVA,: $F_{1,20}$ = 2,21, P = 0.15) and PA-concentration did not change with larval size.

Discussion

In direct contrast to expectations the field experiment indicated that the disappearance rate was not lower when larvae are feeding on the PA-containing *A. alliariae* than when they are feeding on *C. spinosissimum* (Fig. 1). This finding rejects the idea that selection is favouring larval movement from *C. spinosissimum* to *A. alliariae* because it reduces mortality risk. Indeed, the analysis of PA:s of larvae feeding on *C. spinosissimum* in the field and in the laboratory implies that individual larvae in the two-host population do not stay on *A. alliariae* when they reach this host but continue to move and feed on both hosts (Fig. 3). The small amounts of PA:s that were found in the laboratory-reared larvae are most likely remains of the high amounts of plant-derived alkaloids that females transfer to their eggs (Dobler & Rowell-Rahier, 1994).

Larvae disappeared faster from *A. alliariae* than from *C. spinosissimum* and faster when natural enemies were allowed on the plants. The rates of emigration down the stem of plants were similar and cannot explain the species difference in disappearance rate. It should be stressed that the individual plants used in the experiment did not have contact with any other plants, which inhibited the leaf-to-leaf emigration that is known to occur (personal observations). Hence, the emigration observed in the glue traps is unlikely to reflect the natural rates of emigration from the host plants.

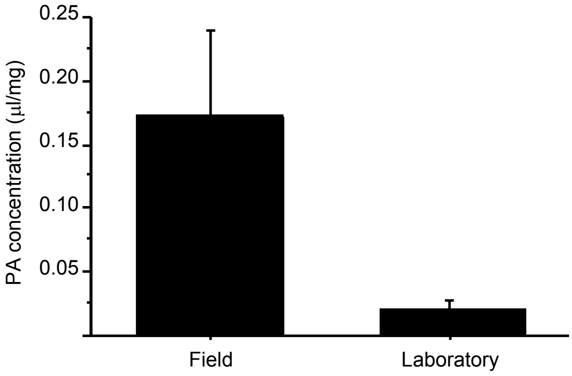

Figure 3. *PA-concentration of larvae collected on C. spinosissimum in the field and larvae reared on C. spinosissimum-only in the laboratory (Means ± 1SE)*

There is a possibility that larvae deliberately fall off their host plants as an emigration or anti-predation strategy. However, field observations suggest that migration between plants typically goes between leafs and that larvae do not fall off easily from the plants when they are disturbed by physical manipulation. Hence, we are left with the explanation that the difference in disappearance rate between host plants is due to a higher predation rate on *A. alliariae* than on *C. spinosissimum*. This was surprising since *A. alliariae* provides larvae with alkaloids that they do sequester and the protective effects of PA:s are well documented in several insects (Brown, 1984; Eisner *et al.*, 2000; González *et al.*, 1999; Masters, 1990) including adult *Oreina* beetles that were subject to bird predation (Rowell-Rahier *et al.*, 1995). Nevertheless, recent evidence clearly shows that the predacious harvestman, *Mitopus morio*, which is common in the two-host population, is able to detoxify PA-containing *Oreina* larvae (Häggström, H., Hartman, T., Rahier, M. unpublished). It is possible that the chemical protection given by PA:s is only effective against some predators and that the alternative host plant *C. spinosissimum* provides protection against more common predators. It has been suggested that the hairy and spiny leaves of *C. spinosissimum* protects eggs against predators (Ballabeni *et al.*, 2001b), and it seems possible that also small larvae are protected by these structures. Moreover, since the proportion of larvae found on *A. alliariae* increases continuously throughout the

the season one may speculate that the benefits of using this plant increase with the size of larvae. Size dependent changes in larval feeding habits are known in other insect groups (Gaston, Reavey & Valladares, 1991).

