Costs and benefits of iridoid glycosides in multitrophic systems

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Voor Jelmer en mijn familie

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Summary

Because plants cannot run away from their attackers, move to more favourable locations or hide, they have to be able to tolerate, adapt and/or defend themselves. Plants have evolved an enormous array of mechanical and chemical defences against herbivores. One can distinguish three types of defence strategies: direct defences that directly affect the herbivores, indirect defences that attract the enemies of the herbivores and tolerance, which reduces the fitness consequences of herbivore damage. It is unlikely that direct and indirect defences act independently in plants. Natural enemies of the herbivores (the indirect defence) can be negatively affected when attacking larvae which feed on plants defended by high levels of allelochemicals (direct defence), causing potential incompatibility between host plant resistance and biological control. On the other hand, herbivores feeding on plants with high levels of allelochemicals may develop more slowly and have an impaired immune function, leading to longer exposure of vulnerable stages to parasitism and predation and causing potential synergism between host plant resistance and biological control. Studies of interactions between direct and indirect defences are almost exclusively based on studies with crop species. It has frequently been observed that domesticated plants have lower levels of defensive chemicals than their wild relatives

In this study I used a natural plant-herbivore-parasitoid complex. The main focus in this thesis is the effect of direct defence chemicals in the plant on higher trophic levels in the system, to detect if there is potential for a conflict between direct chemical defence and indirect defence of the plant.

Natural enemies of herbivores are a potential source of indirect defences for a plant. Plants can attract these enemies by providing shelter (domatia), food or signalling them with volatiles. The efficiency of these indirect defences depends on the effect of the natural enemy on the herbivore. Parasitoids often have an optimal host instar in which to parasitize their hosts. In this optimal instar they develop faster and/or grow bigger and have higher survival. In **chapter 2** I examined this optimal host size for a generalist koinobiont tissue-feeding larval endoparasitoid, *Hyposoter didymator*, and two of its natural hosts, *Spodoptera exigua* and *Chrysodeixis chalcites*. Koinobiont parasitoids attack hosts that continue feeding and growing during parasitism. In contrast with hemolymph-feeding koinobionts, tissue-feeding koinobionts face not only a minimum host size for successful development, but also a maximum host size, since consumption of the entire host is often necessary for successful egression. I hypothesized that the range of host instars suitable for successful parasitism by *H. didymator* would be much more restricted in the large host *C. chalcites* than in the smaller *S. exigua*. In contrast with our predictions, *C. chalcites* was qualitatively superior to *S. exigua* in terms of the survival of parasitized hosts, the number of parasitoids able to complete development and adult parasitoid size. However, in both hosts the proportion of mature parasitoid larvae that successfully developed into adults was low at the largest host sizes. Our results suggest that qualitative, as well as quantitative factors are important in the success of tissue-feeding parasitoids.

One of the direct defence mechanisms of plants against herbivores is the production of allelochemicals. However, the effects of these defence compounds is not necessarily restricted to herbivores but can extend to higher trophic levels in the food chain, including the predators and parasitoids of herbivores. In **chapter 3** I examined the effects of two defence chemicals of *Plantago lanceolata*, the iridoid glycosides (IGs) aucubin and catalpol, on the performance

of two generalist and two specialist herbivores and their endoparasitoids. Furthermore, I studied the sequestration of these chemical compounds in the herbivore-parasitoid complex of the specialist herbivore *Melitaea cinxia*. In general, the performance of generalist herbivores was negatively correlated with the levels of IGs but effects on the performance of their parasitoids were less apparent. Moreover, because herbivores developed more slowly on high IG plants, instars vulnerable to parasitism suffered an increased period of exposure to the parasitoids. On the other hand, effects on specialist herbivores differed between *M. cinxia* and the other specialist herbivore, *Junonia coenia*. The development of *J. coenia* was slower when feeding on plants containing high IG levels, whereas the pattern was reversed in *M. cinxia*. Similarly, development of *Cotesia melitaearum*, a gregarious endoparasitoid of *M. cinxia* caterpillars, benefited when it developed in larvae reared on *P. lanceolata* genotypes with high levels of IGs. Iridoid glycosides were detected in all tissues of the specialist herbivore *M. cinxia*, in its endoparasitoids and in two of their hyperparasitoids. In pupae and adults, the fraction of catalpol, the more toxic of the two IGs, increased with trophic level.

