HOST-PLANT USE IN THE SPECIALIST LEAF BEETLE *Oreina gloriosa*: THE ROLE OF LINEAR FURANOCOUMARINS

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par

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Host-Plant Use in the Specialist Leaf Beetle *Oreina gloriosa :* the Role of Linear Furanocoumarins

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La doyenne:

Martine Rahier

al mè mamin e al mè papi

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GEDULD VERNUNFT UND ZEIT SIND SCHÖN DING AUF ERDEN
WAS HEUTE UNMÖGLICH SCHEINT KANN MORGEN MÖGLICH WERDEN
Patience, raison et temps sont une belle chose sur terre:
ce qui apparaît impossible aujourd'hui peut devenir réalité demain
(Texte allemand tiré de *Imseng, W., 1973. Volkskunst im Saastal. Verlag Werner Imseng, Saas-Fee*)

Peut-être aurait-elle vu ce petit texte entaillé dans la vieille maison paroissiale de Saas-Fee, à quelques centaines de mètres à peine du terrain d'étude? Il fallait encore y croire...

Messieurs **Erich Städler** de la Station fédérale de recherches en arboriculture, viticulture et horticulture de Wädenswil (ZH), **Raffaele Tabacchi** et **Philippe Küpfer** des Instituts de chimie et de botanique de l'Université de Neuchâtel ont accepté de faire partie du jury de thèse. Je les remercie pour leur disponibilité.

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RÉSUMÉ

La chrysomèle alpine *Oreina gloriosa* (Coleoptera: Chrysomelidae) est hautement spécialisée sur l'ombellifère *Peucedanum ostruthium* (Apiaceae), l'impératoire. Dans la nature, adultes et larves sont très sélectifs: les uns et les autres se nourrissent exclusivement de feuilles, mais surtout, ils choisissent certaines plantes et en ignorent complètement d'autres. En outre, des études préliminaires ont suggéré que les larves soient actives la nuit. Comme de nombreuses autres apiacées, *P. ostruthium* synthétise plusieurs furanocoumarines linéaires, métabolites secondaires décrits pour leur activité antiappétante et/ou toxique à l'égard d'insectes phytophages généralistes. Par ailleurs, les furanocoumarines linéaires sont connues pour leur action phototoxique: le rayonnement ultraviolet proche du spectre visible induit chez ces substances une excitation électronique qui peut déterminer différents processus toxiques au niveau cellulaire. Le but de cette étude a été de vérifier l'hypothèse selon laquelle la chimie secondaire de *P. ostruthium* constituerait un facteur explicatif de l'utilisation de la plante hôte par la strictement monophage *O. gloriosa*. Toute la recherche a été menée au sein d'une seule population de la plante et de la population du coléoptère qui lui est inféodée.

Dans un premier temps, nous avons étudié le rythme d'activité de la chrysomèle sur les 24 heures dans son mileu. Ce travail a révélé que les larves se nourrissent exclusivement pendant la nuit et s'enfoncent dans le sol pendant le reste de la journée. Certaines jeunes larves se retirent dans les gaines ou les bourgeons. Au contraire, les adultes ont été observés sur les plantes pendant les 24 heures. Nous avons ensuite vérifié si la croissance larvaire était affectée par la photoactivation des furanocoumarines. Pour cela, les larves ont été placées dans deux environnements lumineux, avec et sans rayonnement ultraviolet. La mortalité a été plus grande dans le deuxième traitement: nous n'avons néanmoins pas pu tirer de conclusions définitives sur l'effet de la photoactivation des furanocoumarines. Dans un travail de terrain, nous avons montré que les invertébrés antagonistes de *O. gloriosa* sont essentiellement actifs pendant la nuit. Au vu du chevauchement entre les périodes d'activité de la chrysomèle et de ses prédateurs, nous suggérons que les ennemis naturels ne constituent pas la raison d'être du comportement des larves. Nous proposons que l'activité nocturne représente une contre-adaptation à la photoactivité des furanocoumarines. Il s'agirait alors d'un rare exemple d'adaptation comportementale chez un insecte spécialisé sur une plante hôte avec furanocoumarines linéaires. En effet, les insectes avec un régime alimentaire strictement lié à ces métabolites secondaires, disposent en général de moyens de détoxification métaboliques. La différence comportementale entre larves et adultes est probablement liée à l'épaisseur et à la pigmentation de la cuticule imaginale, vraisemblablement opaque à la lumière ultraviolette.

Nous avons également vérifié l'hypothèse selon laquelle *O. gloriosa* dans la nature sélectionnerait les plantes en fonction de leur attributs chimiques. Nous avons échantillonné des plantes fortement consommées et des plantes libres de toute attaque d'*O. gloriosa*. Les feuilles ainsi récoltées ont été extraites et analysées par chromatographie liquide pour déterminer la composition qualitative et

quantitative en furanocoumarines linéaires. La chimie secondaire a permis de bien discriminer plantes consommées et non consommées. *O. gloriosa* est probablement à même de reconnaître certaines de ces substances comme signaux ou elle est en mesure de mieux en tolérer certaines plutôt que d'autres dans leur action toxique. De toute façon, la chimie secondaire explique, du moins en partie, l'utilisation de la plante hôte par ce coléoptère strictement monophage au sein d'une population de sa plante nourricière. Cet exemple révèle la nécessité d'approfondir l'investigation, trop souvent négligée, du rôle de la variation chimique intrapopulationnelle dans les études concernant la convenance de l'hôte pour des insectes spécialistes.

RIASSUNTO

Il crisomelide alpino *Oreina gloriosa* (Coleoptera: Chrysomelidae) è altamente specializzato sull'ombellifera *Peucedanum ostruthium* (Apiaceae), l'imperatoria vera o erba rena. Adulti e larve in natura sono estremamente selettivi: gli uni e le altre si nutrono solo di foglie, ma distinguono tra le singole piante cibandosi di alcune e trascurandone completamente altre. Inoltre indagini preliminari hanno suggerito che le larve siano attive di notte. Analogamente ad altre ombellifere, *P. ostruthium* contiene varie furanocumarine lineari, metaboliti secondari con effetto deterrente e/o tossico per insetti fitofagi generalisti. Le furanocumarine lineari sono pure note per la loro fototossicità: la luce ultravioletta di lunghezza d'onda vicina allo spettro visibile induce in queste sostanze un'eccitazione elettronica che può provocare vari processi tossici a livello cellulare. La nostra tesi aveva lo scopo di verificare l'ipotesi secondo cui la chimica secondaria di *P. ostruthium* spiegherebbe l'utilizzazione della pianta ospite - così come l'osserviamo oggi - da parte dello specialista *O. gloriosa*. Per l'insieme della ricerca ci siamo concentrati su una singola popolazione della pianta e sulla popolazione dell'insetto ad essa legata.

Per prima cosa abbiamo studiato il ritmo di attività dei coleotteri in natura. Abbiamo mostrato che le larve si nutrono esclusivamente di notte e passano il resto della giornata nel suolo. Alcune giovani larve si ritirano dentro le guaine o i germogli. Gli adulti invece sono presenti sulle piante durante tutte le 24 ore. In una ricerca successiva abbiamo verificato se la crescita larvale sia compromessa dalla fotoattivazione delle furanocumarine lineari. Abbiamo tenuto le larve in due distinti ambienti luminosi, con e senza radiazione ultravioletta. Quest'ultima ha determinato una maggiore mortalità di *O. gloriosa*, tuttavia non siamo stati in grado di formulare conclusioni definitive sull'effetto della fotoattivazione. Un lavoro sul campo ha mostrato che gli invertebrati antagonisti del crisomelide sono essenzialmente attivi di notte. Dato che i periodi di attività di *O. gloriosa* e dei suoi predatori si sovrappongono, suggeriamo che i nemici naturali non costituiscano il fattore selettivo all'origine del comportamento notturno delle larve. Proponiamo che questa limitazione notturna nell'attività trofica rappresenti un'acquisizione adattativa contro la fotoattività delle furanocumarine. Si tratterebbe allora di un raro esempio di adattamento comportamentale in un insetto specializzato su una pianta contenente furanocumarine lineari. In effetti, in generale gli insetti con un regime alimentare strettamente legato a queste sostanze dispongono di strumenti di detossificazione metabolici. La differenza comportamentale tra larve e adulti potrebbe essere ricondotta al tegumento spesso, colorato e probabilmente opaco alla luce ultravioletta di questi ultimi.

Abbiamo infine verificato l'ipotesi secondo cui la chimica secondaria determinerebbe l'idoneità delle singole piante per la crescita di *O. gloriosa*. Abbiamo proceduto ad un campionamento sul campo di piante fortemente consumate e di piante prive di qualsiasi segno d'attacco del crisomelide. Le foglie così raccolte sono state estratte e analizzate con la cromatografia liquida per determinare la composizione qualitativa e quantitativa delle furanocumarine. La chimica

secondaria ha permesso di ben caratterizzare le piante consumate rispetto a quelle non utilizzate. *O. gloriosa* è probabilmente in grado di riconoscere talune di queste sostanze quali segnali o di tollerarne alcune piuttosto di altre nella loro azione di tossine. In ogni caso, la chimica secondaria permette di spiegare perlomeno in parte l'utilizzazione della pianta in seno ad una popolazione da parte di questo coleottero altamente specializzato. Questo risultato rivela l'interesse di ulteriori ricerche su un aspetto spesso ignorato quale è l'impatto, per artropodi specialisti, della variazione intrapopolazionale nella chimica secondaria sull'idoneità della pianta ospite.

SUMMARY

The alpine leaf beetle *Oreina gloriosa* (Coleoptera: Chrysomelidae) is highly specialized on the umbelliferous plant *Peucedanum ostruthium* (Apiaceae). Adults and larvae feed on leaves and clearly discriminate between individual plants in nature. Preliminary studies suggested that the larvae are active at night. Like many other apiaceous plants, *P. ostruthium* produces several linear furanocoumarins, secondary metabolites shown to be deterrent and/or toxic to generalist herbivorous insects. Moreover, linear furanocoumarins are well known as phototoxins: when irradiated with long-wave ultraviolet light, these compounds enter an excited state which can mediate different within cell toxic processes. The aim of the present thesis was to verify the hypothesis that the secondary chemistry of *P. ostruthium* is a factor explaining the actual host-plant use by the strictly monophagous *O. gloriosa*. All of our work concentrated on a single plant population and its affiliated beetle population.

We firstly investigated the daily activity rhythm of the beetles in the field. Larvae have been shown to feed exclusively at night and to burrow into the soil for the remainder of the day. Some young larvae hid within sheaths or buds during daylight. In contrast, adults were found on the plants over the 24 hours. We further verified if the performance of the larvae was affected by the photoactivation of the linear furanocoumarins. Larvae were forced to remain in two different light environments, with and without ultraviolet radiation. *O. gloriosa* was shown to suffer higher mortality when forced under light with ultraviolet radiation. Nonetheless, we were not able to draw definitive conclusions on the effect of the furanocoumarin photoactivation. In a field study, we showed that invertebrate natural enemies of *O. gloriosa* are mainly active at night. Because of the congruence in time between the beetle and the predators activities we suggest that natural enemies should not be considered as a driving force for the larval night behaviour. We propose that night activity counteracts the photoactive furanocoumarins and should be considered a rare example of behavioural adaptation in a linear furanocoumarin-specialized insect. In fact, specialists are reported to rely on metabolic detoxification. The difference in the behaviour between larvae and adults may be related to the thick and pigmented cuticle of the latter, probably opaque to ultraviolet light.

Finally we tested the hypothesis that individual plants remain free from *O. gloriosa* attack due to their chemical attributes. Samples of plants hosting actively feeding *O. gloriosa* (strongly consumed leaves) and plants exhibiting no damage (unattacked leaves) were collected in the field and examined by HPLC for their qualitative and quantitative linear furanocoumarin composition. The secondary chemistry allowed us to clearly segregate attacked and unattacked plants. We suggest that plant chemistry, relative to the ability of *O. gloriosa* to tolerate or recognise plant chemicals, can to some extent explain the host use of this monophagous leaf beetle within a population of its host plant. This shows the need for further work on the neglected impact, for specialists, of intrapopulation variation in secondary chemistry on host suitability.

INTRODUCTION

The role that plant secondary chemistry plays in governing host use by phytophagous insects is still not unequivocally determined. In some cases secondary compounds undoubtedly function as a plant resistance (Strong et al. 1984, Schoonhoven et al. 1998). Since Ehrlich and Raven's historical paper (1964) secondary metabolites have been for a long time the undisputed protagonists in studies on insect-plant relationships. In fact, they were thought to answer a key question in this field, i. e. what are the driving evolutionary forces behind the predominance of the specialist versus the generalist condition in herbivorous insects (Schoonhoven 1996). Indeed, Ehrlich and Raven proposed secondary plant compounds to be the primary mediator of a coevolutionary interaction between plants and their insect herbivores and were followed in their hypothesis by many other authors who concentrated on the importance of secondary chemicals as toxins. Despite considerable research, definitive evidence for such a coevolutionary process in insect-plant interactions remains very scarce. A compelling example is provided by the Apiaceae (to which also belongs *Peucedanum ostruthium*, studied in this thesis) and their affiliated insects. The biosynthetic pathways leading to different coumarins apparently have been acquired in umbelliferous plants in the following sequence: hydroxycoumarins, linear furanocoumarins, and angular furanocoumarins. Linear furanocoumarins are more toxic to polyphagous insects than their hydroxycoumarin precursors. Angular furanocoumarins, a further step in the evolution of these chemicals appeared only in few advanced genera, are detrimental to oligophagous insects able to tolerate linear furanocoumarins. This pattern has been interpreted as a case of escalation in a coevolutionary arms race (Berenbaum 1983, 2001, Berenbaum and Feeny 1981).

However, again, demonstrations that specialized insects exert an important amount of selection on their hosts are rare. Thus, the use of such a reciprocal warlike scenario to explain all insect-plant relationships would at least be guilty of "insectocentricity". Jermy (1984) introduced the idea of sequential evolution. He argued that the evolution of phytophagous insects merely follows the one of plants, having only in exceptional cases major feedback on plant evolution. Hartmann (1996) stated that secondary metabolites evolved as defence compounds to cope with a competitive environment consisting of herbivores (as proposed by Southwood 1984) among other antagonists (fungi, bacteria, viruses). Bernays (2001) underlined that although the studies of many authors indicate some degree of congruence in insect and plant phylogenies, precise overlap is rare: Becerra (1997) showed that in the system *Blepharida* (Coleoptera: Chrysomelidae) - *Bursera* (Burseraceae) the plant chemical similarity was more important than plant cladogenesis in determining host shifts by the beetle. Poor correlation between insect and host plant clades was also found for the chrysomelid *Ophraella* (Futuyma and McCafferty 1990). According to Bernays (2001) such examples reveal that chemicals should not be considered as deterrent or toxic agents but as positive cues which allow insects to make quick and efficient choices also throughout unrelated plants.

Nonetheless, the importance of chemistry in insect-plant relationships has been questioned by several authors who denounced its overemphatization. Courtney and Kibota (1990) suggested that chemistry should only be considered as one of multiple host-specific cues for arthropod herbivores and claimed no necessary connection between these cues and host suitability per se. Bernays and Graham (1988) argued that biotic interactions are more important than host chemistry in determining host range in spite of the preeminent role of chemistry at the behavioural level. As the latter, Price et al. (1986) and Stamp (2001) evoked the possible central role of herbivore natural enemies. Bernays (2001) also considered predators as a major evolutionary force towards oligo- and monophagy. In her comprehensive theory, she suggested insect nervous system being submitted to a strong evolutive pressure for making quick and efficient host plant choices. Rapidity, reached through recognition of distinctive chemical signals, allows improved vigilance against natural enemies considered as major fitness reducers. Dicke (2000) also considered food selection and predator avoidance to represent a tradeoff that an herbivore has to cope with. He suggested that chemical cues, infochemicals, from natural enemies and competitors affect host plant selection in much the same way as plant cues do. Definitively, in the study of host plant use, chemical ecology, behavioural ecology and evolutionary ecology have evolved from a bitrophic to a tritrophic or even a multivariable context. Abiotic factors as the thermal environment have also been shown to play a major role in the evolution of host-plant use (Scriber and Lederhouse 1992, Scriber 1994). Nowadays it is admitted that a variety of selective forces (e. g. plant chemistry, natural enemies, but also insect size, resource availability, sexual interactions, interspecific competition, environmental variability, ...) must be considered as potential determinants of diet breadth (Jaenike 1990, Bernays and Chapman 1994, Schoonhoven 1996, Schoonhoven et al. 1998). The relative importance of these factors for host specificity is still unclear and probably depends strongly on the insect group (Bernays and Chapman 1994).

This thesis concerned a genus of highly specialized leaf beetles. We focused on the role that the secondary plant chemistry actually plays in the host use. We suggest that the within population variation in concentration of toxic substances, linear furanocoumarins in this case, will determine the host plant suitability for the studied beetle species. The results may thus contradict the commonly admitted rule of adapted insects being insensitive to the secondary chemistry of the plants to which they are affiliated (e. g. Berenbaum 1981, Brower et al. 1988, Chapman et al. 1988, Hsiao 1988, Nitao 1989, Malcolm 1991, 1995, Lee and Berenbaum 1993, Pasteels et al. 1994, 1995). Host plant use was studied in the laboratory and, contrary to most published works, also in the field. This allowed us to assess the crucial role of plant chemistry and of potential biotic interactions in determining the very narrow diet breadth of the beetle.

Oreina Chevrolat (Coleoptera: Chrysomelidae) is a mountain and alpine beetle genus that displays an amazing flexibility in its ecology and life history. Species feed on plants from several tribes of two different plant families, the Asteraceae and the Apiaceae (Dobler et al. 1996, Hsiao and Pasteels 1999). This suggests that a great tolerance to diverse host chemistries is present within the genus as a whole. The host plant is known to influence the beetles' chemical defence, which depending on the beetle species can either be autogenously produced and/or plant derived. In the former case cardenolides are produced from a sterol precursor as cholesterol; in the latter case pyrrolizidine alkaloid N-oxides are sequestered from Senecioneae (Asteraceae) (Pasteels et al. 1994, 1995). These ecological traits per se make the *Oreina* group interesting for investigations of the role of plant chemistry as mediator in host-plant use.

The fact that many phytophagous insect species attack only a part of their host plant population is widely known (Jermy 1984, Price et al. 1986, Bernays and Graham 1988). Intraspecific differences in host plant chemistry have been cited as at least a partial explanation for intraspecific differences in susceptibility to insect attack (e. g. Pereyra and Bowers 1988, Zangerl and Berenbaum 1993, 2003). We do not know of many studies that have investigated the role of plant chemistry in determining the suitability of plants free from herbivores. *Oreina gloriosa*, the studied organism, belongs to a group of species within its genus that switched from the original Asteraceae hosts to Apiaceae (Dobler et al. 1996, Hsiao and Pasteels 1999). As mentioned above, this plant family displays among its members an enormous diversity in its coumarins secondary chemistry (Murray et al. 1982, Berenbaum, 1983, 1991, 2001). *O. gloriosa* shows, only case in its clade, a strict monophagous behaviour on *Peucedanum ostruthium* (L.) Koch (Peucedaneae), which is known to produce several linear furanocoumarins (List et al. 1967, Hadacek et al. 1994, Hiermann et al. 1996, Schinkovitz et al. 2003). Adults and larvae feed exclusively on the leaves and clearly discriminate between individual plants in nature (L. Nessi, pers. obs.).

Linear furanocoumarins have been shown to be deterrent and/or toxic to herbivorous insects (Berenbaum 1978, 1983, 2001, Muckensturm et al. 1981, Luthria et al. 1989, Hadacek et al. 1994, Berdegué et al. 1997, Reitz and Trumble 1997). Moreover, linear furanocoumarins are well known as phototoxins: when irradiated with long-wave ultraviolet light, these compounds enter an excited state which can mediate different toxic processes (Murray et al. 1982, Berenbaum 1991, Arnason et al. 1992). Some authors have shown substantially increased noxious effects on insect herbivores in the presence of light (e. g. Berenbaum 1978, Kagan et al. 1986, Reitz and Trumble 1996). The larvae of *O. gloriosa* have been suggested to be active at night (S. Dobler, pers. comm.). The clear discrimination of host plants in the field and the believed larval activity in the absence of sunlight suggested us that plant chemistry, namely (photoactive) linear furanocoumarins, may be a factor explaining actual host plant use by this monophagous beetle. The aim of the present thesis was to verify this hypothesis. Our work concentrated on a single plant population and its affiliated beetle population, which allowed us to stress the neglected role of plant intrapopulation chemical variation as an explanation for host selection by a highly specialized herbivorous insect. Of course, other factors may be involved in the differential host suitability. We particularly discuss the potential role of natural enemies.

Three major topics have been investigated and are presented in the following chapters. Chapter 1 concerns the larval activity rhythm**.** Abundance and feeding activity of the larvae in the field was recorded on a 24 hours period several times during the summer season for a sample of individual plants. This work allowed us to get information on the behaviour of *O. gloriosa*. Given the linear furanocoumarins phototoxicity, this chapter should be considered as a broad outcome basis for the other sections.

Chapter 2 deals with the host plant choice. We addressed the following question: does the insect's choice correspond to any qualitative and/or quantitative difference in the furanocoumarin content of the host plant? The linear furanocoumarins chemistry of plants attacked and not attacked in the same plant population has been compared.

Chapter 3 concerns the toxicity attributable to the photoactivation of the linear furanocoumarins. Larvae fed on the host plant were exposed or not to ultraviolet light and their performance was compared. A control with a related species has been done. This topic is meant to build a bridge between the former two chapters, namely field observations and the discriminant ability of *O. gloriosa*.

The chapters of this thesis correspond to manuscripts planed to be submitted to scientific journals for publication. Hence we ask the reader to apologise for the repetitions that occur in the text, especially in the introduction section. Meanwhile we have decided to give up submitting.

Literature on plant phototoxins is abundant. However, as far as we know ecological aspects of the production of these compounds by plants are still poorly described. We believe that generally in studies about insect-plant relationships investigations have been conducted with artificial diets. Effects from other plant constituents, other plant characteristics or environmental traits possibly simultaneously influencing host use have been neglected. We think that there is sufficient evidence to suggest that the role of phototoxins in plants is to provide defence against herbivores and pathogens. Nonetheless, a comprehensive theory explaining the phenomenon of light activation of plant defensive chemicals is still lacking. Which selective pressures have led to its evolution in unrelated structurally chemical groups in unrelated plant families? Which constraints do act on its expression in a wide variety of habitats? We hope that our study will provide some insight in this intriguing field.

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CHAPTER 1

NIGHT FEEDING AS A POSSIBLE BEHAVIOURAL ADAPTATION TO PLANT PHOTOTOXIC LINEAR FURANOCOUMARINS IN THE HIGHLY SPECIALIZED *Oreina gloriosa* (COLEOPTERA: CHRYSOMELIDAE)

Luca Nessi and Martine Rahier

Abstract

Oreina gloriosa (Coleoptera: Chrysomelidae) feeds exclusively on *Peucedanum ostruthium* (Apiaceae) in spite of the presence in the host plant of several phototoxic linear furanocoumarins which are deterrent and/or toxic to most other phytophagous arthropods. Insects adapted to feeding on plants containing linear furanocoumarins have mostly developed metabolic strategies to cope with oxidative stress associated with these photoactive compounds. We investigated the daily activity rhythm of *O. gloriosa* in the field. Larvae exclusively fed at night and burrowed into the soil for the remainder of the day. Some young larvae hid within buds or sheaths during daylight. In contrast, adults were found on the plants over the 24 hours. We suggest that night activity of the larvae counteracts the toxicity of the photoactive furanocoumarins and should be considered a rare example of behavioural adaptation in a linear furanocoumarins-specialized insect. The difference in the behaviour between larvae and adults may be related to the thick and pigmented cuticle of the latter. The adult integument is probably opaque to the biological active ultraviolet radiation. We also discuss the potential role of natural enemies in determining the restriction in the time of feeding. Since maximum predators activity occurs during the night we conclude that antagonists should not be considered as a main force driving *O. gloriosa* behaviour.

Key words: *Oreina*, leaf beetle, specialist, *Peucedanum*, Apiaceae, linear furanocoumarins, host use, herbivory, behaviour.

Introduction

It is generally true that adapted insects, monophagous or oligophagous species, are more so tolerant to the secondary toxic and/or deterrent compounds that characterize their host plants. For example, *Ligurotettix coquilleti* (Orthoptera: Acrididae) habitually feeds on *Larrea* (Zygophyllaceae), yet nordihydroguaiaretic acid (NDGA) acts as a deterrent to this grasshopper at the concentrations normally found in the plant (Chapman et al. 1988). Within the Chrysomelidae, some species of the genus *Leptinotarsa* are insensitive to the alkaloids at the concentration occurring in potatoes, although the same compounds are deterrent to other members of the genus (Hsiao 1988). In the genus *Oreina* some species not only have adapted to toxic substances but even exploit them since pyrrolizidine alkaloid N-oxides are sequestered from host plants belonging to the Senecioneae (Asteraceae) (Pasteels et al. 1994, 1995). The monarch butterfly, *Danaus plexippus*, specialized on *Asclepias* species, is most famous among insects for its ability to sequester cardenolides as defensive compounds against bird predators (Brower et al. 1988, Malcolm 1991, 1995). Cardenolides have been interpreted as a part of the plant defence (which also consists of a system of latex bearing canals) against generalist herbivores (Dussourd 1993, Bernays and Chapman 1994). Berenbaum (1981a) in a plant community study showed that an increase in the complexity of toxic chemicals of host plants is associated with a greater degree of specialization of the invertebrate fauna feeding on them. *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail, a specialist restricted to feeding on furanocoumarins-containing plants in the Apiaceae and Rutaceae showed improved growth rate in presence of xanthotoxin, known to be toxic to generalist lepidopterous larvae (Berenbaum 1981b).