In line with the dynamic larval host use in the two-host population, the laboratory assays showed that this population displayed a higher level of exploratory feeding behaviours compared with the two single-host populations (Tables 2,3, Fig. 2). Larvae of all populations showed some degree of preference for *C. spinosissimum* but the strength of this preference varied between populations. Since the *Adenostyles* population never encounters *C. spinosissimum* in the field its feeding preference was surprising, but there are indications that a general preference for *C. spinosissimum* is an ancestral condition in the species (e.g. all populations investigated so far show an oviposition preference for *C. spinosissimum*). With respect to the hypothesis investigated here, however, the most interesting result was that larvae from the two-host population stood out from the two single-host populations in being generally more active (Figs 2a, b), more likely to leave the host they had been feeding on earlier (Figs 2c, d), and by showing a significantly lower feeding preference (Tables 2, 3, Figs 2e, f). These results were consistent between both years of experiment and in line with predictions, as they suggest that larvae from the two-host population are more prone to leave a suitable host plant and to explore their host plant environment compared to larvae from single-host populations. However, the results do not suggest that larvae from the two-host population in this size range preferentially move from *C. spinosissimum* to *A. alliariae*, but rather that they are adapted to use both host species during larval development. The variation among populations suggests that this behavioural difference has a genetic basis and may be due to geographic differences in local selection pressures.

The use of two hosts at the Petit St-Bernard is not a necessity since other populations survive in locations where they only use one host plant and since larvae from all populations in this study can be reared to the adult stage on a single-host diet (Gotthard, K., Margraf, N., Rahier, M. unpublished). Moreover, a dietary regime of alternating *A. alliariae* and *C. spinosissimum* leads to lower larval developmental rates compared to an *A. alliariae*-only diet (Ballabeni & Rahier, 2000a). Hence, any selection for the dual use of two host plant species is likely to be due to ecological factors such as plant-specific mortality risks and the spatial distribution of host. In the two-host environment at Petit St-Bernard *C. spinosissimum* is commonly present practically everywhere while *A. alliariae* is found in dense stands only in a few locations (in these places *C. spinosissimum* is also common). It is only in these mixed stands of hosts that *O. elongata* beetles or larvae are present in noticeable amounts. The distance between the inhabited mixed patches is between 0.5 and 3 km and despite the strong oviposition preference for *C. spinosissimum* there have been no observations of beetles in *C. spinosissimum*-only patches that are situated between the mixed patches (including seven consecutive years of intense field studies). This leads to the paradoxical situation that in the two-host population we can predict the local presence of *O. elongata* mainly from the presence of the host plant that is least preferred for oviposition.

The genetically stronger tendency of larvae from the two-host population to display exploratory feeding behaviours is most likely a consequence and not the cause of the association with mixed host plant patches at this location. The choice of local host patch is not done by larvae but by the adult beetles and it is more probable that the cause of the association between *O. elongata* and mixed host plant patches in the two-host population is due to some adult preference for *A. alliariae* that is unrelated to oviposition preferences. The reason for why adult beetles spends most of their time on *A. alliariae* is unknown but potential adult fitness benefits of using *A. alliariae* include a higher nutritional value, possibility of alkaloid sequestration and better basking sites. Nevertheless, the adult preference for *A. alliariae* and the strong oviposition preference for *C. spinosissimum* provide a plausible explanation for why a majority of eggs in the two-host population are laid on *C. spinosissimum* plants growing in leaf contact with *A. alliariae* (Ballabeni *et al.*, 2001a). This puts larvae in a host plant environment where selection seems to favour alternate larval feeding on both plants.

The role of larval behaviour in the evolution of insect-host plant associations has received relatively little attention although it has been studied in some Lepidopteran groups (Dethier, 1988; Oppenheim & Gould, 2002; Singer & Stireman III, 2001; Tammaru, Kaitaniemi & Ruohomaki, 1995). Conclusions from these studies include that the mixing of many host species is likely to be maintained by other benefits than improved larval growth performance (Singer, 2001; Singer & Stireman III, 2001), that behavioral adaptations may be central to the use of enemy-free space by phytophagous insects and that they may affect the evolution of dietary specialization (Oppenheim & Gould, 2002). These statements are supported by our work on *O. elongata*.

Oreina elongata is specific in its host use and some potential host species that are available in the two-host population are not used (i.e. *Petasites album* that can support the whole larval period, (Ballabeni & Rahier, 2000a). Despite this drive towards specialization natural selection seems to favor the alternate use of two host plant species leading to the evolution of specific oligophagy (see Chambers *et al.*, 1996 for a similar situation). It is interesting to note that a classical laboratory estimation of the oviposition preference-larval performance correlation in *O. elongata* from the two-host population would suggest a remarkable level of maladaptation: females lay almost all eggs on the low-performance host although they are only found in places where the high-performance host is present. This irrationality suggested that important aspects of the relationship between *O. elongata* and its host plants were not captured by the preference-performance correlation. Although still imperfect, the present knowledge of the system suggest that survival rates of eggs and small larvae in the field are highest on the host that is preferred for oviposition, whereas the alternative host support the highest larval growth rate, allow sequestration of protective alkaloids by larvae and adults, and is the host preferred by adult beetles in the field. Practically all larvae in this population start development on the "oviposition host" but the present results suggest that larvae forage actively and move between and feed on both hosts during their development. This type of strategy will tend to even out the highly biased larval distribution between hosts that is present early in the season (Ballabeni *et al.*, 2001a). It seems likely that this host utilization strategy permits larvae to derive different benefits from each host species: protection from predators on one host and better food on the other.