Another characteristic of IGs is that they can be oviposition stimulants for specialist herbivores and feeding stimulants for their larvae, especially when their performance is better on plants that contain IGs. In **chapter 4** I studied the effect of IGs and aspects of plant size (mainly the number of leaves) of the host plant *P. lanceolata*, on the oviposition behaviour of its specialist herbivore *M. cinxia*. A previous study of the same species showed that oviposition was associated with high levels of aucubin in plants in the field, but it did not distinguish whether the higher levels of aucubin were the cause (active choice) or consequence (induction) of oviposition. I conducted a set of dual- and multiple-choice experiments between plants with different levels of IGs, in cages and in the field. In the cages I found a positive correlation between the pre-oviposition level of aucubin and the number of ovipositions, indicating an active oviposition decision for plant with higher aucubin level, rather than plant induction following oviposition. The results also suggest a threshold concentration below which females do not distinguish among levels of IGs. In contrast to the cage experiment, in the field the size of the plant appeared to be a more important stimulus than the IG concentrations, with bigger plants receiving more ovipositions than the smaller ones, regardless of their secondary chemistry. Therefore, the predominant cues used for oviposition may be dependent on environmental conditions.

That not only plant chemicals play a role in oviposition choice, is also clear from **chapter 5**, in which I looked at the oviposition preference, habitat use and food plant suitability of another specialist on *P. lanceolata, M.athalia.* In a big cage experiment I studied the oviposition choice of this butterfly. For the oviposition experiment I used eight different plant species, all containing IGs. The plant species the females preferred for oviposition were *Veronica chamaedrys, V. spicata* and *P. lanceolata*. All of these plants grow in open meadows, which is where I also found the adults flying most frequent in the field. The difference in host plant and habitat use between Åland, Finland (where the field observations where done) and other regions, may reflect local adaptation to land use practices and geology which maintain clusters of small open meadows. Despite the fact that the presence of IGs is an important trait distinguishing host from non-host species used by *M. athalia*, oviposition preference within the group of (potential) host species and among individual plants within host species was largely independent of IG concentration. Although the adult butterflies chose specific plant species for oviposition, the immediate surrounding of these host species was more important than the IG concentrations of these plants. Plants in plots surrounded by bare ground received significantly more egg batches than plants in plots surrounded by vegetation. The larvae of *M. athalia* did not profit from the oviposition choice of their mother. They

performed equally well on all the 13 plant species used for the performance experiment, except for *V. officinalis.*

Many natural plant populations exhibit significant genetic variation in their levels of chemical defence against herbivores and pathogens. In *P. lanceolata* I also found variation in their levels of IGs. One of the factors that could contribute to the maintenance of this variation is the presence of fitness costs of chemical defence. In **chapter 6** I examined if there were fitness costs of having higher levels of IGs. This would imply that in the absence of natural enemies, the production, transport, storage, self-detoxification, activation and/or turnover of secondary plant compounds results in lower plant fitness as resources used for these processes cannot be used for growth, survival or reproduction. In this chapter I describe a regrowth experiment to investigate whether there are trade-offs between resistance and one specific aspect of tolerance, the ability to regrow after defoliation. I let plants with different levels of IGs grow under two nutrient conditions, poor and rich. After 8 weeks I clipped the aboveground biomass, and let the plants regrow for five weeks. The questions I asked were: do high-IG plants (1) suffer allocation costs in terms of shoot and root growth, (2) have reduced regrowth ability (tolerance) after defoliation and (3) are such costs more pronounced under nutrient stress? I found that the total biomass produced by high-IG plants was not lower than that of low-IG plants. However, high-IG plants produced fewer inflorescences (a reproductive cost) and allocated less biomass to roots than low-IG plants. After regrowth, root mass of high-IG plants grown under nutrient-poor conditions was significantly lower than that of low-IG plants. I speculate therefore that if there would be repeated defoliation, high-IG plants would eventually fail to maintain shoot regrowth capacity and that trade-offs between resistance (having high IG levels) and tolerance in this system may not show up until repeated defoliation events occur.