Nonetheless susceptibility to secondary plant metabolites is known also in specialized insects. Angelicin, an angular furanocoumarin of *Pastinaca sativa* (Apiaceae), the wild parsnip, reduces growth rate and fecundity of *P. polyxenes*, which feeds on parsnip leaves and flowering parts (Berenbaum and Feeny 1981). Berenbaum et al. (1986) consider six different furanocoumarins contained in the host plant *P. sativa* as resistance factors against the local specialist *Depressaria pastinacella* (Lepidoptera: Elachistidae). The linear furanocoumarin bergapten alone was shown to decrease its larval growth (Berenbaum et al. 1989).

Much information on the toxicological properties of linear furanocoumarins is available. These substances are benz-2-pyrone compounds with a furan ring fused at the 6,7 positions. The toxic response is directly attributable to the furan ring. These molecules mainly display their toxicity in presence of ultraviolet light by binding to DNA bases. This determines disruption of DNA replication and transcription. Photoactivity is due to the formation of an excited triplet state on absorption of a photon. The excited triplet state can also react directly with proteins, lipids or ground state oxygen possibly forming singlet oxygen, hydroxy radicals or superoxide anion radicals. These molecules can react with proteins (enzymes), lipids and unsaturated fatty acids, causing toxicity (Murray et al. 1982, Berenbaum 1991, Arnason et al. 1992).

Linear furanocoumarins photoactivity has been shown for very different organisms as bacteria, fungi, plants and animals (Murray et al. 1982). The importance of light in toxicity was for example shown by Asthana et al. (1993): pigmented strains of rot fungi resulted to be more resistant to bergapten induced damage than unpigmented ones since pigmentation provided protection against photoactivation due to ultraviolet A radiation. In the case of invertebrates, particularly herbivorous insects, these substances are mainly reported to be deterrent and/or toxic against polyphagous species. Xanthotoxin reduced rates of relative growth and consumption and efficiencies of conversion of ingested and digested food to biomass in the polyphagous *Trichoplusia ni* (Lepidoptera: Noctuidae) (Lee and Berenbaum 1989). Larvae of the not specialized noctuid pest *Spodoptera exigua* significantly reduced the consumption when offered diets containing linear furanocoumarins at concentrations found in the outer leaves of celery (*Apium graveolens*, Apiaceae) plants (Berdegué and Trumble 1997). When offered diets with increasing concentrations of linear furanocoumarins (psoralen, bergapten and xanthotoxin) larval mortality increased (Reitz and Trumble 1997). In both studies ultraviolet light had no consistent detrimental effects. Photoactivation of linear furanocoumarins is not obligatory to elicit herbivore inhibition (Berdegué et al. 1997). For example, Berenbaum (1978) showed xanthotoxin to act as a feeding repellent and a development inhibitor for the polyphagous *Spodoptera eridania* even in the absence of ultraviolet light. Nine different linear furanocoumarins inhibited *Spodoptera littoralis* growth fed on artificial diets (Hadacek et al. 1994). *Spodoptera litura* and another noctuid, *Mythimna unipuncta*, were shown to be deterred by several linear furanocoumarins (Luthria et al. 1989, Muckensturm et al. 1981). The effect of linear furanocoumarins has been mostly studied with caterpillars, in particular noctuids, but inhibitory effects have been shown on members of other insect orders. For example, Muckensturm et al. (1981) showed negative effects on third instar larvae of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) offered treated leaf discs. Kagan et al. (1986) proved high phototoxicity of xanthotoxin against *Aedes aegypti* (Diptera: Culicidae). Larvae were unaffected when grown in the dark but suffered great mortality when irradiated with long wave ultraviolet light. All these reports unequivocally demonstrate the biological relevance of linear furanocoumarins within the defence system of plants accumulating them, which are in particular Rutaceae and Apiaceae (Murray et al. 1982).

Specialization on furanocoumarins containing plants has mainly led to metabolic adaptation. For example, the specialist *P. polyxenes* is capable of metabolising linear furanocoumarins 10 to 100 times faster than do generalist lepidopteran species, such as *Trichoplusia ni* and *Spodoptera frugiperda*, which do not normally feed on plants containing furanocoumarins (Lee and Berenbaum 1993). Linear furanocoumarins metabolization by cytochrome P450 monooxygenases seems to be a widespread means of detoxification (Berenbaum 1995 and references therein, Berenbaum and Zangerl 1994, Hung et al. 1996), also induced after linear furanocoumarins ingestion (Cohen et al. 1989, Nitao 1989).

Among the Coleoptera, specialization on Apiaceae is found in two families, the Curculionidae and the Chrysomelidae, respectively represented in Europe only by the genera *Liophloeus* and *Oreina* (Berenbaum 2001). We studied the daily activity rhythm of a highly specialized species whose larvae have been suggested to feed at night (S. Dobler, pers. comm.). We observed adult and larvae on the host plant and recorded their feeding activity. The leaf beetles of the genus *Oreina* Chevrolat feed on plants from several tribes of two different plant families, the Apiaceae and the Asteraceae (Dobler et al. 1996, Hsiao and Pasteels 1999). *O. gloriosa* belongs to a group of species within its genus that switched from the original Asteraceae hosts to Apiaceae and shows, only case in its clade, a strict monophagous behaviour on *Peucedanum ostruthium* (L.) Koch (Apiaceae, Peucedaneae), which is known to contain several linear furanocoumarins (List et al. 1967, Hadacek et al. 1994, Hiermann et al. 1996, Schinkovitz et al. 2003). Adults and larvae exclusively feed on the leaves and clearly discriminate between individual plants in nature (L. Nessi, pers. obs.). Both the beetle and the plant are common in the Alps mostly at the subalpine and alpine levels. *P.*

ostruthium is frequent in nitrogen-rich meadows and moist gravelly soils on both granite and limestone substrates (List et al. 1967, Lauber and Wagner 1996).

Given this preliminary information, our study was aimed to test whether the behaviour of the larvae and adults of *O. gloriosa* supports the suggestion that they feed and are more active during the night. In contrast to most studies on herbivores feeding on phototoxic plants which concentrated on laboratory tests, our observations were done in nature. The field work allowed us to get some qualitative information on the invertebrate natural enemies of the studied site. We discuss the observed leaf beetle behaviour with regard to the known phototoxic activity of the linear furanocoumarins contained in the host plant.

Materials and methods

Studied population. The *O. gloriosa* studied population is located in the Swiss Alps on the precinct of the village of Saas Balen (Canton Wallis, 46.080 N, 7.570 E) at 1800 m elevation. The host plant, *P. ostruthium*, population extends along a forest roadside in a half-open habitat. The plants grow within patches of one to two square meters. Shoots appear at the end of May. *O. gloriosa* is mainly active from mid June to the beginning of September. Adults emerge from overwintering in the ground and start mating from the beginning to mid June. At this time *P. ostruthium* plants have already grown and are widely fed on. Blooming has not yet occurred and flowers are hidden within buds. The viviparous *O. gloriosa* lays first instar larvae between the end of June and mid July. Larviposition was mostly observed to occur within leaf or flower buds, but also on leaf surface (L. Nessi, pers. obs.). Feeding on the leaves, larvae develop to fourth instar by the beginning of September when they burrow into the ground to overwinter. At mid August a new generation of adults emerges for a short time from pupating larvae of the previous year. In September adults disappear either because they die or because they burrow in the soil to overwinter.

Study of daily activity rhythm. The presence of the larvae and adults of *O. gloriosa* on its host plant and their feeding activity was studied during the summers of 1996 and 1997.

In the summer of 1996 we selected at different locations within the described *P. ostruthium* population 10 different plants at four times along the season (July 2, July 11, July 18, August 10). The selected plants were flower bearing individuals presenting signs of *O. gloriosa* larvae consumption. We have thus selected the plants according to their observed suitability for *O. gloriosa*. At each observation date the selected plants were inspected once each hour for a 24-hr period. The 24-hr period was not continuous, but split into 3 8-hr periods within 3 days, i. e. from 10 p.m. of day 1 to 6 a.m. of day 2, from 2 p.m. to 10 p.m. of day 2, and from 6 a.m. to 2 p.m. of day 3. Each plant was exhaustively examined for the presence of *O. gloriosa* individuals. We performed the night observations with a frontal torch (2.5 V bulb). For each individual we recorded the age (1st, 2nd, 3rd, 4th instar or adult), the feeding activity and the location on the plant relative to plant parts, i. e. leaf, sheath, umbel, and stem. Figure 1 (A, B) shows the architecture of a flower bearing plant. Below-ground parts of plants were not examined.

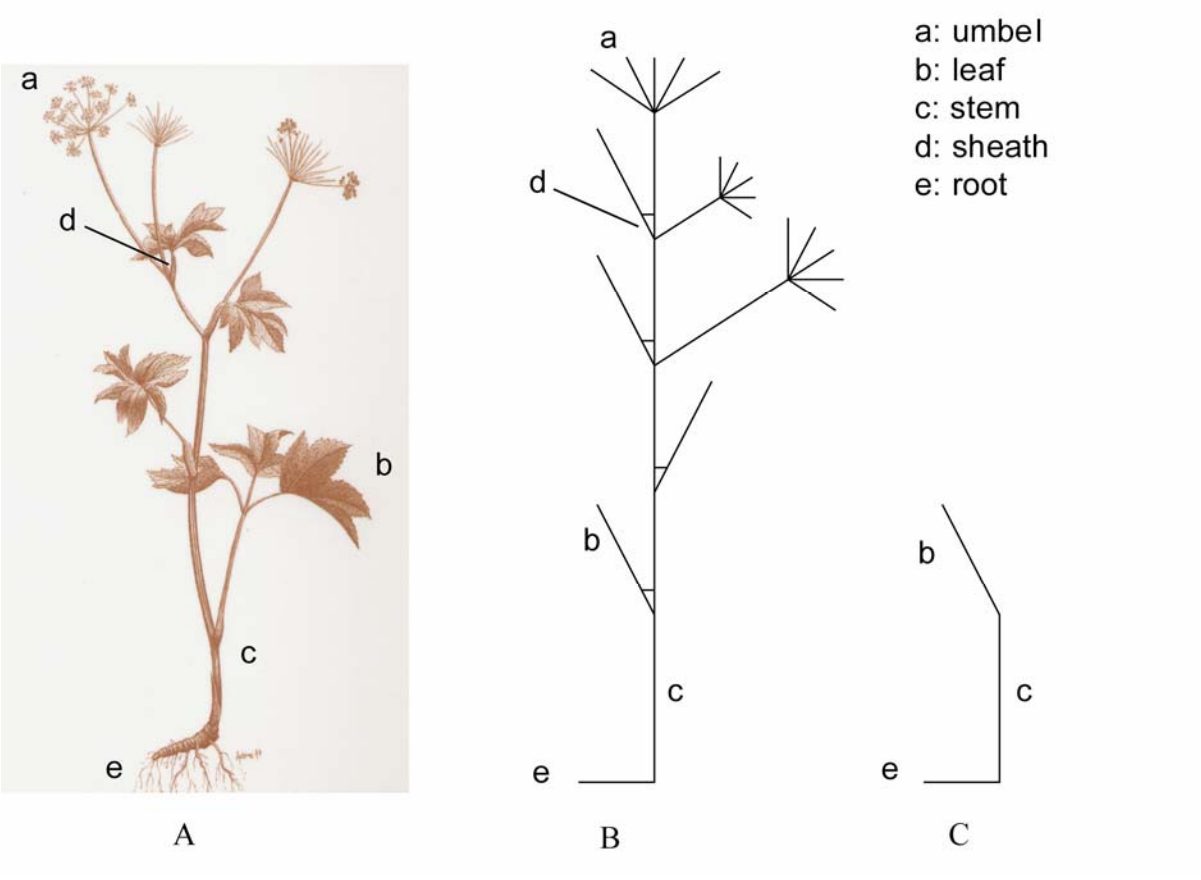


Figure 1. Architecture of representative *Peucedanum ostruthium* plants. A, Drawing of a flower bearing plant (from Brüschweiler 1999). B, Depiction of the same plant. C, Depiction of a vegetative plant.

In the summer of 1997 the study was done in a very similar way. We selected at different locations within the same *P. ostruthium* population as in 1996 60 different plants 10 times along the season (July 2, July 8, July 15, July 22, July 28, August 4, August 12, August 19, August 26, September 4). Plants were vegetative and presenting signs of larval consumption.
At 3 dates (July 8-10, July 28-30 and August 4-6) plants were observed for a 24-hr period as described for 1996. The 7 other times each selected plant was examined once every hour for a 8-hr period within 2 days, between 10 p.m. of day 1 and 6 a.m. of day 2. The same records as in 1996 were done except for the location of the beetles since vegetative plants are structurally very simple, presenting a stem bearing a single leaf (figure 1C). Vegetative plants are more abundant within patches than flower bearing plants. Examination of both types in two successive years allowed some coarse comparisons between the two morphologies. Moreover, investigation of vegetative plant individuals was faster and allowed to avoid disturbance of the beetles. In fact, observation of the flower bearing plants needed some manipulation of the plant parts. At each observation we recorded the temperature above soil surface and the incident light intensity above the plant top. Light intensity was measured with a Minolta illuminance meter T-1.

Natural enemies. A preliminary research aimed to screen the soil-surface invertebrate fauna in the studied site was done in summer 1996. Three Barber traps (Barber 1931) were placed in five different plant patches of the *P. ostruthium* population at three times during a week (July 1-7, July 17-23, August 9-15). The traps were placed within patches with plants which were consumed by the larvae of *O. gloriosa*. At the end of each week of collection the fauna was brought to the laboratory and fixed in 70% ethanol. We did not exploit all the samples and determination of the fauna only occurred at the family or order level. We also got some information about natural enemies from direct observation in the field.

Statistical analyses. Since during most of the 24 hours plants were abandoned by the larvae, we recorded a lot of absences. We decided to group the observations in four periods according to the activity relevance. Data of the 8 hours between 6 a.m. and 2 p.m. were grouped in the period called day 1 (afterwards D1). Day 2 (D2) included the 8 hours period between 2 p.m. and 10 p.m.. The 8 hours night time, when larvae were much more active were split in periods night 1 (N1, from 10 p.m. to 2 a.m.) and night 2 (N2, from 2 a.m. to 6 a.m.). For each period we also grouped all the observations of the season to cope with missing data relative to the instars. This procedure does not permit the calculation of a standard error.

The number of *O. gloriosa* individuals (total number of larvae and adults and feeding individuals) on the plants and on the different plant parts across the four periods (D1, D2, N1, N2) was compared with a Chi square goodness of fit test. If the test revealed significant differences, we used an exact binomial test to compare numbers across all possible pairs of periods of the day to determine between which periods abundance was significantly different. Because of multiple testing of the same periods we adjusted the p values with the Bonferroni method (Sokal and Rohlf

1995). However, data are not independent across periods since we observed the same plants for the entire examination time. In contrast, observations were independent across dates since new plants were selected. All analyses were computed using S-Plus 6 by Insightful Corporation (2001). We also performed logistic regressions for predicting larval/adult presence on the plant with plant parts and period of the day as explanatory variables. For the computation we used the logistic procedure of the SAS Institute (1999). We used a Spearman's rank correlation computed with S-Plus 6 by Insightful Corporation (2001) to examine if larval abundance (all instars) varied with the number of leaves per plant. To perform the analysis we considered the data of the N2 period.

Results

Figure 2 shows the abundance and feeding activity of *O. gloriosa* on *P. ostruthium* plants over 24 hours. The abundance pattern is different between flower bearing plants and vegetative ones, in that the former carry larvae over 24 hours and the latter almost exclusively between 2 and 5 a.m.. The pattern also varies in respect to the night period: flower bearing plants show maximum densities between 10 p.m. and 5 a.m., while maximum densities on vegetative plants are restricted between 2 and 5 a.m.. The fact that flower bearing plants overall carry more larvae than vegetative plants is probably due to their greater structural complexity. Larvae on both plant types feed during the second half of the night. When the larvae leave the plants, they burrow into the ground (L. Nessi, pers. obs.). The study of flower bearing and vegetative plants was done in two different years. We nevertheless believe that some coarse comparison can be drawn.

In contrast to the larvae, adults are found on the plants over the 24 hours on both plant types. On flower bearing plants adults reach the highest abundance in the afternoon and the lowest in the second half of the night. On vegetative plants the number of adults remains constant over the 24 hours. As for the larvae, the greater structural complexity of the flower bearing plants probably allowed more adults to stay on them. Very few adults were observed feeding, particularly on flower bearing plants.

Larvae, all instars combined, are more abundant in the second part of the night, i. e. the period from 2 a.m. to 6 a.m. (N2). This applies as well to flower bearing plants (figure 3A) as to vegetative ones (figure 3B). The data on the four instars larvae examined separately on vegetative plants clearly confirmed this pattern: very few larvae are found on the plants during the three other considered day periods, i. e. from 6 a.m. to 2 p.m. (D1), from 2 p.m. to 10 p.m. (D2), and from 10 p.m. to 2 a.m. (N1) (figure 3B). The situation is different for flower bearing plants, where larvae are found all through the 24 hours (figure 3A). There is a trend of increasing abundances from the morning to the night. N2 shows maximum densities for first and third instars and all of the three fourth instar larvae observed were on the plant during N2. This very small number of observations is due to the fact that when we stopped the investigations in the field on August 10th 1996 fourth instar larvae had just appeared in the population.

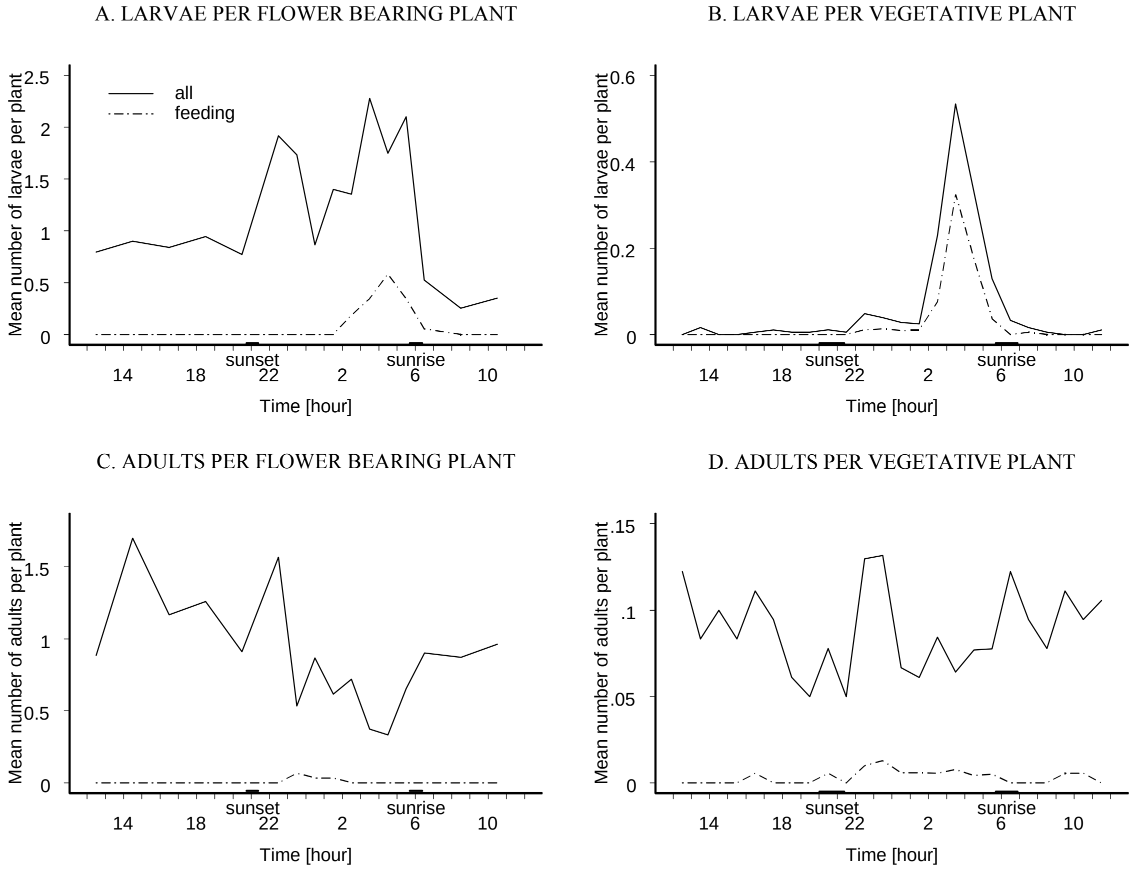


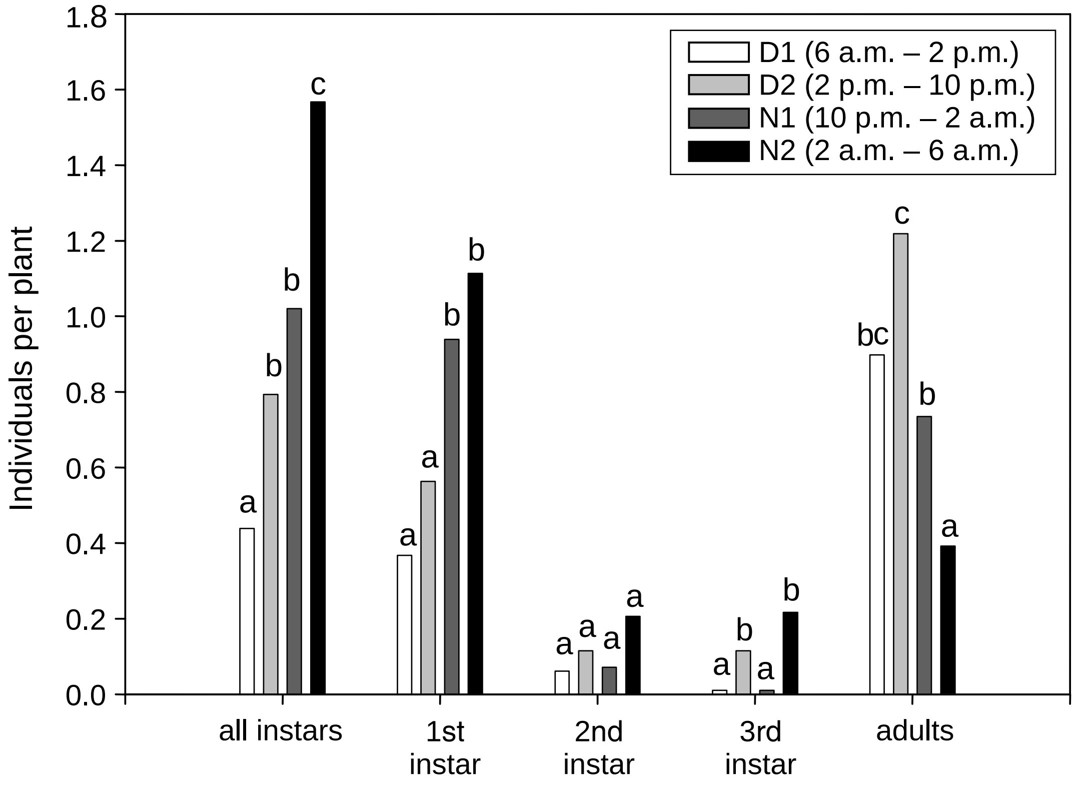
Figure 2. Mean number of *O. gloriosa* individuals per plant over the day. A, mean number of larvae per flower bearing plant (1996). B, mean number of larvae per vegetative plant (1997). C, mean number of adults per flower bearing plant (1996). D, mean number of adults per vegetative plant (1997). Bold lines on the x axis indicate the time of sunset and sunrise.

The distribution of the adult abundance through time completely differs from that of the larvae. On flower bearing plants adults reach the maximum density during daylight and the minimum in the second part of the night (figure 3A). Abundance does not change over the 24 hours on vegetative plants (figure 3B).

In the study of the flower bearing plants, plant parts (i. e. leaf, sheath, umbel, and stem, see figure 1A, B) and period during the day (D1, D2, N1, N2) allowed a good prediction of the presence of *O. gloriosa* larvae and adults (table 1).

Larvae are found on the leaves almost exclusively during the second half of the night (figure 4A). The pattern of abundance during the day of the four instars larvae on the leaves of flower bearing plants corresponds to that one found for the abundance on vegetative plants (figure 3A). This is consistent with their morphology since vegetative plants are composed of a stem and a leaf (figure 1C). The lower numbers are due to the fact that flower bearing plants have more leaves.