It has frequently been proposed that to understand patterns of host plant utilization in insects it is often vital to consider a range of host related selection pressures whose relative importance may vary between life stages of the insect (Janz, 2002; Reavey & Lawton, 1991; Roitberg & Mangel, 1993; Scheirs & De Bruyn, 2002; Scheirs *et al.*, 2000). We believe that the present results support this claim and conclude that the host utilization strategy of *O. elongata* in the two-host situation appears to be maintained by a combination of selection pressures that favour the use of different hosts in different life stages and a larval behaviour that allows alternate use of both hosts.

Acknowledgements

We thank Ted Turlings for help and advise with the chemical analysis and for inspiring discussions. Russell Naisbit provided helpful comments and Mathieu Rapp assisted in the field and in the laboratory. Philippe Kuepfer, Barbara Barisani Ephyse Noussan, Lucie Vaser, the Association Internationale du Jardin Alpin de la Chanousia, and the Ordine Mauriziano, Torino, Italy, provided logistic support. This study was financed by grants 3146850.96 and 31-64’864.01 from the Swiss National Science Foundation.

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APPENDIX appendix

Weather conditions influences host-plant choice in an alpine leaf-beetle

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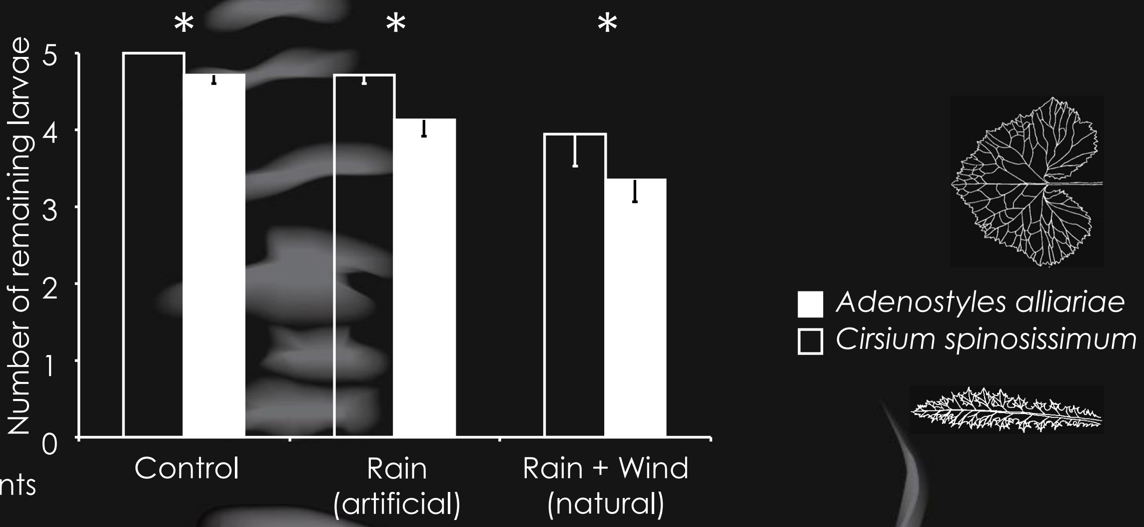
INTRODUCTION

Host-plant choice in herbivorous insects might be influenced by the degree of protection the insect receives on the plant. *Oreina elongata*'s host plants differ in the shape and structure of their leaves and presumably in how they shelter the alpine leaf-beetle against severe weather conditions. We exposed larvae on leaves of both plants to heavy rain and wind and counted the remaining larvae.

CONCLUSION

Larvae fell more often from *A. alliariae* than from *C. spinosissimum* and more often in more severe weather conditions. Together with a higher survival on *C. spinosissimum,* this might explain the oviposition preference for *C. spinosissimum* although *A. alliariae* provides better larval growth and sequestrable defensive compounds.