In **chapter 7** I discuss the results of the effects of IGs on herbivores and their parasitoids and whether these observed patterns differ among generalist and specialist herbivore-parasitoid combinations. I conclude that how direct and indirect defence mechanisms interact depends on the combination of species involved. A possible conflict between these two defence strategies may arise if the plants that are most attractive to natural enemies also possess strong direct chemical defences that exhibit clear negative effects on the performance of predators and parasitoids, or when there are metabolic trade-offs between these two kinds of defences. Future studies with natural plant species should examine wether these conflicts really exist in nature.

Chapter 1

General introduction

Scope and aim of the thesis

The general aim of this thesis is to study effects of direct defence chemicals on the preference and performance of generalist and specialist herbivores and on the preference and performance of higher trophic levels in a plant-herbivore-parasitoid system. Using this information I will speculate on whether selection for increased direct defence could pose a potential evolutionary constraint to selection for increased indirect defence. Most studies that deal with direct and indirect defences are done with crop species. This study will give new insights in the effects of direct defence chemicals and the effect on higher trophic levels in natural systems. In this study I use a natural plant-herbivore-parasitoid complex of *Plantago lanceolata*, to analyse the effects of genetic variation in allelochemistry on plant preference in herbivores and the performance in herbivores and their parasitoids.

I examine the interactions between different genotypes of *P. lanceolata* and several specialist and generalist host-parasitoid associations. Further, I test if there are stronger negative effects on the performance of the herbivores and their parasitoids on plant genotypes selected for high levels of direct chemical defences than on genotypes selected for low defence levels. I investigate if these patterns differ among generalist and specialist herbivore-parasitoid combinations and if there is sequestration of the chemical compounds in any of the species studied. To correlate the performance of the herbivores and parasitoids to the host plant preference of the adult female, I conduct oviposition experiments with specialist herbivores to examine if they have a preference for plant genotypes/species with different levels of secondary metabolites. To investigate the costs involved in having direct defence chemicals I conduct a regrowth experiment with *P. lanceolata* plants with different levels of direct defence chemicals.

In this chapter, I introduce the different types of plant defences: direct defence, indirect defence and tolerance. Next, I describe the positive and negative effects that the different defences may have on plant fitness. Since this may depend on the interactions between direct and indirect defences I discuss the potential conflicts that may arise and how the host range of both herbivores and parasitoids is important to consider. At the end of this chapter I present the study system I use and a short outline of my thesis.

Plant defences, what to do if you cannot run?

Plants in natural populations have to protect themselves against a multitude of natural enemies and cope with a range of abiotic stresses. Because plants cannot run away from their attackers, move to more favourable locations or hide, they have to be able to tolerate, adapt and/or defend themselves (= slow growth-high mortality hypothesis; Nagy and Schäfer 2002, Scheel and Wasternack 2002). Plant defence against herbivory includes a range of adaptations that improve their fitness (e.g., through increased rates of survival, greater seed production, etc.) by reducing the impact of herbivores. Plants have evolved an enormous array of mechanical and chemical defences against herbivores.

One can distinguish three types of defence strategies: direct defence that directly affects the herbivores, indirect defence that attracts the enemies of the herbivores, and tolerance which reduces the fitness consequences of herbivore damage. Defences may be constitutive, operating before herbivore attack, or induced, produced or translocated by the plant following damage or stress (Karban and Myers 1989) and they may vary quantitatively or qualitatively (Feeny 1976, Rhoades 1979, Coley et al. 1985). A given plant species often

exhibits many types of defensive mechanisms, mechanical or chemical, constitutive or induced, that serve to protect the plant, and allow it to escape from its herbivores.

Direct defence

Direct defences are any plant traits that by themselves affect the susceptibility of the plant to and/or the performance of attacking arthropods and thus increase fitness in environments with herbivores. These traits can be divided in antixenosis and antibiosis. Antixenosis is the ability to repel insects, causing a reduction in oviposition or feeding, and it can be based on chemical or mechanical processes. Some chemicals are volatile: they are released from plants into the air and are sensed by insects before they land. Other repellent chemicals are sensed or tasted by insects after they contact a plant or feed on it. Mechanical features that can cause antixenosis include leaf hairiness, stem hardness, sharp spines and trichomes (Cooper and Owen-Smith 1986). Some plants are known to mimic the presence of eggs on their leaves, which discourages oviposition by butterflies (Williams and Gilbet 1981). Antibiosis is the ability to reduce the survival, growth, or reproduction of insects that feed on the plant, and is often caused by the production of toxic chemicals in plant tissues. Hundreds of chemicals that are toxic to insects have been identified from different species and cultivars of plants.