A. FLOWER BEARING PLANT



B. VEGETATIVE PLANT

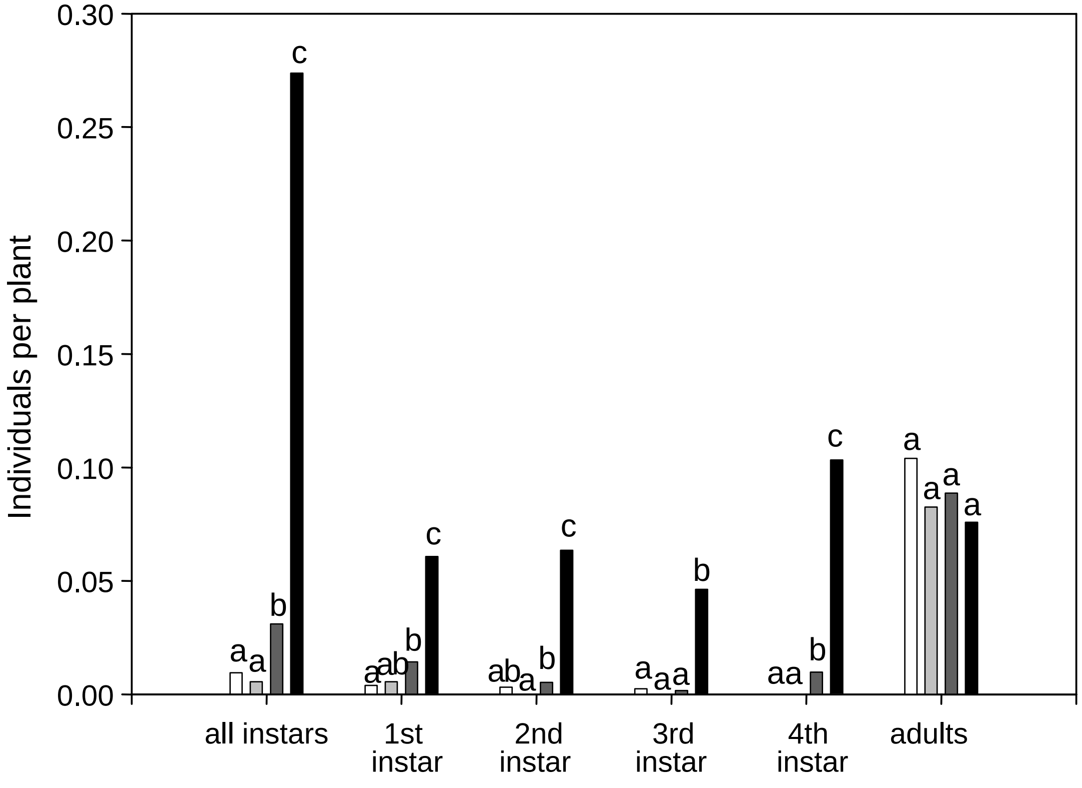


Figure 3. Mean number of *O. gloriosa* individuals per plant at different periods during the day. Different letters indicate significant differences after exact binomial test (used to compare abundance across all possible pairs of periods of the day) and Bonferroni correction. A, individuals per flower bearing plant (1996). Fourth instar larvae were not represented since only three individuals were observed. B, individuals per vegetative plant (1997).

The Spearman's rank correlation for the abundance of all instar larvae and the number of leaves on flower bearing plants was significant ($p = 0.0086$).

No significant differences have been observed for adults abundances during the day.

Table 1. Results from the logistic regression for the effect of day period and plant part on frequencies of presence of *O. gloriosa* on the flowers bearing plants. A, larvae (all instars)*, B, adults**.

A

	d.f.	χ^2	p
period	3	66.08	<0.0001
plant part	3	11.86	0.0079
period x plant part	9	23.74	0.0047

*The model is significant. χ^2 value for likelihood ratio is 149.56, d.f. = 15, $p < 0.0001$.

B

	d.f.	χ^2	p
period	3	62.19	<0.0001
plant part	3	10.94	0.012
period x plant part	9	34.04	<0.0001

**The model is significant. χ^2 value for likelihood ratio is 165.76, d.f. = 15, $p < 0.0001$.

Abundance of larvae within sheaths tends to be constant over time (figure 4B). Significant lower densities observed during D1 at least partially arise from methodological problems and should be looked at with caution. Inspecting the presence of larvae within sheaths certainly disturbed the animals much more than during the observation of other plant parts. Larvae have been observed leaving the sheaths during plant inspection. The disturbing effect increased examination after examination so that major experimental impact was reached during the third 8-hr examination period, which mostly overlaps with D1. Larvae are not equally disturbed: the number of occupied sheaths does not change over time. On the contrary the number of larvae per sheath falls down. For example, the comparison of D1 and N2 larvae abundances by exact binomial test is highly significant ($p = 0$). The comparison of the frequencies of occupation by Fisher's exact test on the contrary is not ($p = 0.107$). Sheaths are the plant part which holds the majority of the larvae observed. In comparison to the total number of larvae staying on the plants, sheaths hold 79.1%, 89.8%, 76%, and 46.7% of the individuals during D1, D2, N1, and N2 respectively. On leaves during N2, the period of maximal occupation of this plant part, only 28.9% of the total larvae were observed.

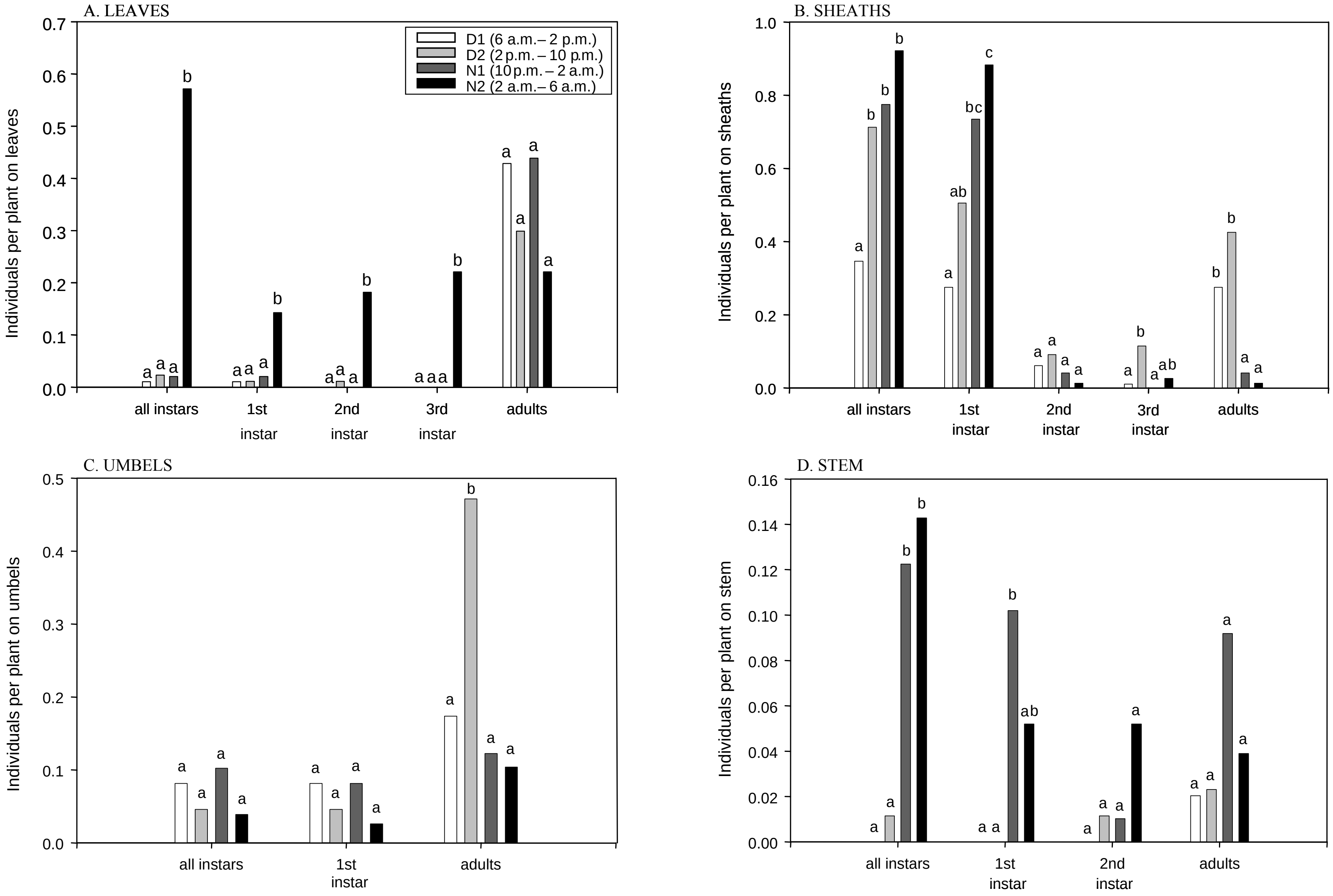


Figure 4. Mean number of *O. gloriosa* individuals per plant on different plant parts at different periods during the day. Different letters indicate significant differences after exact binomial test (used to compare abundance across all possible pairs of periods of the day) and Bonferroni correction. Representations were omitted when less than four individuals were observed. A, individuals on leaves. B, individuals on sheaths. C, individuals on umbels. D, individuals on stem.

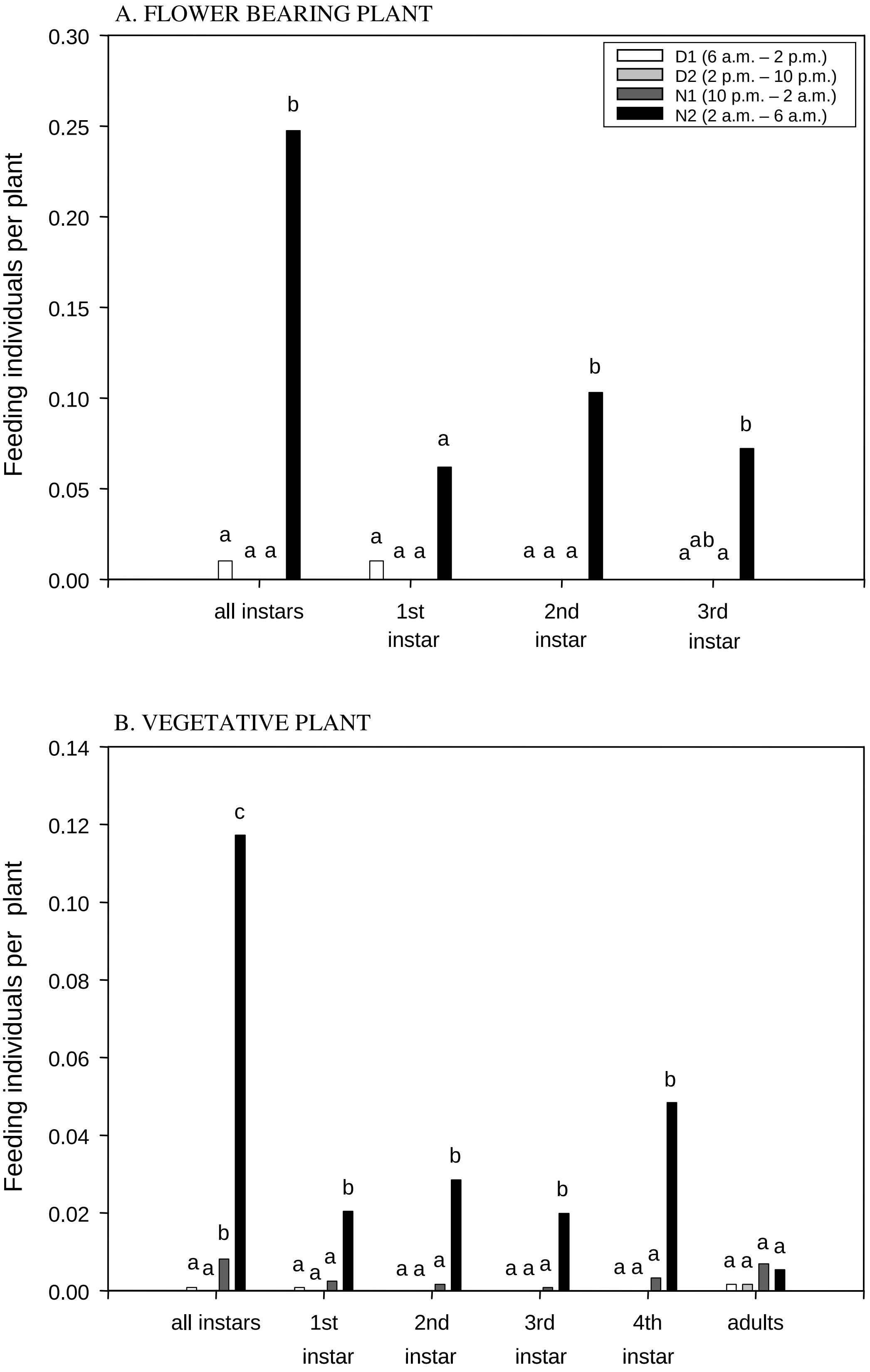


Figure 5. Mean number of *O. gloriosa* feeding individuals per plant over different periods during the day. Different letters indicate significant differences after exact binomial test (used to compare abundance across all possible pairs of periods of the day) and Bonferroni correction. A, individuals per flower bearing plant (1996). Fourth instar larvae and adults were not represented because respectively only one and three individuals were observed. B, individuals per vegetative plant (1997).

Adults are almost exclusively found within sheaths during D1 and D2. Much more mobile than the larvae, adults seemed less disturbed by the observations. Only 22.7% of the total number of adults observed during 24 hours were staying in the sheaths. Nonetheless, if the number of adults counted within sheaths would not be considered, no significant difference would be found between D1, D2, N1 and N2 for the total number on the three other plant parts.

As for the sheaths, abundance of the larvae on umbels is constant over time. Most of the larvae found on umbels are first instar. Adults are much more abundant on umbels during D2. Stems hold larvae only during the night. In contrast, adults are found on stems during 24 hours. For both umbels and stems, relatively few larvae are found (figures 4C and 4D).

The results from the logistic regression revealed that the interaction term, day period x plant structure, is also significant (table 1). For both larvae and adults this results from the divergent pattern for leaves and sheaths occupation over time. Larval presence in the sheaths remains constant over 24 hours while abundance on the leaves increases in the second half of the night. The pattern is somehow reversed for the adults: the number of adults remained constant on leaves but was reduced within sheaths at night.

Larvae feed almost exclusively during the second part of the night (figure 5). Their presence on flower bearing plants during the rest of the day is not directly linked with feeding activity. Distribution pattern of feeding larvae over time corresponds with the presence of larvae on leaves on which *O. gloriosa* exclusively feeds. During the feeding period, larvae do not feed continuously (L. Nessi, pers. obs.), therefore abundance of feeding larvae is lower. Numbers of larvae observed feeding are particularly small in 1996. This reflects in the results of the statistical analysis which for example show consistency in the number of third instar larvae in D2 and N2. Nonetheless, it should be remembered that during the entire summer of 1996 only one larva was observed feeding out of N2. The proportion of feeding and not feeding larvae varies between the plant types but is similar for the four instars in each studied year. We partially attribute the lower proportion of observed feeding larvae in 1996 to greater disturbance during observation. Indeed, inspection of flower bearing plants required manipulation. Only few adults were observed during feeding: no significant difference was observed over time, even if there is a trend for major feeding activity at N1. We do not have definitive explanations for the very few observations of adults while feeding. Possibly adults eat the most before reproduction.

Barber traps allowed to collect a variety of soil invertebrates. We distinguished four major taxa known to be predators (Ekschmitt et al. 1997, Wolters and Ekschmitt 1997, Gobat et al. 1998): the Carabidae (Coleoptera), the Staphylinidae (Coleoptera), the Chilopoda, and the Araneae. On the plants we recorded a single predator, namely *Mitopus morio* Fabricius 1799 (Opiliones: Phalangidae). All of these invertebrates appeared to be constantly present through the summer.

In a single case we observed a *M. morio* individual with a first instar larva of *O. gloriosa* in its mouth parts.

Appendices 1 and 2 show the mean brightness and temperature at the study site over 24 hours as measured during the 1997 season. Appendix 3 shows the abundance of larval and adult *O. gloriosa* over the entire summer in the same year.

Discussion

The larvae of *O. gloriosa* feed during the night and burrow into the soil or hide within the sheaths for the rest of the day. There are many potential explanations for this behaviour, such as avoidance of predators or selection of particular microhabitat conditions. We suggest that night activity of the strict monophagous leaf beetle *O. gloriosa* is an adaptation to avoid the photoactive secondary metabolites, linear furanocoumarins, of its host plant *P. ostruthium*.

Night activity could be considered as one of the rare descriptions of a behavioural adaptation to linear furanocoumarins photoactivity in a specialist insect. Nonetheless, phototoxicity may not be the sole cause for maintaining such a behaviour. Some behavioural means of protection against photoactivity have been described among generalist herbivores that regularly feed on Apiaceae. For example, fifth instar larvae of *Spodoptera exigua* feeding on celery have a clear photonegative behaviour to prevent photoactivation, i. e. they feed in the heart when they are exposed to the light. Younger larvae feed on leaves but are protected by a silken web (Griswold and Trumble 1985). Jones and Granett (1982) report some other adaptations of celery lepidopteran pests: *Heliothis zea* feeds within webbed leaves, *Trichoplusia ni* on the underside of leaves, *Platynota stultana* and *Udea profundalis* in webbed rolled leaves. Berenbaum (1978) stated that concealed feeding is disproportionately common in microlepidopterans (and other insects) associated with furanocoumarins containing plants and suggested this behaviour as a means of avoidance against phototoxicity. Leaf-tying is also reported for non apiaceous plants. Tortricid larvae have adapted this way to the phototoxic *Hypericum perforatum* (Hypericaceae) (Sandberg and Berenbaum 1989). Old instar larvae of the polyphagous *Ostrinia nubilalis* (Lepidoptera: Pyralidae) escape photosensitization due to thiophenes and phenylheptatriyne by spinning silk and by boring into stems or fruits (Champagne et al. 1986).

In rare cases behavioural adaptations have also been described in specialized insects. The larvae of the carrot fly, *Psila rosae* (Diptera: Psilidae), attack numerous wild and cultivated apiaceous plants (Degen 1998). Stanjek et al. (1997) suggested that their strict root feeding behaviour allows entire avoidance of phototoxicity. Guillet et al. (1995) documented induction of shelter-building behaviour in *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) larvae on the phototoxic *Chrysanthemum leucanthemum* (Asteraceae) and preferential oviposition by this species in shaded areas.

An escape behaviour has also been described within the *Oreina* closely related (Bourdonné and Doguet 1991) genus *Chrysolina* Motschulsky. The diurnal burrowing habit of *C. quadrigemina* and *C. hyperici* larvae feeding on *H. perforatum* was suggested to be a response induced by light to reduce photooxydative stress caused by ingestion of the phototoxic anthraquinone hypericin (Fields et al. 1990). All the species of the only other European coleopteran genus, besides *Oreina*, specialized on Apiaceae, *Liophloeus* (Curculionidae) (Berenbaum 2001), are exclusively active at night (Koch 1992).

Among *Oreina* species larval night activity is exclusive only for the Apiaceae feeders. Larvae of the species that rely on asteraceous hosts are found and feed on the plants throughout the day (S. Dobler, pers. comm.). In contrast, larvae of *O. bifrons* (feeding on *Chaerophyllum hirsutum*, Scandiceae, and *P. ostruthium*), *O. speciosa* and *O. variabilis* (both feeding on several plants within Apieae (*Meum*), Peucedaneae (*Angelica*, *Heracleum*, and *Peucedanum*) and Scandiceae (*Chaerophyllum*, and *Anthriscus*)) spend on the host only the night and burrow into the soil at daylight (S. Dobler, pers. comm., diet breadth from Dobler et al. 1996). Since differences in secondary compounds between Apiaceae tribes are few and not defined by the presence or absence of compound classes but rather by differences in their chemical structure (Hegnauer 1971), we suggest that night behaviour in *Oreina* larvae of these different species should be regarded as an adaptation against phototoxicity due to linear furanocoumarins. A further argument is that *O. gloriosa* larvae exposed to visible and ultraviolet light in a laboratory bioassay showed greater mortality than the larvae that were exposed to visible light only (chapter 3).

The larvae that do not leave the *P. ostruthium* plants to burrow into the ground remain hidden within the sheaths, which are opaque to ultraviolet light (chapter 3). In some cases, freshly laid larvae feed in a concealed manner at the very beginning of the leaf buds opening (L. Nessi, pers. obs.). Almost only first instar larvae have been shown to stay within the sheaths. It appears that older larvae are to large to fit within the generally very narrow rolled structure. Sheaths possibly also offer useful microclimatic conditions and definitive protection against predators.

The structure of the umbels dramatically changes during plant development. Young blooming umbels show structural characteristics that resemble very closely to that of the sheaths. Since flowering extends over most of the season, young umbels are available for the larvae for a long time. This should be the reason why we found almost constant numbers of larvae on umbels.
Sheaths and umbels held significantly more adults at daylight than in the night. As mentioned below, adults are probably more tolerant to ultraviolet radiation. In any case, shade from umbels and sheaths may at least partially mitigate some possible toxic effects.

Arthropods that feed on plants containing phototoxic compounds also developed other adaptations. For example, some insects have been shown to avoid tissues with high levels of toxins by selectively feeding on particular sites of the plant (Camm et al. 1976, Champagne et al. 1986, Flemion and McNear 1951 in Berenbaum 1990). Indeed, *O. gloriosa* avoids veins (L. Nessi, pers. obs.), which are reported to contain high levels of linear furanocoumarins (Zangerl and Bazzaz 1992). Physiological means to cope with oxidative stress associated with photoactive compounds have also been described. For example, some evidence suggests that, do to their ability to neutralize singlet oxygen and free radicals, carotenoids sequestered from food may act as defences for herbivorous insects (Carroll et al. 1997), including *C. hyperici* (Aucoin et al. 1990). The thick and strongly pigmented cuticle of the larvae of the furanocoumarins-containing plants feeder *P. polyxenes* is also thought to provide protection against photoactivation (Lee and Berenbaum 1993). Fields et al. (1990) explained the "sun loving" behaviour of *C. hyperici* and *C. quadrigemina* adults with the opacity of the integument which only transmits 0.1-0.2% of the photosensitizing wavelengths. We suggest that *O. gloriosa* adults also at least partially overcome phototoxicity due to physical properties of their cuticle. In any case, our behavioural study does not allow to exclude biochemical and physiological adaptations neither in the larvae nor in the adults.

In an evolutionary perspective, antagonists may also play a relevant role (Bernays and Graham 1988, Feder 1995, Yamaga and Ohgushi 1999, Stamp 2001). In a study on tropical caterpillars, Dyer (1995) concluded that predation could be a substantial selective force in the evolution of narrow diet breadth and that plant chemistry could be the mechanism whereby specialists are better defended. Some authors have suggested that selection through natural enemies can prevent the use of plants on which chrysomelid larvae would be able to develop (e. g. Denno et al. 1990, Ballabeni and Rahier 2000, but see Rank et al. 1998). In *Oreina* species, the diversification of the chemical defences is also suggested to be adaptive against predators (Rowell-Rahier et al. 1995).

O. gloriosa larvae temporal restricted activity could be seen as a way of living that mitigates the beetle vulnerability to predators and parasites, i. e. a habit of enemy-free space sensu Jefferies and Lawton (1984). For example, low night temperatures may prevent parasitic wasps from flying. As stated by Bernays and Graham (1988), insects that gain protection against predators via host-specific crypsis or host-derived defensive chemicals could be kept on these plants through stabilizing selection from their predators. The dominant role of protection offered by the plant for explaining host use has for example been shown by Damman (1987). Pyralid caterpillars consistently preferred the older leaves of their host even though younger ones provided higher food quality for growth: the older leaves were more suitable for rolling and producing a shelter. Sheaths in *P. ostruthium* may play a similar role for *O. gloriosa* larvae.

O. gloriosa larvae and adults are found in high densities on the attacked patches, which may make them readily available as potential preys. Nonetheless, Eggenberger (1989) showed for *O. gloriosa* a quite high survival rate (0.73-0.96 per week). Very little is known about the natural enemies of *Oreina* species (Pasteels et al. 1996), but the harvestman *M. morio* is known as a major predator of different *Oreina* species larvae (Hartmann et al. 2003, Labeyrie and Rahier 2004, H. Häggström, unpublished data). We also recorded other predators (carabids, staphylinids, spiders, chilopods) on the ground around the *P. ostruthium* plants, *M. morio* being almost the only one we also found on the vegetation. All of this invertebrate fauna living on soil surface is known to be mainly active at night to reduce water loss (Ekschmitt et al. 1997, Wolters and Ekschmitt 1997). Thus there seems to be a precise time overlapping with *O. gloriosa* larvae feeding activity. Moreover, most larvae spend daylight time in the soil: we suggest that they may exhibit great vulnerability to predation when crossing the below ground-stem interface before and after feeding. Because of this congruence in time we suggest that arthropod predators should not be considered as a driving force for larval night behaviour. Preliminary research on two populations of *O. cacaliae* and *O. elongata* did not reveal any larval parasitism by flies or hymenopterans (L. Nessi, unpublished data). Larvae of these two species are found on the host plant leaves at night but also during the day at higher temperatures, which a priori make them more readily available for parasitism. We therefore suggest that parasitism should neither be considered as an immediate reason for *O. gloriosa* night activity. A further argument against a natural enemies hypothesis is that no predation on *Oreina* species adults is known. With regard to parasitism, a tachinid fly has been observed on an adult *O. cacaliae* in the field (H. Häggström, pers. comm.). Adult *O. gloriosa* have a highly green to blue aposematic colour with longitudinal blue to red lines on the elytra. As the larvae, they are chemically defended (Rowell-Rahier et al. 1995) through a biosynthesized mixture of cardenolides (Eggenberger and Rowell-Rahier 1991, 1992, 1993a, b, Eggenberger et al. 1992, Pasteels et al. 1992, Pasteels et al. 1996).