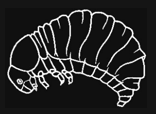
Treatments	Control	Rain (artificial)	Rain + Wind (natural)
N tot	34	48	34
Monte Carlo Sig.	0.040	0.030	0.034

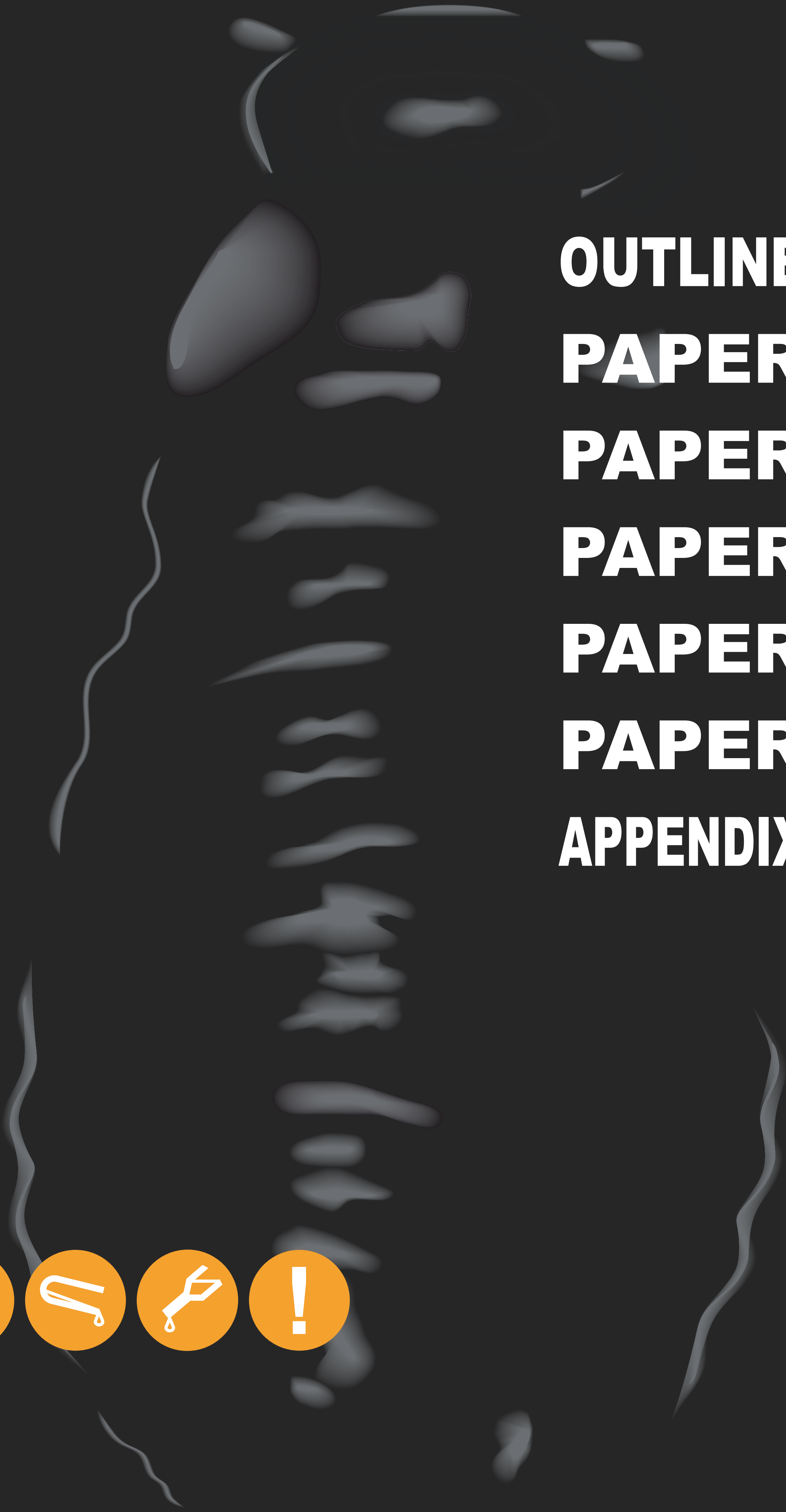
RESULTS

The number of remaining larvae on the plant is significantly affected by treatment and plant. There is a significant difference between plants within each treatment.

ANOVA Table

	Df	F Value	P Value
Treatment	2	13.759	0.000
Plant	1	7.276	0.008
Treatment X Plant	2	0.275	0.760
Residuals	110		





OUTLINE outline

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