Chemical defences

Secondary metabolites

Plants contain a wide variety of chemicals known as secondary metabolites, which are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. These chemicals are often by-products of the synthesis of primary metabolic products (Whittaker 1970). The function and importance of these compounds to the organism is usually of an ecological nature, as they are used as defences against predators, parasites and diseases, in interspecies competition, and in facilitating various reproductive processes (colouring agents, attractive smells, etc). Secondary compounds produced by the plant that influence the behaviour, growth and/or survival of other species are known as allelochemicals. From observations of feeding by herbivorous insects it is known that these compounds can serve as repellents to herbivores or that they are toxic to them (Fraenkel 1959, Ehrlich and Raven 1964).

Qualitative and quantitative chemical defences

Allelochemicals can be characterized as either qualitative or quantitative (Feeny 1976, Rhoades 1979, Coley et al. 1985). Qualitative allelochemicals are defined as toxins that interfere with the herbivore's metabolism, often by blocking specific biochemical reactions (Rhoades 1979). They are present in plants in relatively low concentrations (often less than 2% of dry weight) and can rapidly be synthesised and transported. These chemicals are effective against non-adapted specialist and generalist herbivores.

Quantitative allelochemicals are digestibility reducers that make plant cell walls indigestible to animals. The effects of quantitative allelochemicals are dosage dependent, the higher the proportion of these chemicals in the herbivore's diet, the less nutrition animals gain from ingesting plant tissues. Quantitative defence substances are present in high concentrations in plants (5-40% dry weight) and are equally effective against both specialist and generalist herbivores. Because they are large molecules, these defences are energetically expensive to produce and maintain, and often take longer to synthesize and transport.

Constitutive and induced defences

Defences can further be classified as induced or constitutive. Constitutive defences are those that are always present in the plant species, while induced defences are synthesized at and/or mobilized to the site of attack when a plant is injured. Induced defences need a proximate and reliable cue to be triggered (Harvell 1990). Plant secondary chemicals may be induced by biotic factors e.g., herbivore attack or pathogen infection. Herbivore attack is frequently associated with wounding, and the 'recognition' of herbivore attack (Korth and Dixon 1997, Walling 2000, Baldwin et al. 2001). The induction of secondary chemicals may therefore vary with the identity of the (herbivore) species that damages the plant. Inducible defence will be favoured over constitutive defence when 1) the probability of attack is high but unpredictable, 2) the cues associated with attack are reliable but not fatal for the plant, 3) there is a (fitness) cost to defence, favouring intermittent deployment (Harvell 1990).

Costs of chemical defence

Many natural plant populations exhibit significant genetic variation in their levels of chemical defence against herbivores and pathogens (Simms 1992, Karban and Baldwin 1997, Harvey et al. 2007). This variation is probably maintained by fitness costs of chemical defence (Bergelson et al. 2001). Fitness costs imply that, in the absence of natural enemies, the production, transport, storage, self-detoxification, activation and/or turnover of secondary plant compounds, results in lower plant fitness. This fitness decrease is due to reduced growth, survival or reproduction (Simms and Rausher 1987, Simms 1992, Karban and Baldwin 1997). Different types of costs have been distinguished. The first are described as allocation costs (Simms 1992). These costs result from an internal distribution pattern in which limited resources are used for defence rather than other fitness-enhancing traits (e.g., growth, reproduction). The second type are ecological costs (Simms 1992). These costs occur when plant traits that reduce herbivory have a negative consequence for the plant through interactions with other species. High ecological costs of defence may cause plants to avoid investment in defence altogether, or switch to alternative strategies (e.g., tolerance; Agrawal et al. 2002a). Such ecological costs may favour the evolution of inducible defence because the trade-off between defence and other traits is only expressed when the defence is needed (Agrawal and Karban 1999). Moreover the defences may as well disrupt important mutualistic interactions such as pollination mediated by insects (Adler et al. 2001) and reduced attraction of natural enemies of herbivores on defended plants compared to genetically similar undefended plants (Agrawal et al. 2002b).