Rowell and Blinn (2003) suggested that coumarins may be the immediate reason for an enemy-free space habit since predation on a freshwater amphipode resulted significantly reduced when it consumed the coumarin containing macrophyte *Berula erecta* (Apiaceae). Nonetheless, evidence of avoidance of predation on umbellifer feeders as a response to the coumarins contained in the body is very poor. Some observations have been reported for insectivorous birds and parasitic wasps (Berenbaum 1990 and references therein). The *Trichoplusia ni* parasitoid *Copidosoma floridanum* (Hymenoptera: Encyrtidae) showed increased mortality when offered host larvae fed on diets with increasing concentrations of psoralen, bergapten and xanthotoxin. Reitz and Trumble (1996) showed that these linear furanocoumarins at naturally occurring concentrations clearly mediated the host-parasitoid relationship through direct effects on *C. floridanum* and not merely as a consequence of their effect on the host. Neither larvae nor adults of *O. gloriosa* sequester linear furanocoumarins for their defence (Labeyrie and Rahier 2004). Moreover, the beetle did not gain protection against harvestmen from the plant material in its gut (Labeyrie and Rahier 2004).

It is surprising that an insect vulnerable to a phototoxin "wastes" time and only feeds in the second half of the night. Nonetheless there may be some advantages to feeding close to dawn. Moisture is higher late at night and water content of the food is known to be positively correlated with insect performance in many species (Fields et al. 1990 and references therein). This humidity hypothesis might be particularly relevant for the studied population. Indeed, Saas Balen lies in a region which is characterised by very dry summers and a climate with strong continental characteristics (Werner

1988). The biosynthesis of linear furanocoumarins is activated by ultraviolet light (Beier and Oertli 1983, Berenbaum 1991, McCloud et al. 1992, Masuda et al. 1998) and possibly occurs within the leaves in *P. ostruthium*. The synthesis of coumarins is in fact reported to take place primarily in the leaves (Murray et al. 1982, Berenbaum 1991 and references therein). After sunset, these compounds are possibly in part transferred from the leaf cells where they are produced to other plant parts, for example seeds which are known to contain large concentrations (Berenbaum 1991 and references therein, Zangerl and Bazzaz 1992). This could also explain the delayed consumption in the second part of the night. Day-night variability in the production of allelochemicals is known in other systems: for example De Moraes et al. (2001) found that nocturnal and diurnal herbivore-induced volatiles of tobacco plants were different. *C. hyperici* and *C. quadrigemina* show a temporal feeding pattern similar to that of *O. gloriosa*: Fields et al. (1990) suggest that the leaf beetles may be better able to digest and/or detoxify the host plant allomones during the heat of the day rather than during the cooler night temperatures.

In conclusion, we suggest that night activity allows *O. gloriosa* to avoid ultraviolet-associated damage entirely. We do not have any information about physiological means of protection which may also operate in the *O. gloriosa* strategy of managing linear furanocoumarins toxicity. In any case, this behavioural defence may have allowed the leaf beetle to successfully survive without metabolic defences associated with detoxification of photoactivated substances and therefore to reduce defence costs. On the other hand night activity corresponds to a restriction of the foraging period and to a reduction of body temperature. Both factors may represent important fitness costs since development time is negatively influenced.

Some authors have suggested that adaptations to novel host plants are more likely to be behavioural than biochemical or physiological (Dethier 1941, Feeny et al. 1983, Futuyma et al. 1984, Futuyma and Moreno 1988, Courtney and Kibota 1990, Feeny 1992, Bernays and Chapman 1994, but see Futuyma and Peterson 1985, Carrière and Roitberg 1995). We believe that the study of behavioural adaptations as a response to changes in the environment has been understressed. The very distinct temporal activity pattern in *O. gloriosa* possibly shows the interest of further investigation in this direction.

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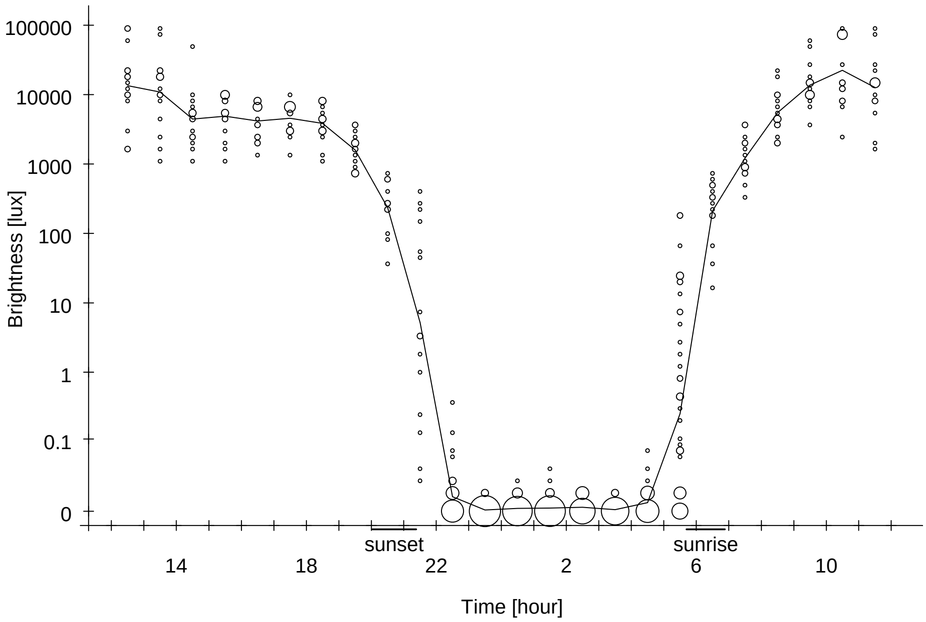
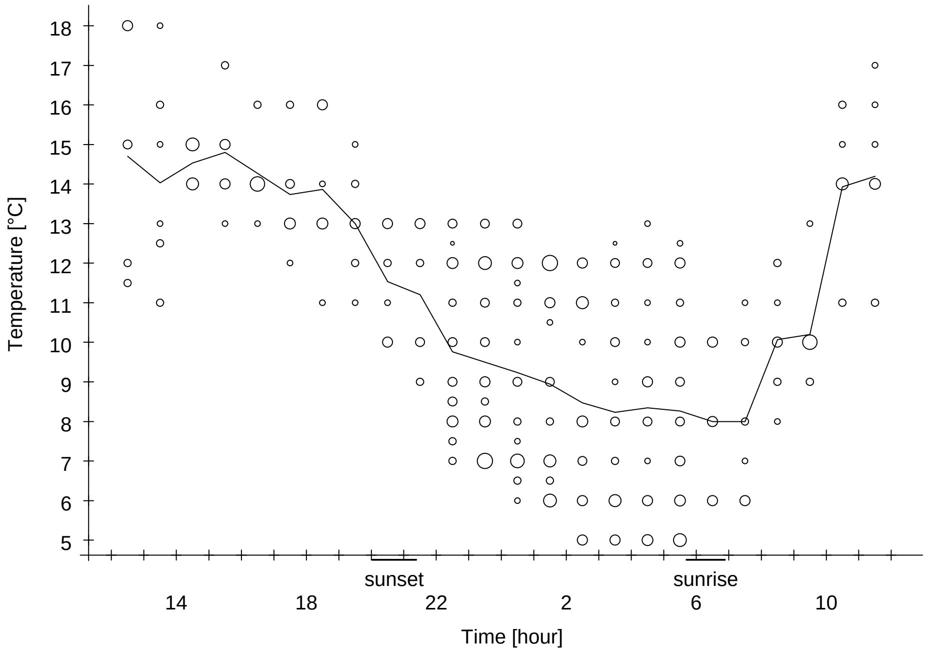
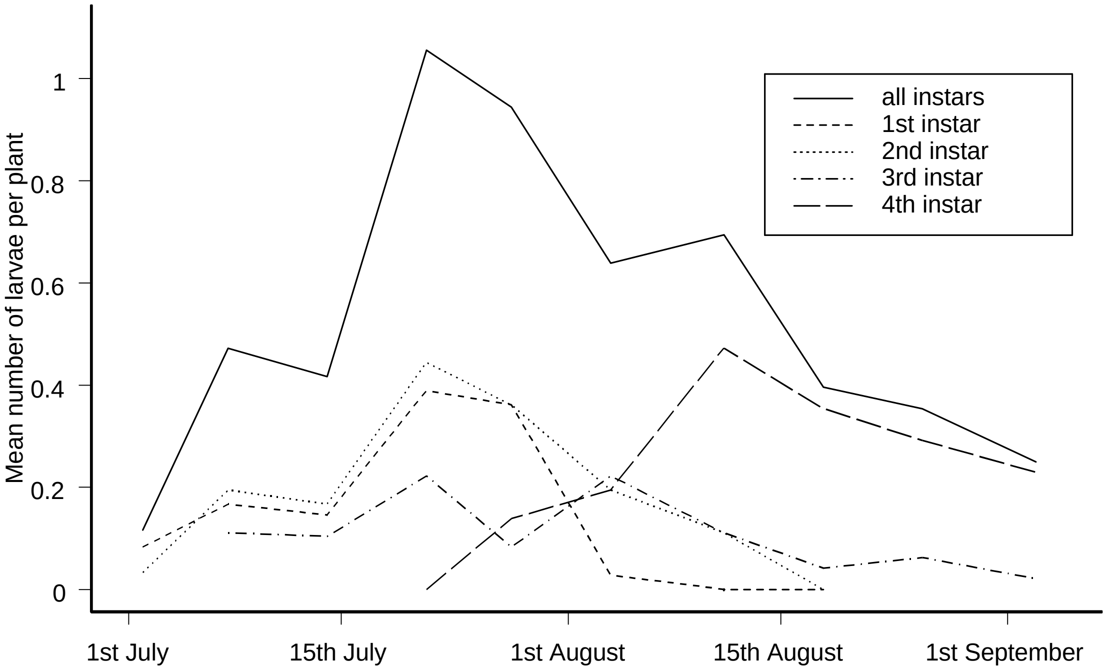
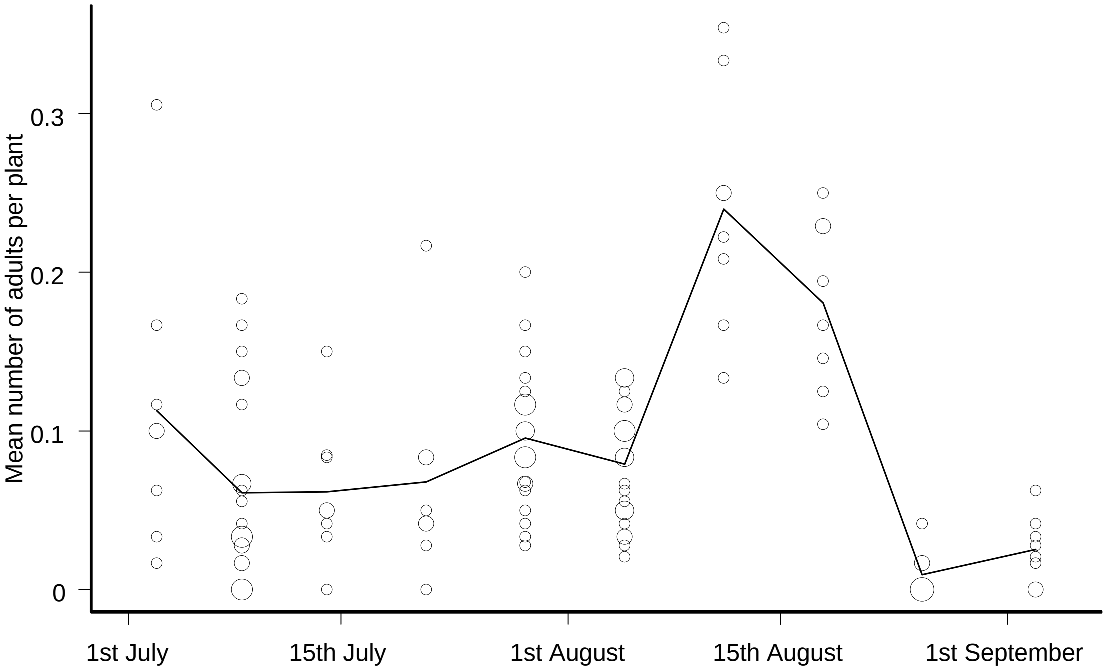


Appendix 1. Brightness at the study site over 24 hours as measured during the 1997 season. The line indicates the mean value. Circles correspond to single measurements. The diameter is a function of the number of observations. Bold lines on the x axis indicate the time of sunset and sunrise.



Appendix 2. Temperature at the study site over 24 hours as measured during the 1997 season. The line indicates the mean value. Circles correspond to single measurements. The diameter is a function of the number of observations. Bold lines on the x axis indicate the time of sunset and sunrise.





Appendix 3. Mean number (lines) of *O. gloriosa* individuals per vegetative plant over the 1997 study season. A, larvae. B, adults. Circles correspond to single inspections. The diameter is a function of the number of observations.

CHAPTER 2

PLANT INTRAPOPULATION VARIATION IN SECONDARY CHEMISTRY MEDIATES HOST SUITABILITY IN THE HIGHLY SPECIALIZED *Oreina gloriosa* (COLEOPTERA: CHRYSOMELIDAE)

Luca Nessi and Martine Rahier

Abstract

The role of plant chemistry in determining the host use in insect-plant relationships is still controversial. Nonetheless, most authors admit that secondary compounds function as a defence device against phytophagous insects. The impact of the intrapopulation variation in the secondary chemistry on host suitability for monophagous insects has been rarely studied. In this work we examined the importance of the linear furanocoumarins as a potential factor governing the distribution pattern of the highly specialist leaf beetle *Oreina gloriosa* in a population of its host plant *Peucedanum ostruthium*. In nature, *O. gloriosa* clearly discriminates between individual plants. *P. ostruthium* produces linear furanocoumarins, compounds that are toxic and/or deterrent to many insects. Samples of plants hosting actively feeding *O. gloriosa* (strongly consumed leaves) and plants exhibiting no damage (unattacked leaves) were collected in the field and examined in the laboratory for their quantitative and qualitative linear furanocoumarin composition. The plant material was extracted and analysed by HPLC, which did not allow definitive identification of the compounds. A canonical discriminant analysis clearly segregated attacked and unattacked plants following the chemical composition. Low correlations between the canonical variable and the chemical variables showed that several compounds simultaneously explain the host-plant use by *O. gloriosa*. We suggest that plant chemistry, relative to *O. gloriosa* ability to tolerate or recognise plant chemicals, can to some extent explain the host use of this narrow monophagous leaf beetle within a population of its host plant. The samples of plants were harvested within different patches, which allowed us to propose some hypotheses about possible genetic variability for linear furanocoumarins synthesis in the studied *P. ostruthium* population.

Key words: *Oreina*, leaf beetle, specialist, *Peucedanum*, Apiaceae, linear furanocoumarins, chemistry, secondary compounds, host use, herbivory.

Introduction

Some authors argue that the plant secondary chemistry is the major force governing host utilization by phytophagous arthropods, either as a proximate cause for plant selection (Renwick and Radke 1987, Pereyra and Bowers 1988, Kolehmainen et al. 1995) and/or as the ultimate cause of the evolution of diet breadth (Berenbaum 1983, 2001, Mitter and Farrell 1991, Becerra 1997, Becerra and Venable 1999, Bernays 2001). Yet, reservations on the preeminence of chemistry in determining patterns of host utilization have been frequently formulated. For example, in respect of plant recognition Jermy et al. (1988) even postulated a random process for *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Several studies have shown attraction of this beetle to volatiles emitted by the potato (*Solanum tuberosum* L., Solanaceae) plant (Jermy et al. 1988, Bolter et al. 1997, Dickens 2000), nonetheless individuals of *L. decemlineata* appeared to "choose" the host by random movement in the field (Jermy et al. 1988). In a recent work, Dickens and Alford (2002) have nevertheless shown that blends of volatiles emitted by potato plants attracted the beetle in field experiments. Jones (1977) showed the main role of plant distribution in the ovipositing preferences of the cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae). On the other hand, with regard to the evolution of host use, for example Courtney and Kibota (1990) suggested that chemistry should only be considered as one of multiple host-specific cues for arthropod herbivores and claimed no necessary connection between these cues and host suitability per se. Bernays and Graham (1988) argued that biotic interactions are more important than host chemistry in determining host range in spite of the preeminent role of chemistry at the behavioural level. As the latter, Price et al. (1986) and Stamp (2001) underlined the possible central role of herbivore natural enemies. This debate opposing the relative importance of plant chemistry and antagonists has lasted a long time and entered into a central topic in the study of insect-plant interactions, namely the evolutionary forces that have led to the predominance of restricted host ranges. At present, it is accepted that a variety of selective forces (e. g. plant chemistry, natural enemies, but also insect size, resource availability, sexual interactions, interspecific competition, environmental variability, plant colour and shape) must be considered as potential determinants of diet breadth (Jaenike 1990, Bernays and Chapman 1994, Schoonhoven 1996, Schoonhoven et al. 1998). When considering the chemistry in studies on host plant use, Dicke (2000) suggested to study also the role of chemical cues from competitors and natural enemies.

The fact that the large majority of phytophagous insects attacks only a fraction of their host plant population is well established (Jermy 1984, Price et al. 1986, Bernays and Graham 1988). Intraspecific differences in host plant chemistry have been cited as at least a partial explanation for intraspecific differences in susceptibility to insect attack (e. g. Pereyra and Bowers 1988, Zangerl and Berenbaum 1993, 2003). However only very few studies have investigated the role of plant chemistry in determining the suitability of herbivore free individuals in a plant population.

At present most authors agree that plant secondary compounds play an important role as a resistance against herbivorous arthropods (Strong et al. 1984, Schoonhoven et al. 1998). At first glance, it seems unlikely that extremely specialized insects are not adapted to the host plant chemistry, for example by means of adequate detoxification enzymes. Several studies have shown the ability of monophagous or oligophagous insects to cope with secondary compounds which are toxic and/or deterrent to polyphagous (Berenbaum 1981, Brower et al. 1988, Chapman et al. 1988, Hsiao 1988, Nitao 1989, Malcom 1991, 1995, Lee and Berenbaum 1993, Pasteels et al. 1994, 1995). But, if Blau et al. (1978) claim that specialized insect herbivores are not affected by secondary chemistry, they admit that chemicals that constitute qualitative toxins to most species can have quantitative effects on adapted ones. Oligophages, presumably well adapted to host plant chemicals, were shown to be adversely affected by high concentrations of some compounds (Siemens and Mitchell-Olds 1996). The specialist monarch butterfly, *Danaus plexippus*, has also been reported to incur a cost to feeding on its host-plants: both the amount of latex produced by the *Asclepias* host species and its cardenolide level adversely affected growth rate and survival of young caterpillars (Zalucki and Malcolm 1999, Zalucki et al. 2001a, b). Angelicin, an angular furanocoumarin of *Pastinaca sativa* (Apiaceae), the wild parsnip, reduces growth rate and fecundity of *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail, a specialist on parsnip leaves and flowering parts (Berenbaum and Feeny 1981). The local specialist *Depressaria pastinacella* (Lepidoptera: Elachistidae) was shown to be sensitive to furanocoumarins occurring in its host plant *P. sativa* (Berenbaum et al. 1986, Berenbaum et al. 1989).

Plants in the family Apiaceae produce linear furanocoumarins, secondary metabolites with well known toxic effects against a variety of organisms (Murray et al. 1982, Berenbaum 1991) and with specific deterrent and/or toxic effects on herbivorous insects (Berenbaum 1978, Muckensturm et al. 1981, Luthria et al. 1989, Hadacek et al. 1994, Berdegué et al. 1997, Reitz and Trumble 1997). Moreover, linear furanocoumarins are well known as phototoxins: when irradiated with ultraviolet light, these compounds enter an excited state which can mediate different toxic processes (Murray et al. 1982, Berenbaum 1991, Arnason et al. 1992). Linear furanocoumarins are benz-2-pyrone compounds with a furan ring fused at the 6,7 positions (figure 1) which is responsible for the toxicity of these compounds. Linear furanocoumarins mainly display their toxicity by binding to DNA bases. This determines disruption of DNA replication and transcription. Photoactivity is due to the formation of an excited triplet state on absorption of a photon. The excited triplet state can also react directly with proteins, lipids or ground state oxygen possibly forming singlet oxygen, hydroxy radicals or superoxide anion radicals. These molecules can react with proteins (enzymes), lipids and unsaturated fatty acids, causing toxicity (Murray et al. 1982, Berenbaum 1991 and references therein). Some authors have shown substantially increased noxious effects on insects in the presence of light (e. g. Berenbaum 1978, Kagan et al. 1986, Reitz and Trumble 1996) and concealed feeding habits (leaf rolling, leaf tying, leaf mining, stem or seed boring) have been considered as adaptive against linear furanocoumarins photoactivation (Berenbaum 1978, Jones and Granett 1982, Griswold and Trumble 1985).

Specialization on furanocoumarins containing plants has mainly led to metabolic adaptation. Linear furanocoumarins metabolization by cytochrome P450 monooxygenases seems to be a widespread means of detoxification (Berenbaum and Zangerl 1994, Berenbaum 1995 and references therein, Hung et al. 1996), also induced after linear furanocoumarins ingestion (Cohen et al. 1989, Nitao 1989).

The leaf beetles of the genus *Oreina* Chevrolat (Coleoptera: Chrysomelidae) feed on plants from several tribes of two different plant families, the Apiaceae and the Asteraceae (Dobler et al. 1996,

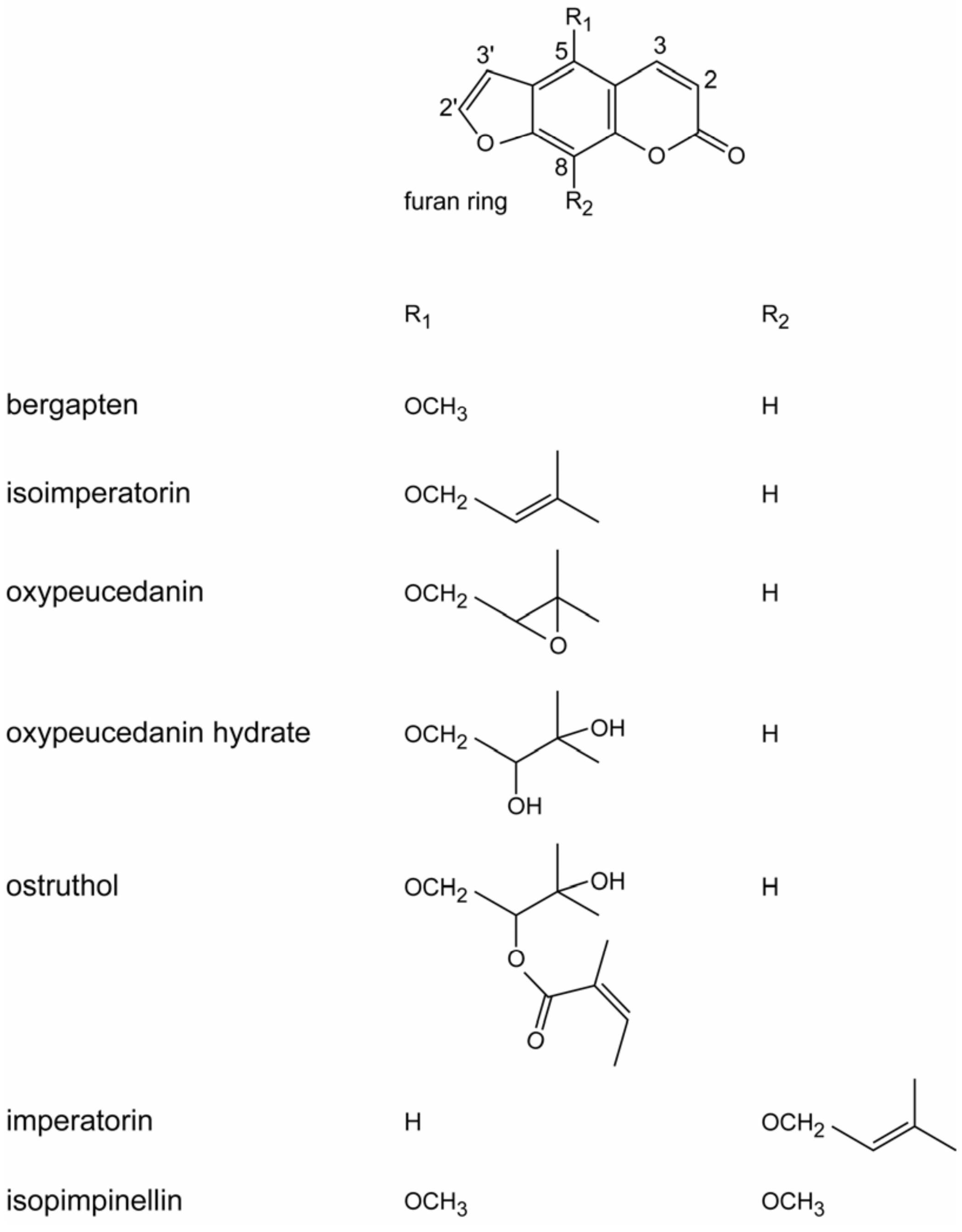


Figure 1. Chemical structure of the linear furanocoumarins found in the leaves of the Saas Balen *P. ostruthium* population.

Hsiao and Pasteels 1999). *Oreina gloriosa* belongs to a group of species within its genus that switched from the original Asteraceae hosts to Apiaceae and shows, only case in its clade, a strict monophagous behaviour on *Peucedanum ostruthium* (L.) Koch (Apiaceae, Peucedaneae), which is known to produce several linear furanocoumarins (List et al. 1967, Hadacek et al. 1994, Hiermann et al. 1996, Schinkovitz et al. 2003). Both the beetle and the plant are common in the Alps mostly at the subalpine and alpine levels. *Peucedanum ostruthium* is frequent in nitrogen-rich meadows and moist gravelly soils on both granite and limestone substrates (List et al. 1967, Lauber and Wagner 1996). Adults and larvae feed exclusively on the leaves and clearly discriminate between individual plants in nature (L. Nessi, pers. obs.). We suggest that plant chemistry, namely (photoactive) linear furanocoumarins, may be a factor explaining actual host plant use within the studied population and the species as a whole. The major role of these secondary metabolites in governing *O. gloriosa* host plant suitability is also suggested by the strict night feeding of the larvae (chapter 1) and by higher mortality of the larvae exposed to visible and ultraviolet light rather than visible light alone (chapter 3). Our study stresses the neglected role of plant intrapopulational chemical variation as an explanation for host selection by highly specialized herbivorous insects. Nonetheless, differential suitability based on chemistry is not mutually exclusive with other potential mechanisms (predation or other biological interactions, microclimatic conditions, ...). We studied a *P. ostruthium* population located in the Swiss Alps. We tested the hypothesis that individual plants remain free from *O. gloriosa* attack due to their chemical attributes by comparing the linear furanocoumarins chemistry (qualitatively and quantitatively) of plants with marks of high larval consumption with that of plants in the same population that were uninfested by the beetles.

The nutritive quality of the plants is known to vary in time as a function of different factors such as the plant phenology or insect attack (Zobel and Brown 1990a, Zangerl 1990, Zangerl and Berenbaum 1990). We examined if *O. gloriosa* larvae respond to these suggested variations by examining the evolution of their numbers on attacked plants over the season.

Genetically based variation in defence characters is the fundamental prerequisite for evolutionary change to occur in a plant population exposed to herbivore selection pressure. Large amounts of phenotypic variation in plant secondary compounds have been described to exist in natural populations (e. g. Berenbaum et al. 1986, von Borstel et al. 1989, Zangerl and Berenbaum 2003), and at least part of this variation has been found to have a genetic base (Berenbaum et al. 1986, Zangerl and Berenbaum 1990, van Dam and Vrieling 1994, Hägele and Rowell-Rahier 1999). *Peucedanum ostruthium* plants grow within patches characterized by a webbed rhizome. Sampling several plants in different patches allowed us to propose some hypotheses about possible genetic variability for linear furanocoumarins synthesis in the studied population of *P. ostruthium.*

Materials and methods

Studied population. The population of *P. ostruthium* is located in the Swiss Alps on the precinct of the village of Saas Balen (Canton Wallis, 46.080 N, 7.570 E) at 1800 m elevation. It extends along a forest roadside in a half-open habitat. The plants grow within patches of one to two square metres. Shoots appear at the end of May and plants wither at mid September. *Oreina gloriosa* is mainly active from mid June to the beginning of September. Adults emerge from overwintering in the ground and start mating at the beginning to mid June. At this time *P. ostruthium* plants have already grown and are widely fed on. Blooming has not yet occurred and flowers are hidden within buds. Most of the plants in the population are vegetative (chapter 1, figure 1C). These plants are just composed of a stem and a leaf. The viviparous *O. gloriosa* lays first instar larvae between the end of June and mid July. Feeding on the leaves, larvae develop to fourth instar by the beginning of September when they burrow into the ground to overwinter.

Plant collection. We collected 30 *P. ostruthium* plants attacked by larvae and 30 unattacked. Each of both 30 plants samples was collected in 5 different patches, 6 plants per patch. Attacked plants came from highly attacked patches and unattacked plants came from unattacked or extremely weakly attacked patches. The 10 sampled patches were located within 700 metres along the forest roadside with a minimum between-patch distance of 10 metres (patches 8 and 9 which were located on both sides of the road). We believe that the rhizome of a selected patch could not extend to another. All patches were similar in size (2 square metres) and were growing in similar environmental conditions (brightness, moisture). All harvested plants were vegetative. The collection took place the 6th of August 1997 when seeds had just been produced in the population. At this time first instar *O. gloriosa* larvae had almost disappeared and fourth instar larvae were already present (see chapter 1, appendix 3A). Plants unattacked by larvae may have shown little damage caused by adult beetles. The percentage of the leaf surface lacking because of larval consumption was estimated and recorded. The plants were cut, put into dark paper bags and stored in a barn close to the field site where they dried. The dried plants were than transported to the laboratory (where they were kept at room temperature) for extraction and chemical analysis.

Extraction and chemical analysis. Extraction and chemical analysis methods were inspired by the study of Hadacek et al. (1994) who investigated the linear furanocoumarins diversity within the genus *Peucedanum*. The definitive methodology was decided after personal advise of Hadacek and coworkers at the University of Vienna. We quantified the linear furanocoumarins from the leaf powder. We powdered the leaf (without the petiole) of each plant with a centrifuge mill and stored the powder in plastic bags, shaded, until extraction. For the extraction we weighed 25 mg (± 0.2 mg) powder into a glass tube and added 4 ml hexane. We closed the tube and stirred it at 420 revolutions/min for a two hours period. The extract was then filtered (LS 14 1/2 filter papers) into a rotavapor flask. The extraction tube was rinsed out through the filter paper into the flask with 1 ml hexane. The hexane extracts were then dried with a rotavapor. The residues were dissolved in 2 ml

HPLC quality methanol (MeOH) and the solutions were transferred into 11 ml brown glass vials. The extracts were concentrated: first the vials were dried and then 500 µl HPLC quality MeOH were added. For the high performance liquid chromatography analysis we prepared a 2 ml brown glass sample vial with 100 µl of a 5-methoxypsoralen (bergapten, see figure 1) solution (0.1 mg/ml MeOH) as internal standard. After our analyses were completed, Perret (2000) identified bergapten in *P. ostruthium* leaves from Saas Balen. She only found traces of this compound, we therefore believe that its utilisation as internal standard remains acceptable. After evaporation of the solvent out of the 2 ml vial we transferred the 500 µl extract solution into it through a reversed phase packing C18 tube (Supelclean™ ENVI™-18 SPE Tube, 1ml) for a fine filtration. For compound separation we used a reversed phase column (Chrompack, Hypersil BDS C18, 5 µm, 250 x 4.6 mm) on a Varian HPLC system. The column was thermostatted at 40°C. The mobile phase was MeOH and an aqueous buffer (*o*-phosphoric acid 0.015 mol, tetrabutylammonium hydroxide 0.0015 mol, pH 3). The elution gradient was: 0 - 22 min MeOH 50 - 70%, 22 - 25 min MeOH 70 - 80%, 25 - 30 min MeOH 80%, 30 - 36 min MeOH 80 - 100%, 36 - 43 MeOH 100%, flow rate 1 ml/min. 20 µl of solution were injected by the autosampler and the peak area was determined from the 229 nm chromatogram with the Varian Star Chromatography Workstation software, version 4.0 (Varian Inc. 1999). Thirteen substances were identified as linear furanocoumarins but precise identification was not possible. The analysis on the Varian device appeared to be more discriminant than that realized in Vienna since single peaks were split in two or even more peaks. Perret (2000) further investigated the linear furanocoumarins chemistry of *P. ostruthium* from Saas Balen. Her work could not be exhaustive and produced results in contrast with both the data obtained in Vienna and on the Varian workstation. We selected thirteen peaks that presented retention times and absorbance spectra very close to the substances identified in Vienna. The substances were denoted with alphabetical letters (from A to M) following the retention time. We could not calculate the amount of the thirteen linear furanocoumarins in the sample. We expressed the amount as bergapten equivalents. The linear furanocoumarins composition of *P. ostruthium* is nonetheless well known. Investigations mainly concentrated on roots. The plant is reported to contain isoimperatorin, (±)-oxypeucedanin, (+)-oxypeucedanin hydrate, (+)-ostruthol, imperatorin, 3'-acetate of oxypeucedanin hydrate (List et al. 1967, Hadacek et al. 1994, Hiermann et al. 1996, Schinkovitz et al. 2003).

Larvae on attacked plants. The presence of the larvae of *O. gloriosa* on its host plant and the extent of the consumption of the leaves was studied during the summer 1997. We selected at different locations within the described *P. ostruthium* population 60 different vegetative plants 10 times along the season (July 2, July 8, July 15, July 22, July 28, August 4, August 12, August 19, August 26, September 4). The selected plants were vegetative individuals presenting signs of *O. gloriosa* larvae consumption. We have thus selected the plants according to their observed suitability for *O. gloriosa*. At each observation date the plants were inspected once each hour for a 8-hr period within 2 days, between 10 p.m. of day 1 and 6 a.m. of day 2, which corresponds to the period of highest larval activity (chapter 1). At each inspection we noted the number of larvae of each instar.

Prior to the inspections we estimated and recorded the percentage of the leaf area consumed.

Statistical analyses. Two related techniques of multivariate statistics were performed to visually separate groups of plants from their traits, namely a canonical discriminant analysis (CDA, also cluster analysis) and a principal component analysis (PCA). Given the damage status (classification variable) and the concentrations of the different linear furanocoumarins (quantitative variables) the CDA derives canonical variables (linear combinations of the quantitative variables) that summarize between-class variation. In much the same way, PCA derives principal components that summarize total variation. In both analyses the classification variable had a binomial form, i. e. undamaged versus damaged. Both analyses were computed with the SAS release 8.2 by SAS Institute Inc. (1999). To predict consumption considered as a continuous variable (leaf area lacking) we performed a stepwise multiple regression analysis of the chemical variables using S-Plus 6 by Insightful Corporation (2001).

To study the variation of the number of larvae on attacked plants over the season we first grouped the data (eight inspections) of each observation for each plant. Thus, for each of the 10 observations dates and each plant we got one total number for each instar. We computed a linear multiple regression analysis of the time past since the beginning of the season and the total number of each instar larvae with consumed leaf area (expressed as the log transformed-percentage of total area) as the dependent variable. The analysis was performed using S-Plus 6 by Insightful Corporation (2001).

Results

Figure 2 shows a representative chromatographic profile of leaves of the Saas Balen *P. ostruthium* population. In table 1 we compiled the interpretations of the profile following the analysis in our laboratory, in Hadacek's laboratory at the University of Vienna, and in Perret's (2000) work.

As shown by the figure 3A the cluster analysis (Canonical discriminant analysis, CDA) based on the concentrations of the thirteen linear furanocoumarins allowed to clearly segregate attacked and unattacked plants. The canonical correlation was 0.786 ($p = 0.0001$). Only one plant within patch 1 (unattacked) was characterized by a chemistry completely fitting with that of attacked patches and only few plants of both attacked and unattacked patches were close to zero on the canonical axis.

Patch 3 was characterized by a very particular chemistry. As shown by figure 4A, the comparison of the samples by principal component analysis (PCA) shows that patch 3 plants are located far away from the others and are strongly characterized by the first principal component, which is positively correlated with substances H, C and A (figure 4B). The correlations between these three chemicals and the first principal component are 0.921, 0.910 and 0.906 respectively. Because of its eccentric chemistry, we left this patch out of the subsequent analyses.

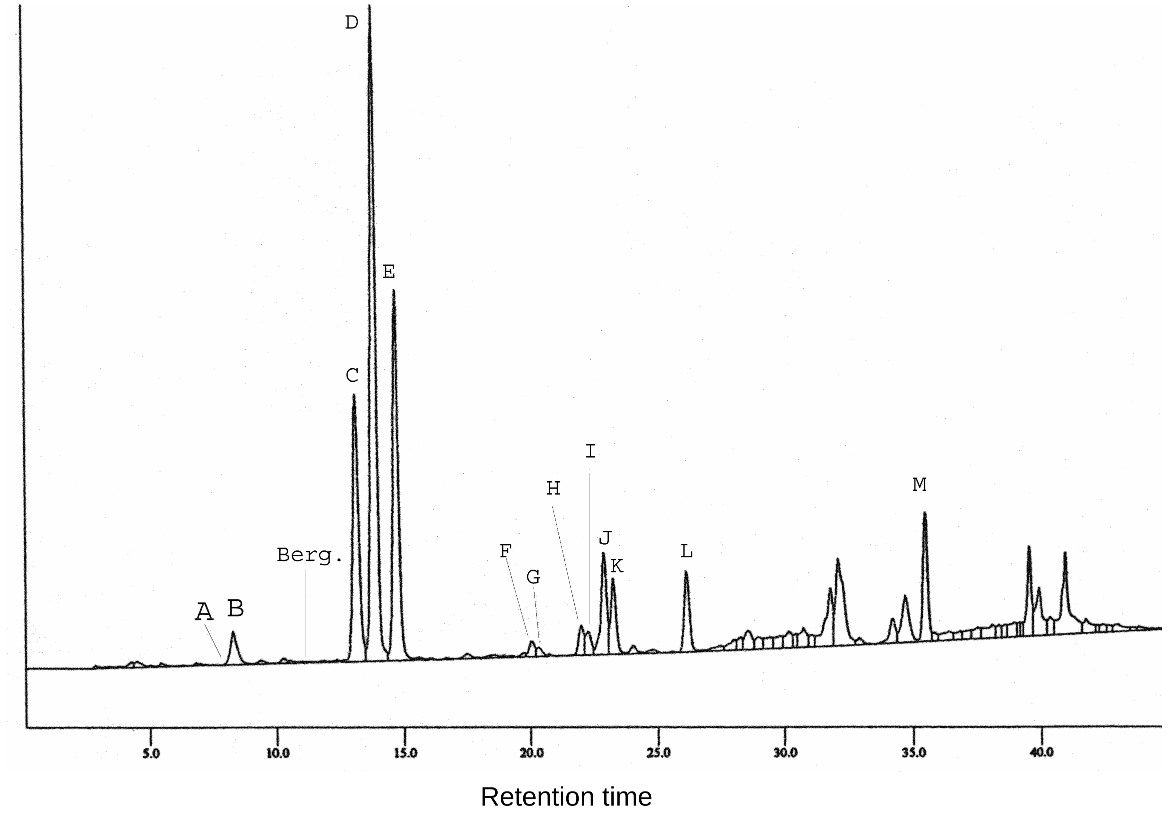


Figure 2. Representative chromatogram of leaves of *P. ostruthium*. This profile was obtained with a mixture of plant material collected in summer 1996. We noted the position of compound A and bergapten (Berg., internal standard), both absent in the sample.

Table 1. Interpretation of the chromatographic profile of leaves of the studied *P. ostruthium* population following the analysis in our laboratory, in Hadacek's laboratory in Vienna, and in Perret's (2000) work. Letters in the first column ("our analysis") refer to the profile shown in figure 2.

our analysis	Hadacek's analysis	Perret's analysis
A to B region	one peak: oxypeucedanin hydrate	unidentified compound unidentified linear furanocoumarin
bergapten region	no peaks	isopimpinellin and bergapten (traces)
C	isopimpinellin	unidentified linear furanocoumarin
D to E region	one peak: oxypeucedanin	oxypeucedanin unidentified linear furanocoumarin
F to G region	one peak: ostruthol	imperatorin unidentified linear furanocoumarin
H to K region	one peak: demethylsuberosin	unidentified linear furanocoumarin unidentified compound unidentified compound unidentified compound
L	isoimperatorin	isoimperatorin
M	aurapten	unidentified compound

Figure 3B shows the distribution of attacked and unattacked plants after cluster analysis excluding patch 3 data: the result is very similar to that obtained for all plants, i. e. good segregation (canonical correlation 0.776, p = 0.0002) of damaged and undamaged samples. None of the chemical variables strongly characterizes the canonical variable. B is the first variable fitting the model but the Pearson correlation coefficient between the canonical variable and B is only -0.432 (p = 0.002). Appendices 2 and 3 show the between linear furanocoumarins correlations. High correlation can possibly indicate the sharing of biosynthetic pathways.

Consistently with the results of the canonical discriminant analysis, B also explained the highest amount of variance of consumed leaf surface in a stepwise multiple regression model (table 2). The canonical model is logistic since the dependent variable was the state of the plant, i. e. attacked versus unattacked. On the contrary, in the regression model the dependent variable was continuous as it was expressed as percentage of consumed leaf area. A significant overall regression accounts for a relatively low amount, 55.74%, of the variation in leaf damage.

Larvae seem to be conservative in respect of the host plant they eat. In the field, plants fed on at the beginning of the season still were consumed at the end (L. Nessi, pers. obs.). Regression of consumed leaf area against time past since the beginning of the season and the total number of each instar larvae (table 3) revealed that damaged surface significantly increased with time, 4th and 3rd instar larvae (variables are cited in the order of decreasing significance). This result agrees with the well known pattern of old larvae having greater impact on host plant than young ones. Growth between successive instars is logarithmic and usually over 60% of the total food required for larval development is consumed during the final instar (Hodkinson 1982). The multiple linear regression model states at least the conservative feeding habits across the older stages as observed in nature, which means that damaged plants remain suitable for third and fourth instar larvae.

The content of the linear furanocoumarins also allowed to discriminate patches. Figure 5 shows the result of a cluster analysis (CDA) without patch 3 plants. When used for the computation, these samples resulted strongly correlated to the first canonical axis and far away from all other patches. Their exclusion allows better segregation of all other samples. The patches clustering contrasts with the results of the canonical analysis concerning damage status. For example, patch 4, attacked, is strongly positively correlated on the first canonical axis with patches 9 and 10, unattacked. In contrast, it is strongly negatively correlated with patches 5 and 6, both attacked. In fact, the first canonical axis is only weakly correlated with all of the chemicals variables. The higher correlation is shown by compound B with a Pearson correlation coefficient of only -0.33, p = 0.02.

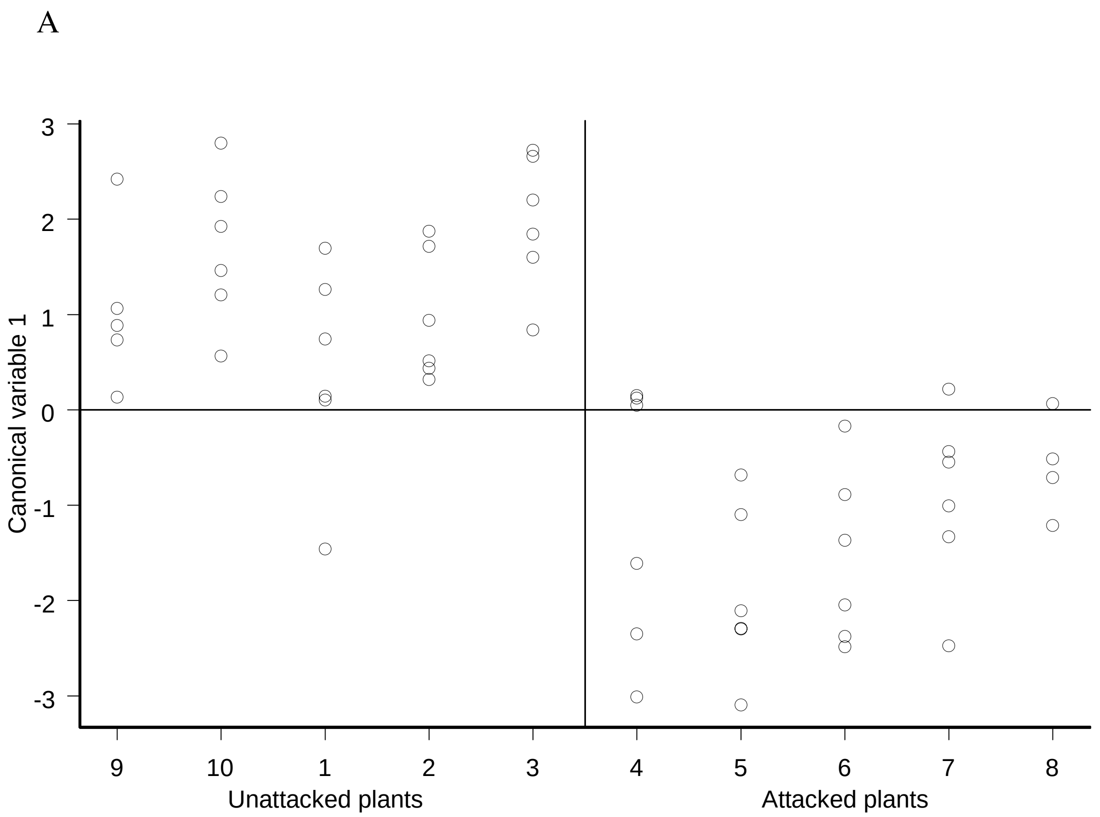


Figure 3. Distribution of attacked and unattacked plants after cluster analysis based on linear furanocoumarins concentrations. Numbers on the x-axis denote the patches the plants belong to. A, plants of all patches (canonical correlation 0.786, p = 0.0001). B, plants of patch 3 not considered (canonical correlation 0.776, p = 0.0002).

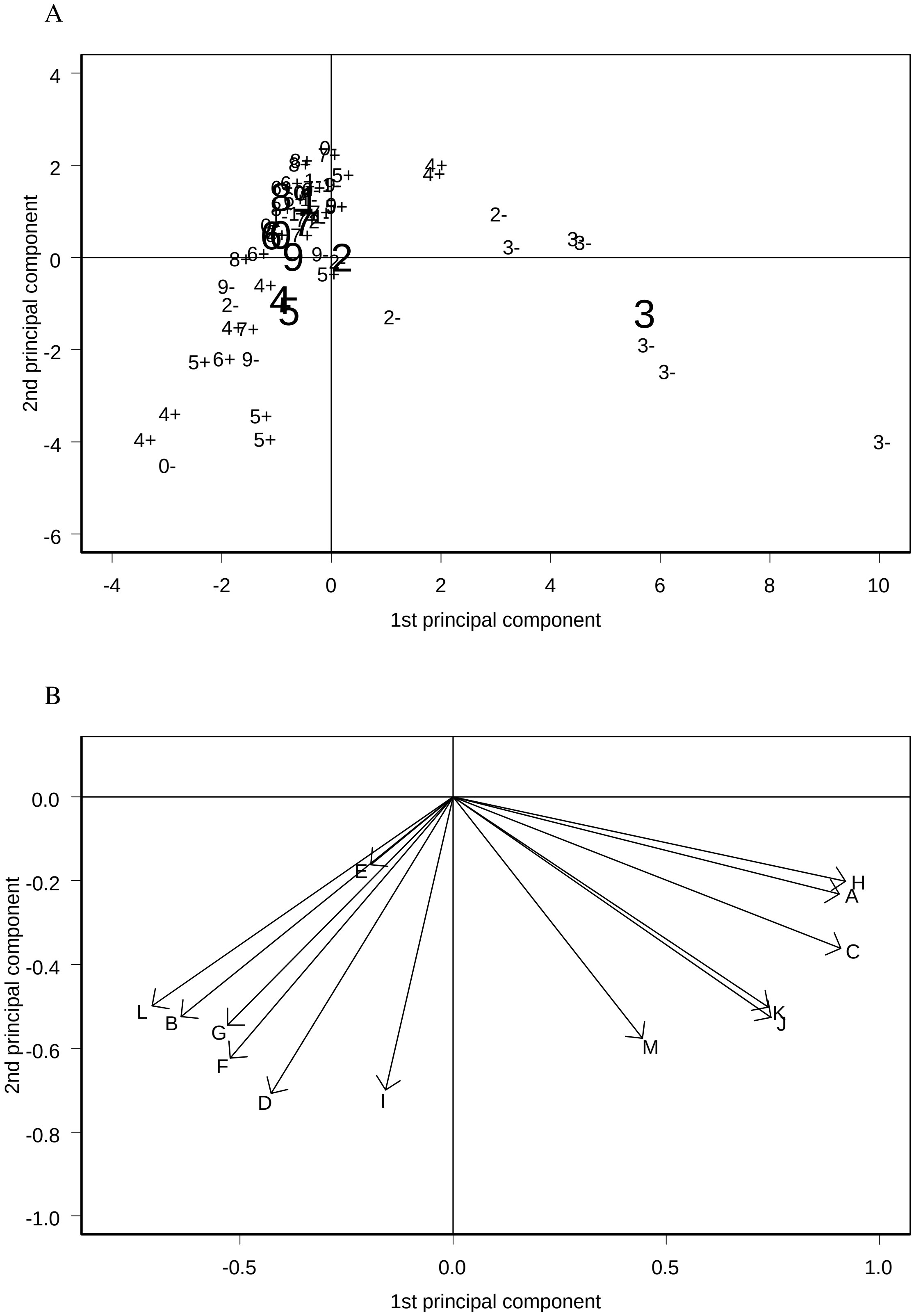


Figure 4. Distribution of the plants (A) and of the thirteen linear furanocoumarins (B) after principal component analysis based on the chemicals concentrations. Numbers denote the patches (patch 10 = 0). Signs + and - denote attacked and unattacked plants. Large numbers denote the geometrical average of the patches. First and 2nd principal component explain 42% and 25% of the variance.