Indirect defence

Another category of plant defences are features that indirectly protect the plant by enhancing the probability of attracting the natural enemies of herbivores and increase the carnivore's foraging success and thereby facilitate top-down control of herbivore populations (Price et al. 1980, Dicke 1994, Turlings et al. 1995, Karban and Baldwin 1997, Dicke 1999a). There are many and varied ways in which plants may promote the effectiveness of natural enemies of herbivores. One of these ways is to provide protection for them. Some plants produce specialised structures, called domatia, that are inhabited by predatory arthropods and fungivores, but rarely by herbivores (Beattie 1985, Odowd and Willson 1989, Pemberton and Turner 1989, Jolivet 1996, Walter 1996). Domatia are thought to provide protection against adverse weather conditions or relatively larger hyperpredators. Another way to attract the third trophic level is to provide food. There is evidence for the arrestment of predatory

arthropods when nutrient supplements, such as pollen, are available. Extrafloral nectaries provide nutrition for predators that increases their foraging efficiency in certain areas of a plant (Heil et al. 2001). The provisioning of protection and food by plants mainly represents examples of indirect, constitutive defence. Many plants also possess inducible indirect defences. They can increase their rate of nectar secretion after herbivore attack and release volatile chemicals in response to attack by herbivores. These volatile signals attract and arrest the natural enemies of the herbivores (Sabelis et al. 1999). In addition to attracting natural enemies of the herbivores, the volatiles can function as a direct defence by repelling the ovipositing herbivores (De Moraes et al. 2001, Kessler and Baldwin 2001), but they can also attract adapted herbivores (Dicke and Vet 1999). Finally, they may be involved in plant-plant interactions (Farmer 2001).

Tolerance

If a plant is not able to actively defend itself against its herbivores it may be beneficial to be tolerant. A plant genotype is classified as being tolerant if it can sustain tissue loss with little or no decrease in fitness relative to that in the undamaged state (Stowe et al. 2000). Certain morphological traits, such as storage in below-ground and stem structures, in addition to physiological responses, such as herbivore-induced increases in photosynthetic capacities and nutrient uptake, are correlated with compensatory growth following herbivore attack (Stowe et al. 2000).

If tolerance does not negatively affect rates of plant consumption by enemies, an increase in the enemy load might possibly increase the amount of damage, thus reducing the host's capacity for tolerance. It is reasonable to expect that under low levels of damage tolerance would increase (Hutha et al. 2003, Del-Val and Crawley 2005). As the amount of damage increases, tolerance will finally reach its maximum and any further increase in the amount of damage will reduce the benefits of tolerance because of internal/external constraints. Recent studies have indicated that host plants probably face limits on their maximum tolerance because of resource limitation (Fornoni et al. 2004) and/or physiological and morphological constraints (Hochwender et al. 2000).

Genetic variation for resistance and tolerance can be maintained by selection and by trade-offs. These trade-offs can be in the form of a fitness cost (Frank 1992, Antonovics and Thrall 1994, Frank 1994) or trade-offs between different systems of defence (Tiffen 2000, Eubanks et al. 2005). As resistance is likely to require the mobilization of limited resources, it is believed to be an expensive strategy for a potential host-plant. The costs of resistance would manifest themselves in the reduced fitness of resistant plants to relative susceptible plants in the absence of herbivores. Plants also vary in the degree to which they can tolerate damage. Genetic variation for tolerance could also be maintained by a cost (Simms and Triplett 1994). Selection may generate trade-offs between tolerance and resistance (van der Meijden et al. 1988, Simms and Triplett 1994, Fineblum and Rausher 1995, Strauss and Agrawal 1999). As resistant genotypes should experience less damage, selection for the ability of these genotypes to tolerate damage would likely be weak. In less resistant, more heavily damaged plants, there would likely be strong selection for tolerance. As a consequence, it is expected to observe a negative genetic correlation between resistance and tolerance (Carr et al. 2006).