Table 2. Stepwise multiple regression analysis of the concentrations of thirteen linear furanocoumarins with consumed leaf area (expressed as percentage of total area) as the dependent variable.

Variables	regression coefficient	SE	t	p	increase in variance explained (%)
B	438.043	72.427	6.048	< 0.001	27.8
G	-108.401	25.13	-4.314	< 0.001	8.95
I	283.733	63.008	4.503	< 0.001	14.85
M	-18.042	8.185	-2.204	0.032	4.14

Statistic for the model : d.f. = 4, 52, F = 16.37, p = 9.6 e^{-9}, 55.74% of variance explained.

Table 3. Linear multiple regression analysis of time past since the beginning of the season and the total numbers of each instar larvae with consumed leaf area (expressed as log–transformed percentage of total area) as the dependent variable.

Variables	regression coefficient	SE	t	p
time	0.029	0.002	14.428	< 0.001
3rd instar larvae	0.286	0.096	2.988	0.003
4th instar larvae	0.42	0.078	5.375	< 0.001

Statistic for the model: d.f. = 5, 521, F = 70.31, p = 0, 40.29% of variance explained. Total numbers of 1st and 2nd instar larvae failed to meet 0.5 significance level for entring into the model.

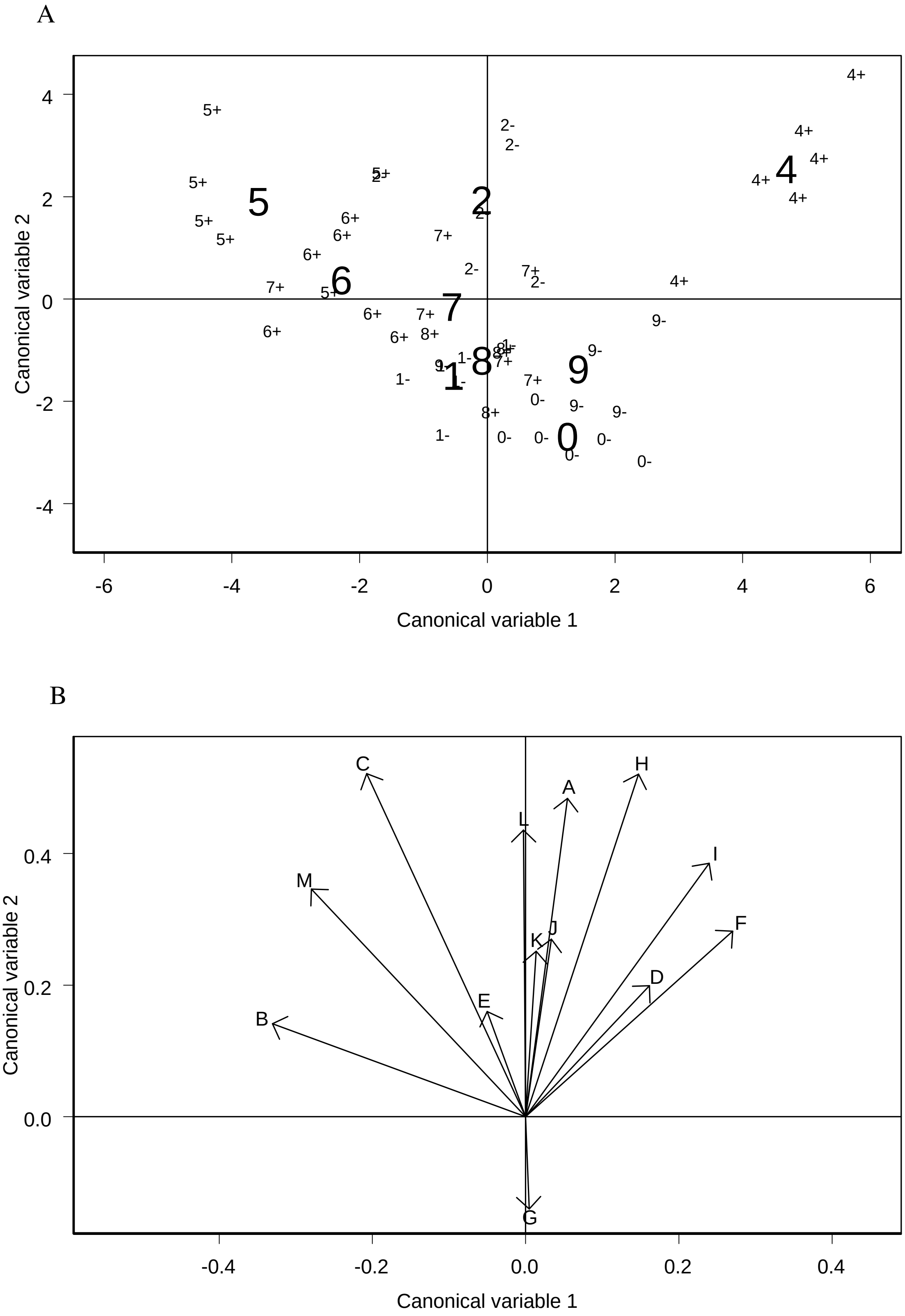


Figure 5. Distribution of the patches (all but patch 3) (A) and of the thirteen linear furanocoumarins (B) after cluster analysis based on the chemicals concentrations. Numbers denote the patches (patch 10 = 0). Signs + and - denote attacked and unattacked plants. Large numbers denote the geometrical average of the patches. The canonical correlations for the first and the second axis are respectively 0.929 (p = 0.0001) and 0.888 (p = 0.0001). The other canonical variables do not meet significance.

Discussion

Damaged and undamaged leaves differ in their content of linear furanocoumarins. Individuals of *O. gloriosa* clearly discriminate between host-plant patches in the field. At the behavioural level, some of the linear furanocoumarins found in *P. ostruthium* possibly act as sign stimuli ("token stimuli") for the monophagous *O. gloriosa*, i. e. they drive the larviposition and feeding of the beetle. In extreme specialists, known for their "fine tuning" ability (Bernays 2001, but see Bernays et al. 2003), great sensitivity to one or a few host-specific chemicals appears to result in such compounds being the dominant factor in host selection (Städler and Buser 1984, Pereyra and Bowers 1988, Hurter et al. 1999). Plant secondary metabolites, volatile and nonvolatile ones, have been shown of great importance for identification of the host by specialized insects (Städler 1992, 2002, Bernays and Chapman 1994). For example, linear furanocoumarins have also been described as oviposition stimulants (Städler and Buser 1984, Stanjek et al. 1997, Degen et al. 1999). There may be interactions among linear furanocoumarins (and with other primary and secondary compounds) that may provide cues for *O. gloriosa* to consume plants of a particular patch. Indeed, the beetle possibly recognises a mixture of compounds, as it happens for many if not all herbivorous insects including oligophagous species (Degen and Städler 1998, Städler et al. 2002) and specialists (Städler 1992, 2002). Nutritional factors may also play a central role beside secondary chemicals in host selection (Cates et al. 1987, Derridj et al. 1987, Hard 1987).

If the hypothesis of a signal function is true, then it has to be coupled with a relative lack of inhibitory effects of these chemicals for *O. gloriosa*. Nonetheless, linear furanocoumarins are secondary metabolites with known insecticidal properties. Hadacek et al. (1994) have shown that all but one linear furanocoumarin isolated from roots of *P. ostruthium*, namely imperatorin, isoimperatorin, (±)-oxypeucedanin, (+)-oxypeucedanin hydrate, inhibited the growth of the polyphagous *Spodoptera littoralis* (Lepidoptera: Noctuidae). This led Hadacek et al. (1994) to state: "these results confirm the classification of linear furanocoumarins, as they can be found in *P. ostruthium* (...) as potent allomones against herbivores in broad sense". Little is known about herbivores, either specialists or generalists, associated with *Peucedanum*. In Saas Balen besides *O. gloriosa* no other herbivores but an unidentified species of *Cercops* (Homoptera: Cercopidae) has been observed. *Peucedanum palustre*, *P. cervaria*, and *P. oreoselinum* are known as a food plant of *Papilio machaon* (Lepidoptera: Papilionidae) (Ligue suisse pour la protection de la nature 1987). The lack of information on the herbivore community feeding on these plant species difficults the drawing of conclusions on the plants suitability, as it has been proposed by Berenbaum (1981).

The toxic properties of these secondary metabolites have been shown for many herbivorous insects, but almost only polyphagous ones. We nevertheless suggest that intraspecific differences in *P. ostruthium* chemistry at least partially explain the differences in susceptibility to *O. gloriosa* attack. Other examples of specialized insects sensible to the host plant chemicals are rare, particularly among Apiaceae feeders, but the linear furanocoumarin isopimpinellin is known to defend the wild parsnip, *P. sativa*, from the locally specialized parsnip webworm, *D. pastinacella* (Berenbaum et al. 1986). High concentrations of two linear furanocoumarins, xanthotoxin, bergapten, and an angular furanocoumarin, sphondin, significantly reduced growth and consumption rates of its ultimate instar larvae (Zangerl and Berenbaum 1993).

The hypothesis that linear furanocoumarins represent toxic substances to *O. gloriosa* is consistent with the activity rhythm of the beetle, since larvae are known to feed exclusively at night (chapter 1). Adults feed also at daylight, but are possibly protected from ultraviolet radiation by the thick and coloured cuticle. Moreover, larval performance was significantly reduced for beetles fed *P. ostruthium* in presence of ultraviolet light as compared to a control without this radiation (chapter 3). The proposed biochemical mechanism of toxicity through light is also consistent with the ecology of the studied *P. ostruthium* population. The patches develop on a forestry roadside, i. e. a quite open habitat with sunlight easily reaching the soil. In Apiaceae linear furanocoumarins are known to occur on or near leaf surfaces (Städler and Buser 1984, Zobel and Brown 1990b, Stanjek et al. 1997): this distribution may be seen as a means of enhancing photoactivation.

The effect of linear furanocoumarin was shown to increase with increasing concentration in the fed artificial diet for herbivorous insects. Older larvae of the celery (*Apium graveolens*, Apiaceae) pest *Spodoptera exigua* are deterred from feeding by high levels of linear furanocoumarins found in older foliage of celery (Berdegué et al. 1997), but they show no such sensitivity to the lower concentrations found in the petioles (Berdegué et al. 1997) where the older larvae tend to feed (Griswold and Trumble 1985). In addition, low concentrations of linear furanocoumarins do not impact on development and survival of *S. exigua* (Brewer et al. 1995, Reitz and Trumble 1997). Moreover, increasing concentrations of linear furanocoumarins (psoralen, bergapten and xanthotoxin) increased *S. exigua* larvae mortality (Brewer et al. 1995, Reitz and Trumble 1997, but see Diawara 1993). If this pattern also fits *O. gloriosa*, then the most inhibitory and/or toxic substance(s) should be searched among compounds G and M which are negatively correlated with larval consumption (table 2). Linear furanocoumarins in mixture are known to be more toxic than isolated compounds. Feeding deterrency, development and survival of *S. exigua* larvae were more affected when exposed to combined psoralen, xanthotoxin, and bergapten (Brewer et al. 1995, Berdegué et al. 1997, but see Diawara 1993). Synergism was also shown for the mixture of xanthotoxin, bergapten and the angular furanocoumarin angelicin in the diet offered *P. polyxenes* (Berenbaum and Zangerl 1993). Synergistic interactions have also been demonstrated against *Heliothis zea* (Lepidoptera: Noctuidae) between the five furanocoumarins (the four linear xanthotoxin, bergapten, imperatorin, isopimpinellin and the angular angelicin) that naturally co-occur in fruits of *P. sativa* (Berenbaum et al. 1991). The importance of synergism has also been denied. Increased dietary concentrations of each psoralen, xanthotoxin, and bergapten significantly induced higher *S. exigua* mortality, but some combinations of these linear furanocoumarins resulted in significantly antagonistic effects on survival. Diawara et al. (1993) suggested that combining the different substances could increase feeding deterrency. In view of these examples, one can state that more than a single compound should play a role in host use by *O. gloriosa*. This seems consistent with the fact that the correlation is weak between the chemical variables and the canonical variable in the discriminant analysis about damaged and undamaged plants. Anyway, caution is needed since in nature insect response to secondary compounds varies as a function of different factors such as insect physiological state, biological interactions or environmental variability (Schoonhoven et al. 1998).

Plant secondary compounds are not uniformly distributed through leaf tissue (Schoonhoven et al. 1998, Städler 2002). Within the Apiaceae Camm et al. (1976) and Eckey-Kaltenbach et al. (1994) report furanocoumarins being localized in the leaf oil ducts. Zangerl and Bazzaz (1992) showed that half of all the furanocoumarins in a leaflet of *P. sativa* is located in the primary vein and one third is in the secondary veins. Possibly *O. gloriosa* partially reduces additive and/or synergistic toxic impact of furanocoumarins by selectively feeding on parts of the leaves. Indeed, the four instar larvae and, to a lesser degree, the adults eat around the veins (L. Nessi, pers. obs.). Selective feeding on less or non-toxic plant parts is well known. For example, first instar larvae of the polyphagous *Trichoplusia ni* (Lepidoptera: Noctuidae) avoid even the smallest veins of wild parsnip, *P. sativa*. Later instars, unable to avoid the smallest veins, continue to avoid large ones. Stronger toughness of the veins tissue in comparison to the rest of the leaf was suggested to be an inadequate explanation for the avoiding behaviour (Zangerl and Bazzaz 1992). Young larvae of *Ostrinia nubilalis* (Lepidoptera: Crambidae) feed on leaves but avoid sites with high levels of phototoxins as resin canals and glands of its asteraceous hosts (Champagne et al. 1986). Sucking insects are able to avoid furanocoumarins by selectively feeding on plant tissues with low concentration or lack of toxins. *Lygus lineolaris* (Miridae) feeds on embryos of umbelliferous seeds to avoid vittae and oil glands (Flemion and McNear 1951 in Berenbaum 1990). The aphids *Aphis heraclella* and *Cavariella pastinacae* were able to avoid xanthotoxin when hold on cut leaves put in a solution of this compound (Camm et al. 1976). Polyphagous caterpillars were also shown to actively reduce the defence potential of plants containing toxic metabolites. Dussourd and Denno (1994) suggested that the noctuids *Anagrapha falcifera* and *Autographa precationis* can normally grow on *Petroselinum crispum* (Apiaceae) due to their leaf trenching ability. We are aware that determining the leaf chemistry irrespective to tissue parts may arouse criticism. Nevertheless, it should be remembered that finding the amounts of chemicals effectively encountered by arthropod herbivores or pathogens is a very difficult task. For example, Eckey-Kaltenbach et al. (1994) report fungal attack leading to furanocoumarin accumulation near the infection sites and to secretion of the metabolites into droplets. These examples show the importance of considering scale in studies on host plant use.

It is well known that the production of allelochemicals in plant tissues is affected by developmental and environmental factors. For example, furanocoumarin content in umbelliferous species has been shown to vary with the season (Zobel and Brown 1990a) and with light and nutrition (Zangerl and Berenbaum 1987). Likewise, changes in furanocoumarin content may arise in response to stress by ultraviolet radiation (Beier and Oertli 1983, McCloud et al. 1992, McCloud and Berenbaum 1994, Masuda et al. 1998), low temperatures (Beier and Oertli 1983), ozone (Eckey-Kaltenbach et al. 1994), pathogen attack (Heath-Pagliuso et al. 1992, Van Gijsegem et al. 1995, Masuda et al. 1998) and insect feeding (Zangerl 1990, Zangerl and Berenbaum 1990). Therefore, it is also probable that the damage inflicted to the *P. ostruthium* plants by *O. gloriosa* induced linear furanocoumarin production and may even have determined most of the observed chemical difference between damaged and undamaged patches. However, this seems of secondary relevance here since the highly mobile larvae do not leave the attacked plants for less damaged ones. Even if the different chemistries of attacked and unattacked plants resulted from *O. gloriosa* feeding, damaged plants still are recognised as more useful. Larvae of different instars feed on the same leaf in the field (L. Nessi, pers. obs.): responses to the plant chemistry may be more or less consistent across instars, contrary to what has been observed for different *Spodoptera* species with regard to different linear furanocoumarins (Berdegué et al. 1997). Alternatively, the experience of the induced furanocoumarins may result in adaptation, habituation and/or induction of preference for the larvae (Bernays and Chapman 1994, Schoonhoven et al. 1998).

Young leaves, more abundant in the field when first instar *O. gloriosa* larvae appear, are known to contain higher constitutive levels of furanocoumarins than older ones (Zobel and Brown 1990a, b, McCloud et al. 1992, Asthana et al. 1993). Feeding induction possibly maintains high chemical concentrations that all instar larvae have to be able to manage.

The optimal defence theory predicts that plant tissues that are unlikely to be attacked should have low constitutive amounts of defence and high inducibility, while tissues with similar fitness value that are likely to be attacked should have high levels of constitutive defence and low inducibility (McKey 1974, 1979, Rhoades 1979 in Zangerl and Rutledge 1996). Following this scenario the *P. ostruthium* leaves possibly present consistent concentrations of secondary chemicals over plant development so that all instars of *O. gloriosa* larvae encounter similar furanocoumarin concentrations.

It is highly likely that fourth instar larvae overwinter in the soil very close to the plants they have fed on. Since pupation takes place where larvae overwinter (L. Nessi, pers. obs.), young adults possibly select as food and/or larvipositing plant *P. ostruthium* individuals they experienced as larvae the year before. The possibility that some learning behaviour is associated with host selection and use thus has to be considered. Such a memory effect may result from a strict preimaginal chemical conditioning (i. e. the Hopkins' host-selection principle *sensu strictu*) as it has been described for dipteran flies (Tully et al. 1994, Ray 1999) and curculionid beetles (Rietdorf and Steidle 2002). But the restricted preference for some plants may also result from other mechanisms, possibly acting synergistically, as genetic or inherited environmental effects (Barron 2001) or imaginal chemical conditioning (Corbet 1985) which has frequently been described (Barron 2001 and references therein). We may also consider the selection of the host-plant patches partially as the result of the apparent little dispersion of *O. gloriosa*. In any case, as already suggested by several authors (e. g. Wiklund 1981, Courtney and Kibota 1990, Jallow and Zalucki 1998) who claimed the centrality of oviposition choices in insect plant relationships, *O. gloriosa* females may play an important role in host plant choice and use.

The phenotypic differences between patches in linear furanocoumarins content possibly has genetic bases. The *P. ostruthium* studied plants grow in patches with a very thick rhizome. Collection of the plants in nature showed that different stems within a patch arise from the same root (L. Nessi, pers. obs.) so that it seems likely that a patch is made up of a single or a few genotypes. Patches from which plants were harvested for chemical analysis were located far away from each another, which makes unlikely any genetic identity between them. If this pattern is true, this *P. ostruthium* population may harbour genetic variability for linear furanocoumarins expression and thus the potential for response to selection may be present. We lack any reliable information about the pressure herbivores exert on the fitness of the studied population of *P. ostruthium*. Nonetheless the patches attacked by *O. gloriosa* may undergo an intensive defoliation before fruits become ripe. Very little leaf damage was shown to massively reduce plant growth (Zangerl et al. 1997) so that we can not exclude a great impact on *P. ostruthium*. Van der Meijden (1996) suggested genetic polymorphism for plant defences as a result of differential selection by specialist and generalist herbivores, but specialists alone were shown to constitute a strong evolutionary force. For example, the local specialist *D. pastinacella* maintains genetically based variability (significantly additive) in linear furanocoumarins expression in its host, *P. sativa*. In this case polymorphism results from the negative genetic correlation between defensive and fitness characters (Berenbaum et al. 1986). The insects can act as a selective agent on *P. sativa* chemistry (Berenbaum et al. 1986) and in turn the plant furanocoumarin chemistry can act as a selective agent on the webworm's (*D. pastinacella*) detoxification metabolism, which is also characterized by continuous heritable variation (Zangerl and Berenbaum 1993), in a coevolutionary scenario which fits at least some interacting insect and plant populations (Zangerl and Berenbaum 2003). Any conclusion about *O. gloriosa* evolutionary pressure on the studied *P. ostruthium* population would be risky. The evolution of the linear furanocoumarins defence, if one, possibly occurred also under selection pressure from other factors as pathogens, other herbivores or even allelopathic protection against other plants. In fact, the cluster analysis allowed to separate the patches but the segregation was not primarily attributable to one or more chemical variables which allowed to group the patches as a function of their damage status.

Patch 3 showed a very particular chemistry. We do not have any definitive explanation for this pattern. Differences in comparison with the other sampled patches may arise from genetic and/or environmental characteristics. However, the latter were not detectable when the plants were harvested, since patch 3 appeared to be similar to the others in respect of brightness, moisture and soil surface.

In conclusion, we suggest that plant chemistry, relative to larval ability to tolerate some linear furanocoumarins and/or to recognise some of them as stimulants, can in part - 55.7% of the variation in leaf damage is explained by a stepwise regression - explain the distribution of this insect within a population of its host plant. Differential suitability based on chemistry is not mutually exclusive with other possible mechanisms (e. g., microclimatic and soil conditions, natural enemies and competitors, vegetation arrangement, plant phenology, microorganisms) but the linear furanocoumarin chemistry possibly contributes to differential attack rates even if other mechanisms are operative. In other words, from an evolutionary point of view, the actual extreme narrow host range would result from the combination of different fitness parameters.

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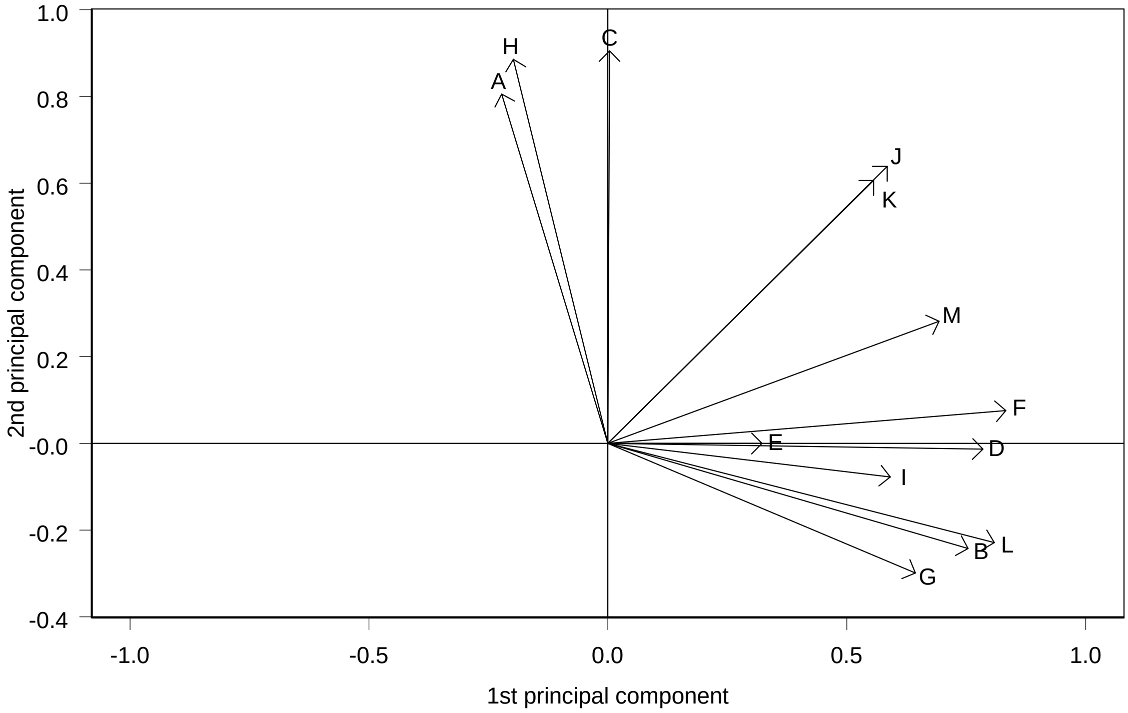
Linear furanocoumarins	A	B	C	D	E	F	G	H	I	J	K	L	M
Pearson correlation coefficient	-0.04395	-0.43177	0.00977	-0.06037	-0.31998	0.10072	0.08806	0.03071	-0.30411	0.1235	0.15585	-0.30593	-0.03557
p	0.7594	0.0016	0.9457	0.6739	0.0221	0.4819	0.5389	0.8306	0.03	0.3879	0.2748	0.029	0.8043

Appendix 1. Correlation between linear furanocoumarins and canonical variable (CDA without patch 3, see text).

		A	B	C	D	E	F	G	H	I	J	K	L	M
A	pcc	1.000	-0.222	0.785	-0.152	-0.169	-0.236	-0.172	0.802	-0.007	0.222	0.174	-0.358	0.098
	p		0.118	<.0001	0.286	0.235	0.096	0.227	<.0001	0.961	0.117	0.223	0.010	0.492
B	pcc	-0.222	1.000	-0.152	0.457	0.373	0.407	0.742	-0.332	0.428	0.219	0.175	0.556	0.566
	p	0.118		0.286	0.001	0.007	0.003	<.0001	0.017	0.002	0.123	0.220	<.0001	<.0001
C	pcc	0.785	-0.152	1.000	0.018	-0.100	0.045	-0.204	0.803	0.062	0.449	0.418	-0.183	0.272
	p	<.0001	0.286		0.903	0.487	0.754	0.151	<.0001	0.663	0.001	0.002	0.200	0.054
D	pcc	-0.152	0.457	0.018	1.000	-0.227	0.667	0.576	-0.132	0.620	0.398	0.394	0.593	0.413
	p	0.286	0.001	0.903		0.109	<.0001	<.0001	0.356	<.0001	0.004	0.004	<.0001	0.003
E	pcc	-0.169	0.373	-0.100	-0.227	1.000	0.244	-0.111	-0.137	-0.026	0.296	0.271	0.359	0.390
	p	0.235	0.007	0.487	0.109		0.084	0.438	0.337	0.856	0.035	0.055	0.010	0.005
F	pcc	-0.236	0.407	0.045	0.667	0.244	1.000	0.391	-0.069	0.471	0.532	0.519	0.674	0.526
	p	0.096	0.003	0.754	<.0001	0.084		0.005	0.632	0.001	<.0001	<.0001	<.0001	<.0001
G	pcc	-0.172	0.742	-0.204	0.576	-0.111	0.391	1.000	-0.316	0.472	0.094	0.062	0.397	0.345
	p	0.227	<.0001	0.151	<.0001	0.438	0.005		0.024	0.001	0.510	0.664	0.004	0.013
H	pcc	0.802	-0.332	0.803	-0.132	-0.137	-0.069	-0.316	1.000	-0.063	0.334	0.277	-0.363	0.144
	p	<.0001	0.017	<.0001	0.356	0.337	0.632	0.024		0.662	0.017	0.049	0.009	0.314
I	pcc	-0.007	0.428	0.062	0.620	-0.026	0.471	0.472	-0.063	1.000	0.109	0.045	0.489	0.207
	p	0.961	0.002	0.663	<.0001	0.856	0.001	0.001	0.662		0.446	0.756	0.000	0.144
J	pcc	0.222	0.219	0.449	0.398	0.296	0.532	0.094	0.334	0.109	1.000	0.975	0.284	0.457
	p	0.117	0.123	0.001	0.004	0.035	<.0001	0.510	0.017	0.446		<.0001	0.043	0.001
K	pcc	0.174	0.175	0.418	0.394	0.271	0.519	0.062	0.277	0.045	0.975	1.000	0.281	0.406
	p	0.223	0.220	0.002	0.004	0.055	<.0001	0.664	0.049	0.756	<.0001		0.046	0.003
L	pcc	-0.358	0.556	-0.183	0.593	0.359	0.674	0.397	-0.363	0.489	0.284	0.281	1.000	0.501
	p	0.010	<.0001	0.200	<.0001	0.010	<.0001	0.004	0.009	0.000	0.043	0.046		0.000
M	pcc	0.098	0.566	0.272	0.413	0.390	0.526	0.345	0.144	0.207	0.457	0.406	0.501	1.000
	p	0.492	<.0001	0.054	0.003	0.005	<.0001	0.013	0.314	0.144	0.001	0.003	0.000	

Appendix 2. Pearson correlation coefficient (pcc) between linear furanocoumarins and its significance.



Appendix 3. Distribution of the thirteen linear furanocoumarins after principal component analysis. Calculations were performed with all but patch 3 plants. First and 2nd principal component explain 36% and 26% of the variance.

CHAPTER 3

EFFECT OF ULTRAVIOLET LIGHT ON LARVAL PERFORMANCE OF *Oreina gloriosa*, A LEAF BEETLE SPECIALIZED ON THE PHOTOTOXIC LINEAR FURANOCOUMARINS-CONTAINING *Peucedanum ostruthium* (APIACEAE)

Luca Nessi and Martine Rahier

Abstract

Specialist insects are known to be relatively insensitive to the secondary toxic and/or deterrent compounds that characterize their host plants. The phototoxic linear furanocoumarins are reported to lack toxicity to insects that feed exclusively on Apiaceae, a plant family producing a wide variety of these chemicals. *Oreina gloriosa* (Coleoptera: Chrysomelidae) is strictly monophagous on an apiaceous plant, *Peucedanum ostruthium*, and the larvae exclusively feed at night. Our research aimed to determine if the performance of the leaf beetle is affected by the photoactivation of the linear furanocoumarins produced by *P. ostruthium*, as it is suggested by its night activity. Larvae were forced to remain in two different light environments, with and without ultraviolet radiation, which corresponds to the biologically active wave lengths. Both light environments had dramatic negative effects on larval development, nonetheless larvae of *O. gloriosa* suffered higher mortality when forced under light with ultraviolet radiation. It was not possible to draw definitive conclusions on the effect of furanocoumarins photoactivation. Strong vulnerability to photoactivation in *O. gloriosa* would have suggested metabolic defences to be scarce or absent, which would have represented a rare scenario among insects specialized on phototoxic plants. We may only state that ultraviolet radiation significantly reduces *O. gloriosa* fitness, possibly partially explaining its night feeding behaviour. A quantitative genetics study did not reveal any genetic variation in resistance to ultraviolet light.

Key words: *Oreina*, leaf beetle, specialist, *Peucedanum*, Apiaceae, linear furanocoumarins, ultraviolet light, phototoxicity.

Introduction

Several studies have shown that specialized phytophagous insects are adapted to secondary compounds which are toxic and/or deterrent to generalists (e. g. Berenbaum 1981a, Brower et al. 1988, Chapman et al. 1988, Hsiao 1988, Nitao 1989, Malcolm 1991, 1995, Lee and Berenbaum 1993, Pasteels et al. 1994, 1995). Yet, some studies at least evoke possible negative effects of secondary metabolites on monophagous or oligophagous species (Blau et al. 1978, Berenbaum and Feeny 1981, Berenbaum et al. 1986, Berenbaum et al. 1989, Siemens and Mitchell-Olds 1996, Zalucki and Malcolm 1999, Zalucki et al. 2001).

Members of the plant family Apiaceae produce linear furanocoumarins, secondary metabolites with well known toxic effects against a variety of organisms (Murray et al. 1982, Berenbaum 1991). With regard to herbivorous arthropods, linear furanocoumarins have been shown to be deterrent and/or toxic (Berenbaum 1978, Muckensturm et al. 1981, Luthria et al. 1989, Hadacek et al. 1994, Berdegué et al. 1997, Reitz and Trumble 1997). Moreover, linear furanocoumarins are well known as phototoxins, i. e. they have substantially increased noxious effects in the presence of light. Linear furanocoumarins are benz-2-pyrone compounds with a furan ring fused at the 6,7 positions (figure 1, chapter 2). The detrimental response is directly attributable to the furan ring. When irradiated with ultraviolet light, linear furanocoumarins enter an excited state: the toxicity is mainly due to the fact that the energized molecules bind to DNA bases. This determines disruption of DNA replication and transcription. Photoactivity is due to the formation of an excited triplet state on absorption of a photon. The excited triplet state can also react directly with proteins, lipids or ground state oxygen possibly forming singlet oxygen, hydroxy radicals or superoxide anion radicals. These molecules can react with proteins (enzymes), lipids and unsaturated fatty acids, causing toxicity (Murray et al. 1982, Berenbaum 1991, Arnason et al. 1992).

Most studies on phototoxicity against arthropods have been aimed to the development of pesticides in agricultural systems (e. g. Arnason et al. 1992, Krasnoff et al. 1994). Only a few studies have concentrated on natural occurring photoactive compounds. With respect to linear furanocoumarins only a very few systems have been investigated. Still, some authors have shown increased detrimental effects on herbivorous insects in the presence of light (e. g. Berenbaum 1978, Reitz and Trumble 1996) and concealed feeding habits (leaf rolling, leaf tying, leaf mining, stem or seed boring) have been considered as adaptive against photoactivation (Berenbaum 1978, Jones and Granett 1982, Griswold and Trumble 1985).

Linear furanocoumarins are reported to lack toxicity to insects that feed exclusively on Apiaceae. *Papilio polyxenes* (Lepidoptera: Papilionidae), that feeds primarily on members of this plant family, showed even improved growth rate in presence of xanthotoxin, known to be toxic to generalist lepidopterous larvae (Berenbaum 1981b). Specialization on furanocoumarins-containing plants has mainly led to metabolic adaptation. Linear furanocoumarins metabolization by cytochrome P450 monooxygenases seems to be a widespread means of detoxification (Berenbaum and Zangerl 1994, Berenbaum 1995 and references therein, Hung et al. 1996), described also for *P. polyxenes* (Lee and Berenbaum 1993). But other defence mechanisms have also been described to reduce photooxidative stress. For example, the local specialist parsnip webworm *Depressaria pastinacella* (Lepidoptera: Elachistidae) which feeds on the furanocoumarins producing *Pastinaca sativa* displays a variety of mechanisms to cope with this secondary compounds. Besides the P450 mediated metabolism (Berenbaum and Zangerl 1992), which represents the principal counteradaptation, the caterpillars excrete a small amount of unmetabolized furanocoumarins in the silk (Nitao 1990). In addition, constitutive levels of antioxydant enzymes, possibly involved in neutralizing toxic oxyradicals generated as a result of furanocoumarins photoactivation, are high relative to insects not normally associated with phototoxic plants (Lee and Berenbaum 1990). Moreover, Carroll et al. (1997) suggested that lutein sequestered from the food plant in the yellow fat body which surrounds the larval gut also reduces furanocoumarin phototoxicity. As other carotenoids, lutein may neutralize singlet oxygen and free radicals. Finally, webworms may also rely to a certain extent on behavioural mechanisms of protection since they are often found in a silken web woven with parts of plant umbels (Carroll et al. 1997). Larvae of *P. polyxenes* are also reported to partially rely on their relatively thick and pigmented cuticle (Lee and Berenbaum 1993).

The leaf beetles of the genus *Oreina* Chevrolat (Coleoptera: Chrysomelidae) feed on plants from several tribes of two different plant families, the Apiaceae and the Asteraceae (Dobler et al. 1996, Hsiao and Pasteels 1999). *Oreina gloriosa* belongs to a group of species within its genus that switched from the original Asteraceae hosts to Apiaceae and shows, only case in its clade, a strict monophagous behaviour on *Peucedanum ostruthium* (L.) Koch (Apiaceae: Peucedaneae), which is known to produce several linear furanocoumarins (List et al. 1967, Hadacek et al. 1994, Hiermann et al. 1996, Schinkovitz et al. 2003). Both the beetle and the plant are common in the Alps mostly at the subalpine and alpine levels. *Peucedanum ostruthium* is frequent in nitrogen-rich meadows and moist gravelly soils on both granite and limestone substrates (List et al. 1967, Lauber and Wagner 1996). Adults and larvae feed exclusively on the leaves and clearly discriminate between individual plants in nature. The linear furanocoumarins chemistry allowed to explain to some extent the host suitability (chapter 2). The major role of these secondary metabolites in governing *O. gloriosa* host plant use is also suggested by the strict night feeding behaviour of the larvae (chapter 1). We suggest that *O. gloriosa* evolved the night activity as a defence against the photooxidative stress associated with its host plant secondary chemistry. Our objective was to determine if the larval performance of *O. gloriosa* is affected by the photoactivation of the linear furanocoumarins produced by *P. ostruthium*. Strong vulnerability to photoactivation would suggest metabolic defences to be scarce or absent, which would represent a rare scenario among insects specialized on phototoxic plants.

No artificial diet for *O. gloriosa* was found. This would have allowed us to test independently for the effect of both the chemicals and of their photoactivation. We therefore performed the experiment with the beetle food plant in presence and absence of ultraviolet light. Simultaneously a close related species which feeds on no furanocoumarins-containing plants was used as "control". We performed the experiment with *Oreina elongata* Suffrian which is specialized in the use of only four host plants that belong to two genera of the family Asteraceae, *Adenostyles* and *Cirsium* (Margraf 2003), none of which produces furanocoumarins (Hegnauer 1977, Murray et al. 1982). The population from where *O. elongata* were collected is affiliated to two host plants, *A. alliariae* and *C. spinosissimum*. It is located in an open habitat, above the tree line, at approximately 2200 m above sea level, elevation at which ultraviolet radiation is at clear days known to be strong. The larvae are found on their host plants throughout the day, also in sunlight (P. Ballabeni, S. Dobler, pers. comm.). During the experiment *O. elongata* was fed *A. alliariae* in the presence and absence of ultraviolet light. *Oreina gloriosa* and *O. elongata* belong to the two major clusters within the genus (Dobler et al. 1996). The *gloriosa*'s clade species switched to Apiaceae from an ancestral association with Asteraceae, actually kept by the other cluster. Given its phylogenetic proximity and the absence of phototoxic compounds in its host plants, *O. elongata* was given a control status in the experiments. Again, *O. elongata* allowed us to overcome the lack of an artificial diet for *O. gloriosa*.

The study also concerned genetic differences in growth ability under ultraviolet radiation within the beetle population . If present, such differences are a measure of how much variation is available for selection to act upon, which in turn, if the performance characters are heritable, can determine the potential for evolutionary change. Several authors have demonstrated genetic bases to be involved in some host use modifications (Futuyma and Peterson 1985). Futuyma et al. (1984) showed genetic bases in an incipient divergence in plant utilization by the polyphagous moth *Alsophila pometaria* (Lepidoptera: Geometridae). Genetic differences in preference for hawthorn or apple were shown to have led to the formation of host races in the apple maggot fly *Rhagoletis pomonella* (Diptera: Tephritidae) (Prokopy et al. 1982). Brewer et al. (1995) suggested that differential selection for furanocoumarin tolerance among populations of the celery pest *Spodoptera exigua* (Lepidoptera: Noctuidae) may have resulted in part from varying chemistry of the encountered host plants.

Genetic differences are also known for specialized insects. Berenbaum and Zangerl (1992) showed that the enzymatic detoxification activity of furanocoumarins in the local specialist *D. pastinacella* is genetically variable and subject to selection. Thompson (1988) experimentally found significant variation in host choice within a population of the monophagous *Papilio oregonius*, clearly illustrating genetic bases for potential host shifts. When experimentally exposed to other plants than the only host in nature, isofemale strains of *P. oregonius* showed both within and between strain variation in host choice. The *Papilio machaon* species complex, to which *P. oregonius* belongs, feeds on plants in the Apiaceae, Rutaceae and, more rarely, Asteraceae. The use of the latter plant family seems to have evolved recently (Sperling and Feeny 1995).

A pattern in the evolution of host use involving the families Asteraceae and Apiaceae is also known for the *Oreina* group (Dobler et al. 1996, Pasteels et al. 1996) where host plant switches have occurred and genetic variation in host suitability is known to have been maintained at least for some species. For example, a *O. elongata* population showed genetic variation for performance traits when reared on each of the two hosts plants (Ballabeni and Rahier 2000a). This possibly corresponds to some genetic variation for tolerance to varying chemistry. In fact, *O. elongata* accepts plants from both Asteraceae tribes to which *Oreina* species are associated, Senecioneae and Cardueae, which are phylogenetically distant and chemically distinct (Hegnauer 1977). Moreover, larvae from the same population were able to develop on a third composite species, *Petasites albus*, which is abundant on the population site but is never used as a host (Ballabeni and Rahier 2000b).

A further indication of the little restriction to plant chemistry by the specialized *Oreina* species is also given by Hägele and Rowell-Rahier (2000). The performance of *O. cacaliae* and *O. speciosissima* was not different in experiments where larvae were reared on leaf discs treated with two chemicals alone or combined, one of which, the sesquiterpene cacalol, is lacking in the host plant. Little restriction was also shown by the fact that both species were indiscriminate towards cacalol in choice experiments. These examples of flexibility in host use show that specialization may not necessarily eliminate genetic variation in handling different host plant chemistries. In our study, we wanted to verify if the studied population of *O. gloriosa* showed genetic variation for survival when exposed to ultraviolet light. Nevertheless, the experiment does not allow to discriminate the effects of light per se and furanocoumarin activation. Genetical aspects of the population studied have already been investigated. Eggenberger and Rowell-Rahier (1992) showed that a considerable part of the variation in the self-synthesized chemical defence of the beetle is genetically determined.

Genetic variation in resistance to ultraviolet radiation toxicity could be of great evolutive significance in the system *O. gloriosa*-*P. ostruthium.* As shown in chapter 2, some phenotypic variation in linear furanocoumarins chemistry exists in the *P. ostruthium* population from where the beetles were collected for this study. Some of this variation possibly has genetic bases. Adults and larvae of *O. gloriosa* clearly discriminate between plants and patches with different secondary chemistries. Natural selection may possibly drive, or has already driven, the beetle population towards the ability to choose less toxic plants. To cope with linear furanocoumarin phototoxicity most specialized insects rely on enzymatic metabolism (Cohen et al. 1989, Nitao 1989, Berenbaum 1995 and references therein, Berenbaum and Zangerl 1994, Hung et al. 1996). In some insects metabolic detoxification has been shown to be genetically variable and therefore available for selection to act upon (Berenbaum and Zangerl 1992). If any metabolic defence exists in the *O. gloriosa* population, our experiment would allow us to reveal genetic variation for this fitness trait.

Materials and methods

Studied populations. The *O. gloriosa* studied population is located in the Swiss Alps on the precinct of the village of Saas Balen (Canton Wallis) at 1800 m elevation. The host plant, *P. ostruthium*, extends along a forest roadside in a half-open habitat. The plants grow within patches of one to two square meters. Shoots appear at the end of May. *Oreina gloriosa* is mainly active from mid June to the beginning of September. Adults emerge from overwintering in the ground and start mating at the beginning to mid June. The viviparous females lay first instar larvae between the end of June and mid July. Larvae develop to fourth instar by the beginning of September when they burrow into the ground to overwinter.

Beetles of *O. elongata* were collected from a population located in the western Alps, on the pass of the Petit Saint-Bernard, at the border between the French region Savoie and the Italian region

Vallée d'Aoste. The pass lies at 2188 meters elevation. The host plants, *A. alliariae* and *C. spinosissimum*, grow in close patches. *Oreina elongata* is mainly active in July and August. Adults emerge from overwintering in the ground and start mating between the end of June and mid July when plants appear in the field. Eggs are laid until the beginning of August. Larvae develop to fourth instar by the end of August-beginning of September when they burrow into the ground to overwinter.

Collection and rearing of organisms. Females of *O. gloriosa* and *O. elongata*, which had mated in the field with unknown males, were collected when recognizable to be gravid, i. e. with an extended abdomen. Individuals of *O. gloriosa* were collected in June and those of *O. elongata* in July 1998. The beetles were collected randomly and brought to the laboratory in cooled boxes. They were kept individually in round plastic containers (15 cm diameter, 4 cm height) until they produced offspring. To provide necessary humidity, the bottom of each box was covered with a moistened chalk layer of about 5 mm thick. The chalk was covered with a round filter paper of the same diameter as the box. Containers were distributed randomly within an incubator where the beetles were maintained at 14 ± 2°C during the day and 8°C ± 2°C at night with a photoperiod (LD) 17:7. The females were reared on the food plants, *P. ostruthium* for *O. gloriosa* and *A. alliariae* for *O. elongata* . Newly collected plants were shipped twice a week to the laboratory. There are *O. elongata* populations that occur in places in which only one or the other host plant (*A. alliariae* or *C. spinosissimum*) is present and others that occur in the presence of both hosts (Dobler and Rowell-Rahier 1994, Dobler et al. 1996, Pasteels et al. 1996). The Petit Saint-Bernard population is sympatric with both host plants and uses both for oviposition and feeding (Ballabeni and Rahier 2000a, b). *O. elongata* larvae from the Petit Saint-Bernard population were shown to perform better on *A. alliariae* than on *C. spinosissimum* (Ballabeni and Rahier 2000a). We therefore chose for our experiment the former plant which also has the advantage to have large flat leaves which can easily be cut and used for rearing and experiments. *Oreina gloriosa* is viviparous: first instar larvae were collected for the experiment within 24 hours after having been laid. Whereas, *O. elongata* is oviparous and larvae were collected within 24 hours after emerging from the eggs.

Light treatment. We planed a crossed experimental design with both *O. gloriosa* and *O. elongata* larvae. Larvae of *O. gloriosa* were exposed to four different light regimes (environments). In the first environment, called *UV*, larvae were exposed to ultraviolet light. Fiftyfive first instar larvae were placed individually within a plastic petri dish (5.5 cm diameter), transparent to ultraviolet light, with a plaster bottom. Each larva was offered a *P. ostruthium* leaf disc (2.5 cm diameter) and placed in an incubator at 8°C ± 2°C in the dark. After 7 hours of incubation the larvae were taken out from the incubator. The leaf disk was removed and the plaster was humidified. The larvae were then placed in the light for a 17 hours period. Preliminary experiments showed that, as it happens in nature (chapter 1), larvae do not feed in daylight. Thus, removing the leaves does not modify larval consumption rate. The light regime was obtained as follows in this environment: a cool white tube (lamp F36T8 Cool White, Sylvania Lighting SA, Meyrin, Switzerland) and a black-light blue tube (lamp F36T8 BLB, Sylvania Lighting SA) were suspended 40 cm above the petri dishes. The cool white tube did not emit below 380 nm. The black-light blue tube had a maximal emission at 356 nm and covered the range 300 to 420 nm. After Berenbaum (1978), the wavelengths that activate the furan double bond are in the range 320 to 360 nm. Camm et al. (1976), Reitz and Trumble (1997), and Masuda et al. (1998) indicate respectively wavelengths of 320 to 400, 300 to 400 nm and of 300 to 380 nm as the most active for furanocoumarins. The light period took place at room temperature. After the light period each larva was offered a 2.5 cm diameter *P. ostruthium* leaf disc and placed back in the "dark" incubator for a new 7 hours period. Alternating the dark and light periods, the experiment was run until the day following the third moult or until the death of the larvae. Before placing the larvae in the light the position of the petri dishes was always randomized.

In the second environment, called *no UV*, 55 larvae were exposed to white light only. The experimental design for this environment was the same as for *UV* but light source was only a cool white tube. Light intensity, as measured with a Minolta illuminance meter T-1, was approximately of 4000 lux in both *UV* and *no UV* environments, which is similar to the brightness conditions as measured during daylight on the *P. ostruthium* canopy in Saas Balen (appendix 1, chapter 1). During the light period all the larvae were arranged on the same table which was divided into two sides (*UV* and *no UV*) by suspending a wood board between the different light sources. This separation entirely shielded the *no UV* side from the black-light blue tube radiations.

In the third and fourth environments, called *UV+leaf* and *no UV+leaf* respectively, 15 larvae were offered a leaf disc for the entire experimental period. Constantly present leaf discs allowed the larvae to hide from light. Leaves have the advantage to better mimic the natural situation than any other light opaque material. Indeed, larvae have been shown to spend daylight time hidden in the host plant sheaths (chapter 1), which are structurally very similar to leaves. In the third environment (*UV+leaf*) light regime was the same as in the first environment (*UV*); in environment 4 (*no UV+leaf*) it was the same as in environment 2 (*no UV*). Larvae were offered fresh leaf discs every 24 hours, just before the 7 hours darkness period. Since larvae do not feed in the light, we could offer leaves as light screen without any risk to modify consumption rate as compared with larvae in environments 1 and 2.

A similar bioassay design was used to determine the performance of *O. elongata* under ultraviolet light. Three different light regimes were given to the larvae, corresponding to the environments 1, 2 and 4 given to *O. gloriosa*. The test was run as described for *O. gloriosa* but sample sizes were 30 for *UV* and *no UV* and 15 for *no UV+leaf*. During the experiment larvae were offered leaf discs of *A. alliariae* of the same diameter as described for *O. gloriosa*. *Oreina elongata* feeds over the entire day (P. Ballabeni, pers. comm.), so that light regimes 1 (*UV*) and 2 (*no UV*) result in an unnatural situation. Nonetheless, preliminary tests showed *O. elongata* to feed normally when offered the host plant only during a restricted period as planed for the present bioassay. Treatment 4 (*no UV+leaf*), more congruent with the natural conditions for both tested species, allows us to compare their performance (expressed as the ability to reach the last larval instar). It can not be considered merely as a light treatment for *O. elongata* because of its extended period of feeding.

We expect the performance of the two beetles to be similar, which in absence of an efficient artificial diet would make *O. elongata* a useful organism to be used as a control for studies on the development of *O. gloriosa*.

Ultraviolet absorbance of the plastic petri dishes was verified with a Uvikon 930 (Kontron Instruments, Munich, Germany) spectrophotometer. To mimic potential condensation on the petri cover we vaporized some distilled water. Water droplets did not increase opacity to ultraviolet radiation. The Uvikon 930 device was also used to verify the opacity of the leaves.

Larvae were checked daily, before each light period, for mortality and developmental stage. Larvae were weighed after larviposition for *O. gloriosa* and after emergence for *O. elongata* and then after the third moult, when the experiment was stopped. The third moult is the last one before pupation. Fourth instar larvae in the laboratory feed for a variable time before burrowing into the ground (L. Nessi, pers. obs.). This variability makes it difficult to use the last instar for performance studies in response to light regimes and we therefore gave up using it. We did not always weigh the larvae on the exact day of larviposition/hatching and of the last moult. Freshly larviposited/emerged or moulted larvae have a soft and white cuticle. In the period when integument hardens and darkens water loss is important. Larvae were weighed after this interval of time to assure that weight had stabilized.

For each larva we registered the following performance characters: survival (instar at death), growth rate (mg of weight increase per day from larviposition/hatching to third moult) and development time (number of days from hatching to third moult). In the comparison between *O. gloriosa*-*O. elongata* we only looked at the survival. Different sample sizes within and between species were due to limited space in the "dark" incubator.