Effect of plant defences on plant fitness

Does the plant benefit from direct defences against herbivores?

Mechanisms that produce negative effects on the herbivore do not automatically lead to positive effects on the plant. It has to be seen from the perspective of the individual plant fitness. Digestibility-reducers reveal such a paradox for plant-herbivore interactions. Insects reared on diets of low digestibility or plants with digestibility-reducers, have a strong tendency to compensate through increased consumption (Barton Browne 1975). This means that the per capita damage by those insects that complete maturation on digestibility-reducing plants is actually larger because of those compounds.

Increased direct defence may have positive and/or negative effects on the impact of natural enemies of the herbivores (e.g., parasitoids). On the positive side, natural enemies can exploit herbivore populations more effectively when the herbivores have a reduced growth rate. In many host-parasitoid associations, a parasitoid can attack its host only at certain stages (Godfray 1994). If these vulnerable stages are prolonged, a host will be exposed longer to the danger of being parasitized (= slow growth-high mortality hypothesis; Clancy and Price 1987, Damman 1987). From a plant-fitness perspective, the fact that digestibility-reducers can increase the effectiveness of the natural enemies of the herbivores may be more important than was originally assumed. Instead of being a supplement to the positive selective value of the digestibility-reducing trait, increased enemy efficiency may be essential for making the value positive in the first place (Price et al. 1980).

Plant allelochemicals may also impede the optimal functioning of the herbivore's immune system. One of the mechanisms employed by herbivores to defend themselves against parasitoids is by encapsulating the parasitoid eggs or larvae. The success of the encapsulation reaction often depends on the health of the herbivore, and this can be affected by stress, imposed by the host plant on the herbivore as a result of poor nutritional quality, starvation, or allelochemicals. A plant with toxins or low nutritional quality may suppress the insect's immune system and thereby benefit the endoparasitoids (Kraaijeveld and Godfray 1997, Ojala et al. 2005).

On the negative side there are plant defences that have a stronger impact on the natural enemies of herbivores than on the herbivores themselves. Such a negative balance arises when herbivores passively take up or actively sequester toxic plant compounds. Sequestering herbivores probably use stored allelochemicals to defend themselves against their natural enemies (Hunter 2000). It is therefore in the plant's interest to produce substances that have a negative impact on the herbivore without impeding the natural enemies of the herbivore. In cases where plants do better when herbivores are parasitized, it can be expected that natural selection has favoured individuals that employ chemical defences, which have a minimal negative impact on the natural enemies of herbivores (Turlings and Benrey 1998).

Does the attraction of parasitoids benefit the plant?

Fitness benefits for plants resulting from indirect defences have been well documented for plants that attract ants with domatia and/or food (Janzen 1966, McKey 1988, Oliveira 1997). Also the attraction of predators by volatile infochemicals can benefit the fitness of the plant, because a predator kills its host immediately, thus preventing further damage to the plant (Sabelis and de Jong 1988). However, the effect of parasitoid activity on herbivory strongly depends on the particular herbivore-parasitoid combination. It is probably by reducing the numbers of specialist herbivores that natural enemies will play the most important role, from the plant's point of view (van der Meijden and Klinkhamer 2000). This reduction can be

direct, if parasitism reduces the amount of immediate damage of the plant tissues caused by herbivores, or indirect, by reducing the numbers of herbivores recruited to future generations.

Numerous studies have revealed that the effects of parasitism on host growth and development often vary between solitary and gregarious koinobiont parasitoids (Harvey 2000). Most solitary koinobiont parasitoids reduce the level of herbivory, because parasitized hosts consume significantly less plant tissues than uparasitized cohorts (Jones and Lewis 1971, Vinson 1972, Smilowitz and Iwantsch 1973, Harvey et al. 1999, Harvey et al. 2004). Most solitary koinobiont parasitoids arrest host growth prior to the final instar whereas gregarious koinobionts frequently stimulate host feeding behaviour (Tanaka et al. 1984, Sato et al. 1986). This is because gregarious parasitoids must compete with siblings for resources within a single host. Consequently, host damage levels may increase in response to a corresponding increase in parasitoid load (Harvey 2000), with heavily parasitized (or superparasitized) hosts consuming considerably more plant tissue than hosts with small parasitoid loads. This results in a negative effect on the first trophic level, the food plant. However, experimental studies have shown that hosts parasitized by gregarious koinobionts may still cause less damage to the plant than hosts that are not parasitized (Elzinga et al. 2003).