Genetic differences in growth ability under ultraviolet radiation. The effects of the genotype (family), the environment (light regime) and their interaction on *O. gloriosa* larval performance were studied simultaneously to the performance experiment described above. Indeed, the 55 larvae of both *UV* and *no UV* environments were formed from each 11 families. Each family was produced from a female. Once the larvae were laid, they were transferred individually to the petri dishes and alternatively assigned to each environment. For every family, 5 larvae were assigned to the *UV* and 5 to the *no UV* light regimes.

Statistical analyses. In the environments where the larvae were exposed to the light (whether with ultraviolet radiation or not), they underwent great mortality. We could not perform an analysis of variance for some of the performance characters (development time, growth rate) because the data did not meet the model assumptions. We treated the data in a binomial form, namely surviving versus not surviving, and performed logistic regressions using the procedure of the SAS Institute (1999). Light regime and initial weight were considered as explanatory variables. Initial weight was never significantly different between the treatments as tested by t-test or 1 factor Anova computed using StatView and SuperAnova by Abacus Concepts (1992, 1989).

Larval performance was higher when leaf discs were offered during the entire experiment. In such cases we did an analysis of covariance to test the effects of environment, with initial weight as a covariable, on growth rate and development time. Statistics were calculated with SuperAnova by Abacus Concepts (1989). Growth rates and development time were transformed by their natural logarithm to meet the assumptions of homogeneity of variances (Sokal and Rohlf 1995). We used type III sum of squares which tolerates unbalanced sample sizes, considering that differential mortality effects made the data unbalanced. Effect on survival was studied by logistic regression using the procedure of the SAS Institute (1999). Survival data were coded as 0 (larva died before third moult) or 1 (larva was alive at third moult).

Individuals of *O. elongata* in the *UV* and *no UV* environments was even more affected than *O. gloriosa*, since no larvae were able to moult. Therefore enormous caution is needed in interpreting the results. We nevertheless compared the performances, expressed as the number of days until death, in the two light regimes by the mean of survival analysis computed with a log-rank test (Kleinbaum 1996) using S-Plus 6 by Insightful Corporation (2001).
Fisher's exact tests using S-Plus 6 were performed to compare genotype performances, i. e. the number of larvae per family surviving to the first moult.

Results

The performance of *O. gloriosa* larvae, expressed as the ability to moult, significantly varied under the different light regimes. Initial weight also explained the success in larval development, although to a lesser extent than the four light conditions (table 1). Larvae performed clearly better when they were offered the possibility to hide, independently of exposure to ultraviolet radiation (figure 1). When forced under light, larvae succeeded in moulting significantly better in the absence of ultraviolet radiation (figure 1 and table 2).

Table 1. Logistic regression for the effect of ultraviolet light regimes (*UV, no UV*, *UV+leaf, no UV+leaf*, see text) and the initial weight on *O. gloriosa* larval performance (expressed as the ability of moulting to second instar).

	d.f.	χ^2	p
Treatment	3	34.98	< 0.0001
Initial weight	1	9.97	0.0016

The model is significant. χ^2 for likelihood ratio is 71.33, d.f. = 4, $p < 0.0001$. The treatment x initial weight interaction could not be calculated since to little data were available within each treatment. Indeed, the treatment effect is dominant so that larvae either moult or die almost independently of initial weight.

Table 2. Logistic regression for the effect of the light regimes *UV* and *no UV* (see text) and the initial weight on *O. gloriosa* larval performance (expressed as ability to moult to second instar).

	d.f.	χ^2	p
Treatment	1	12.58	0.0004
Initial weight	1	6.53	0.011

The model is significant. χ^2 value for likelihood ratio is 20.78, d.f. = 2, $p < 0.0001$. The treatment x initial weight interaction could not be calculated since to little data were available within each treatment. Indeed, the treatment effect is dominant so that larvae either moult or die almost independently of initial weight.

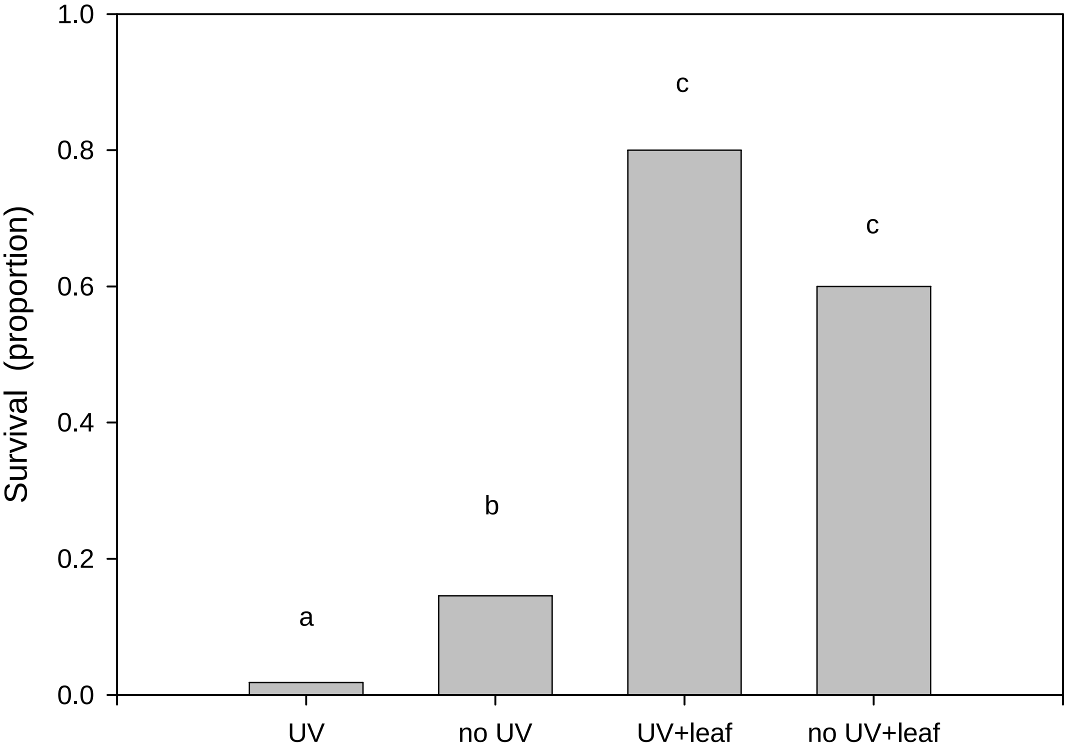


Figure 1. Proportion of *O. gloriosa* larvae reared under four ultraviolet light regimes (*UV*, *no UV*, *UV+leaf*, *no UV+leaf*, see text) that survived until the third moult. Different letters indicate significant differences after Fisher's exact test. *UV*, *no UV*: N = 55; *UV+leaf*, *no UV+leaf*: N = 15.

Figure 2 shows the performance of different *O. gloriosa* families in the environments *UV* and *no UV*. No family (genotype) effect was observed in the treatment *UV* (Fisher's exact test computed with the proportion of larvae surviving until the first moult, p = 1). In contrast, families performed in a significantly different way in the light regime *no UV* (p = 0.0046). We were not able to introduce the family effect in the logistic model shown in table 2 since to little data were available. In fact, many larvae in each family did not moult. However, as shown by Fisher's exact tests, we may state that some genetic variation exists in family performance in the absence of ultraviolet radiation and that this variation disappears when larvae are forced in ultraviolet light, where all genotypes perform equally bad.

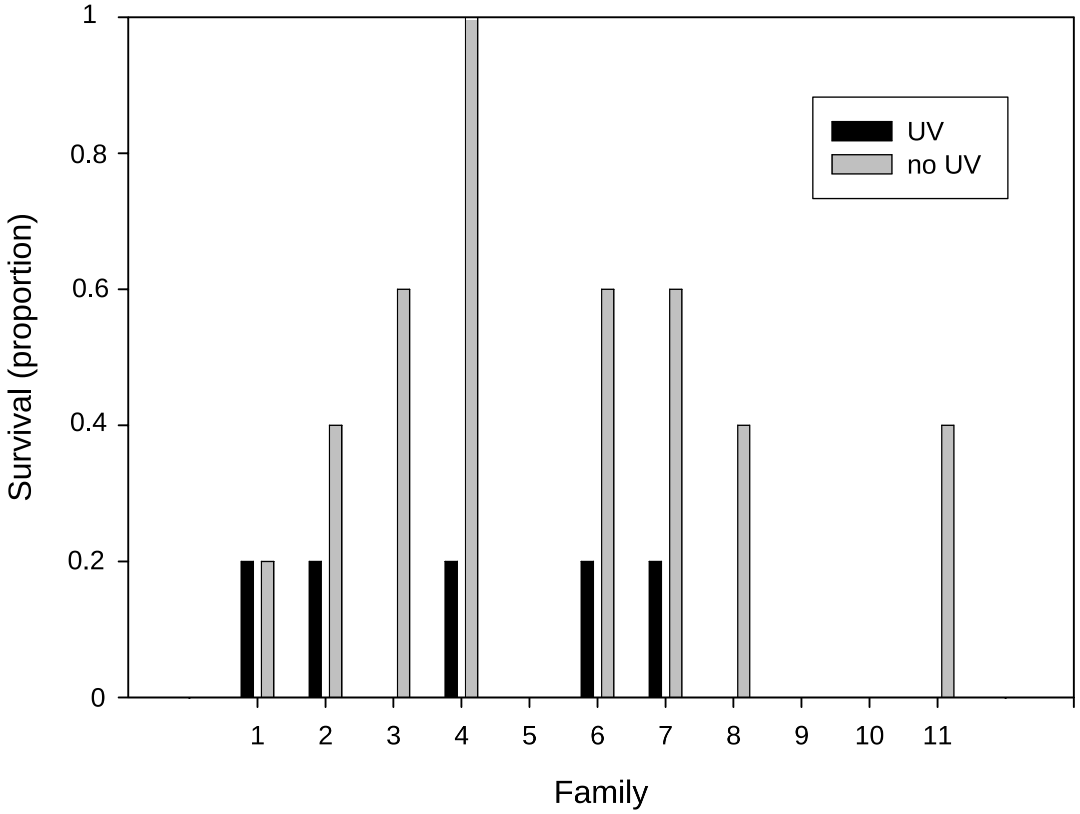


Figure 2. Proportion of *O. gloriosa* larvae of different families (genotypes) reared under the two light regimes *UV* and *no UV* (treatment described in the text) that survived until the first moult.

Results shown in table 3 confirm figure 1 data in that larval performance (expressed as growth rate and development time) was not different in the presence or absence of ultraviolet light when the beetles were allowed to hide. This is consistent with leaf opacity to these radiations. The differential survival of the larvae between the *no UV* and both *leaf* treatments (figure 1) shows that protection against ultraviolet radiation alone is not a satisfactory explanation to interpret larval performance. Leaf discs probably provide in addition better microclimatic conditions allowing the larvae to reduce water loss.

Table 3. One factor ANCOVA for *O. gloriosa* larval growth rate (A) and development time (B) with initial weight as covariable. Treatment corresponds to the two light regimes *UV+leaf* and *no UV+leaf* described in the text.

A

	d.f.	ss	F	p
Treatment	1	0.097	0.71	0.41
Initial weight	1	0.0099	0.072	0.79
Treatment x initial weight	1	0.054	0.39	0.54
Residual	17	2.326		

B

	d.f.	ss	F	p
Treatment	1	0.0025	0.10	0.75
Initial weight	1	0.00007	0.0027	0.96
Treatment x initial weight	1	0.00066	0.027	0.87
Residual	17	0.41		

Importance of the leaf disc for larval survival was also seen in the *O. elongata* experiment. Indeed, none of the 60 larvae forced in the light, independently of ultraviolet radiation, survived to the first moult, but 10 of 15 larvae in the treatment *no UV+leaf* did.

Survival curves for *O. elongata* larvae reared under the two light regimes *UV* and *no UV* (figure 3) were slightly not significant after log-rank statistic (Chi square = 3.5, d.f. = 1, p = 0.060). Even if we need to be cautious in interpreting the results since larvae of the two experimental groups performed very badly, there seems to be a trend for ultraviolet light to have a more detrimental effect.

The performance of *O. gloriosa* and *O. elongata* was not different when larvae were allowed to hide under the leaf (table 4). This result is nonetheless unsatisfactory to make *O. elongata* as a comparative organism to control the growth in *O. gloriosa* studies with respect to resistance to ultraviolet light. Indeed, *O. elongata* larvae performed badly in the entire set of experiments presented here. Severe effects on *O. gloriosa* larvae may be the result of major faults in the experimental design as a whole.

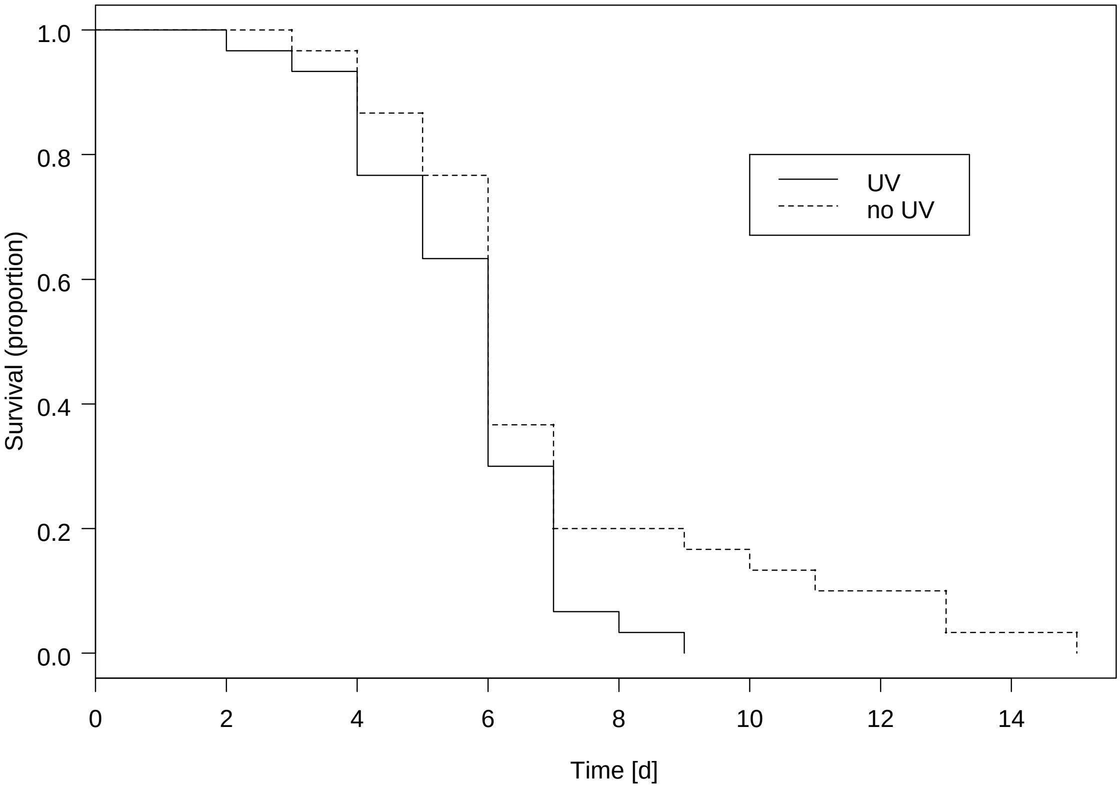


Figure 3. Kaplan-Meier survival curves for *O. elongata* larvae reared under the two light regimes *UV* and *no UV* described in the text.

Table 4. Logistic regression for the effect of the species (*O.gloriosa* and *O. elongata*) and the initial weight on larval performance (expressed as the ability of surviving to the third moult).

	d.f.	χ^2	p
Species	1	0.67	0.41
Initial weight	1	0.37	0.54

The model is not significant. χ^2 value for likelihood ratio is 0.92, d.f. = 2, p = 0.63. Initial weight varies between species. Within species performance is independent of initial weight.

Discussion

Larvae of *O. gloriosa* clearly suffer from the exposure to ultraviolet radiation and possibly lack any ability to metabolically manage the photoactivated furanocoumarins. Was this scenario true *O. gloriosa* might be considered an exception among insects specialized on furanocoumarin-containing plants. Indeed, adapted species are known to widely rely on metabolic means to detoxify these chemicals (Berenbaum and Zangerl 1994, Berenbaum 1995 and references therein, Hung et al. 1996). However, the results of the experiment make it difficult to draw conclusions about the differential effect of ultraviolet radiation toxicity per se and the toxicity resulting from linear furanocoumarins photoactivation.

Ultraviolet radiation can be detrimental per se and melanism shown by many insect species at high elevation is in fact commonly explained by providing better ultraviolet protection. For example, the colour of *O. cacaliae* shows a strong correlation with altitude (Knoll and Rowell-Rahier 1998). In the case of *O. gloriosa* negative effects of ultraviolet light may also result from linear furanocoumarins phototoxicity. Some authors have shown furanocoumarin-enhanced toxicity by ultraviolet radiation. Larvae of *Trichoplusia ni* (Lepidoptera: Noctuidae) offered diets with psoralen, bergapten, and xanthotoxin and exposed to ultraviolet light slowed their development (Reitz and Trumble 1996). Even a low concentration of xanthotoxin in the diet resulted to be highly toxic to the polyphagous armyworms *Spodoptera eridania* under ultraviolet radiations found in sun light (Berenbaum 1978). Nonetheless, noxious effects due to linear furanocoumarin photoactivation are not obligatory in herbivorous arthropods. For example, ultraviolet radiation had no consistent detrimental effects on the celery pest *Spodoptera exigua* in presence of diets with psoralen, bergapten and xanthotoxin in concentrations found in the plant leaves (Berdegué and Trumble 1997, Berdegué et al. 1997, Reitz and Trumble 1997). The aphids *Aphis heraclella* and *Cavariella pastinacae* forced to take up xanthotoxin and kept exposed to ultraviolet light were unaffected over short term (24 hours). Camm et al. (1976) explained this tolerance with the limited cell division during the experiment.

Contrasting to *O. gloriosa*, insects specialized on furanocoumarins-containing plants seem to tolerate ultraviolet radiation. Larvae of *P. polyxenes* (which is restricted to feeding on Apiaceae and Rutaceae) were unaffected when offered diets with xanthotoxin, bergapten and imperatorin in the presence of ultraviolet light (Lee and Berenbaum 1993). Ultraviolet radiation was shown to elicit avoidance behaviours in the locally specialized *D. pastinacella* only if the larvae were artificially fed diets without lutein (Carroll et al. 1997). This carotenoid, directly sequestered from the host plant *P. sativa*, protects the caterpillars from furanocoumarins-associated photooxydative damage.

Contrary to our expectation, *O. elongata* larvae performed equally bad whether or not exposed to ultraviolet light with a trend of better survival in the absence of ultraviolet radiation. The beetle's host plants do not produce furanocoumarins (Hegnauer 1977, Murray et al. 1982). Since photoactivation of these chemicals can definitively be ruled out we expected *O. elongata* to better tolerate ultraviolet radiation. Some caution is nonetheless needed since photoactivation of polyacetylenes can on the contrary not be excluded (Arnason et al. 1992). The comparison of the survival ability (larvae alive at third moult) across light regimes between *O. gloriosa* and *O. elongata*, was done to allow us to draw some coarse conclusions about ultraviolet toxicity per se and furanocoumarins photoactivation. Thus, although *O. gloriosa* and *O. elongata* larvae performed in a very similar way under the *no UV+leaf* light regime, the control status of *O. elongata* was unsatisfactory.

Dryness during the period of exposure to light possibly is the main reason for the low survival of the two species. Indeed, performance experiments with leaf discs have been used before with larvae of different *Oreina* species, but in these cases discs were constantly present during the bioassay (Ballabeni and Rahier 2000a, b, Hägele and Rowell-Rahier 2000). Hiding under the discs possibly allowed the larvae to reduce water loss.

The offspring of all the *O. gloriosa* females performed equally bad when exposed to ultraviolet light. However, we found differential survival among families in the absence of ultraviolet radiation. Again, we do not know if the ultraviolet toxicity relies on linear furanocoumarin photoactivation or not, but its detrimental effect widely overcomes the genotypic component. As mentioned before, the studied *O. gloriosa* population seems to entirely lack any ability to cope with ultraviolet radiation and there is no evidence for genetic variation on this trait.

If we had included into our experiment more families, more genetic variability might perhaps have become evident. In such a scenario a prerequisite for adaptation to ultraviolet radiation would be met. But linear furanocoumarin-phototoxicity possibly represents a very strong selective force. Larvae of *O. gloriosa* have been shown to feed only at night (chapter 1). Field observations of the Saas Balen population showed indeed no variation among individual beetles in respect of the time of foraging. All instars larvae were shown to feed exclusively in the second half of the night. No larvae were observed in the sunlight: in fact, after foraging most larvae burrow into the soil and some young larvae hide within the flower or leaf sheaths which are opaque to ultraviolet light. This behaviour allows the beetle to entirely avoid chemicals photoactivation and could be seen as the main result of natural selection. Night activity could be genetically fixed in the Saas Balen population. Selection may have lead to night behaviour rather than to the ability to deal with allelochemicals.

Some authors postulate that the function of secondary compounds, acting as deterrent cues, may be to modify the herbivore behaviour rather than to function as toxic defences (Jermy 1984, Bernays and Graham 1988). We suggest that linear furanocoumarins and their photoactivation in *P. ostruthium* surpasses deterrency and leads to severe detrimental effects.

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OUTLOOK

Further work is absolutely necessary to definitively isolate and quantify the linear furanocoumarins present in *P. ostruthium* leaves. To better understand *O. gloriosa* host use the investigation of the secondary chemistry has to be followed by bioassays with the chemicals in presence and absence of the activating ultraviolet radiations. In absence of an artificial diet, pure compounds in varying concentrations could be applied on leaf discs with known chemistry. Prior to these investigations we propose easy choice and performance experiments with larvae fed on leaves of attacked and unattacked plants harvested in the field.

It should be kept in mind that host plant compounds that are known to occur in a specific plant taxon may not necessarily play a crucial role in host plant use and particularly in host plant selection. For example, the specialist *Depressaria pastinacella* (Lepidoptera: Elachistidae) seems to assess the furanocoumarins (which are toxic to it) content of the fruits of *Pastinaca sativa* (Apiaceae) by assessing the octyl butyrate content (Cianfrogna et al. 2002). To get more reliable information on the *O. gloriosa-P. ostruthium* system we suggest a comparison of a larger chemical profile of attacked and unattacked plants. A multitude of other factors may play a relevant role in determining host suitability. Among them we would first investigate soil humidity and texture. *O. gloriosa* spends most of its life in the ground where pupation occurs. Natural enemies should receive further attention.

Other aspects are worth of research. We propose easily feasible quantitative genetics studies in both furanocoumarin production (plant clones can be produced from the rhizome) and larval performance on different diets to shed light on possible evolutive change. Further work on linear furanocoumarin induction and costs of defence (for example measured as seed production in artificially damaged and undamaged plants) should also earn more attention.

Besides these aspects immediately concerning the *O. gloriosa-P. ostruthium* system we would suggest further investigation aimed to shed light on the evolutionary forces behind the host shifts and specialization in the *Oreina* group. Host shifts are believed to be facilitated by the chemical similarity among the old and new host (e. g. Dethier 1941, Ehrlich and Raven 1964, Berenbaum 1983, 2001, Feeny et al. 1983, Feeny 1992, Bernays 2001). Indeed, insects attempting shifts of diet may on the one hand recognise "old" feeding or oviposition stimuli and, on the other hand, show metabolic preadaptation for the "new" plant. The Asteraceae are considered to be chemically related to the Apiaceae (Berenbaum 1990, 2001). Among the *Papilio machaon* (Lepidoptera: Papilionidae) species complex some switches are documented from the ancestral Apiaceae to the Asteraceae (Thompson 1988, Sperling and Feeny 1995, Berenbaum 2001). *P. machaon hudsonianus* is reported to feed on *Petasites* (Sperling and Feeny 1995), a composite genus also used by some *Oreina* species. Degen (1998) suggested the Apiaceae also to be the ancestral host plant family of the genus *Psila* (Diptera: Psilidae). The use of the Asteraceae by *P. rosae* (uncertain, E. Städler, pers. comm.), *P. nigricornis*, and *P. bicolor* was suggested to result from two possibly independent host shifts. Moreover, almost every umbellifer specialist taxon is closely associated with taxa specialized on composites (Berenbaum 1990): detailed phylogenetic relationships and host-plant associations are for example available for the depressariine Elachistidae (Lepidoptera), the genus *Papilio*, the genus *Papaipema* (Lepidoptera: Noctuidae), and the genus *Euleia* (Diptera: Tephritidae) (Berenbaum 2001 and references therein). All these examples suggest that the similarity in the composition of the two plant families as stated by the chemists is actually also recognised by herbivorous insects and reinforce the hypothesis of chemical similarity as a major force driving host switches between composites and umbellifers.

Dobler et al. (1996) stated that in the *Oreina* group "switches among host plants may rather be dictated by the available choice in high-altitude habitats than by plant relatedness and chemical similarity". We suggest that further investigations are needed before any definitive conclusions can be drawn. For example, it would be interesting to examine whether umbelliferous plants that more closely match the chemistry of host-plants would be selected by asteraceous feeders in bioassays, and vice versa. Studies on chemical (and non chemical) plant cues determining host choice in different beetle species could also be a useful approach for a better understanding of speciation in *Oreina*. The unavoidable basis of such a research would be the isolation and the quantification of the chemicals functioning as stimuli. Moreover appropriate behavioural tests would be necessary.

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Études

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