Interactions between direct and indirect defence

It is unlikely that direct and indirect defences act independently in plants. Their potential incompatibility has been a concern in crop protection, because it would limit the opportunities for combining variety selection for increased direct chemical defence with biological control of herbivores by parasitoids (Bottrell et al. 1998). Parasitoids can be negatively affected when they attack larvae that feed on plants defended by high levels of allelochemicals, causing an incompatibility between host plant resistance and biological control (Gunasena et al. 1989). Moreover, knowledge of indirect defences through the release of herbivore-induced plant volatiles (HIPV) that attract parasitoids is almost exclusively based on studies with crop species. Domesticated plants, which have been artificially selected, may have altered allelochemistry and nutrient content. It has been frequently observed that domesticated plants have lower levels of defensive chemicals than their wild relatives (Roddick 1986, Sotelo et al. 1995, Lindig-Cisneros et al. 2002, Lindig-Cisneros et al. 2004, Harvey et al. 2007). As a result of this process, cultivars may incur greater damage than wild plants, either by becoming more vulnerable to herbivores or by reducing the effectiveness of natural enemies. Studies from natural systems are required to better understand the importance and evolution of this type of indirect defence (Benrey et al. 1998).

Is there a potential conflict between direct and indirect defence?

It is essential to understand the potential conflict between direct and indirect defence in order to fully understand the evolution of defence strategies in plants. This would enable us to assess whether direct and indirect defence mechanisms should be considered as alternative defence strategies that are constrained by trade-offs, or, under which conditions both strategies could be simultaneously favoured by selection.

Constitutive secondary metabolites and volatiles can be produced through the same biosynthetic pathway. This is the case for terpenoid defence compounds such as IGs and volatile terpenes. A conflict is possible when there is a trade-off between the production of chemicals used in direct and volatiles used in indirect defence.

In wild plant species and populations, direct plant defences are known to be very effective in reducing levels of damage caused by poorly adapted generalist herbivores. On the other hand, parasitoids and predators in the third trophic level may play an important role in reducing damage caused by specialist herbivores that are adapted to specific secondary plant compounds (Waage and Mills 1992, Turlings et al. 1995, van Lenteren et al. 1997).

The success of different (and potentially conflicting) defence strategies in plants may partially rest on the degree of dietary specialisation (and adaptation to more toxic plant species or genotypes) by herbivores and their natural enemies. Specialist herbivores are often not negatively affected by an increase in levels of phytotoxins in plant tissues, and may even require them as oviposition and/or feeding stimulants (van Loon and Schoonhoven 1999). Also, some specialist herbivores are seldomly attacked by generalist parasitoids and predators, because they contain sequestered plant defence chemicals. Therefore, specialist natural enemies of specialist herbivores may play a more important role in decreasing the amount of plant damage, because they are able to cope with more toxic plant types or toxins that are sequestered in their herbivore host or prey (Dicke 1999b, Mattiacci et al. 2001). On the other hand, because generalist herbivores develop more poorly on well-defended plant species or genotypes, a pattern that is often reflected in the performance of their predators and parasitoids, the strength of indirect defences between plants and their natural enemies is likely to be less strong than in specialist systems. An increase in plant defence level will have a negative effect on generalist natural enemies, which may result in a lower level of predation of parasitism of both specialist and generalists, and thus may have a negative effect on plant fitness. At the same time generalist herbivores will be more deterred by the direct defences themselves, which leads to an increase in plant fitness. A decrease in defence chemicals will have the opposite effects. The evolutionary outcome of this conflict between defence chemicals and their effects on the second and third trophic level, clearly depends on the balance of the relative impacts on plant fitness by generalist herbivores and generalist natural enemies of specialist herbivores (van der Meijden and Klinkhamer 2000). It is important to realise that biochemical, genetic and/or trophic-level mediated trade-offs between levels of secondary metabolites and parasitoid attraction/performance do not necessarily result in a conflict between direct and indirect defences. This strongly depends on the biotic environment, i.e. the composition of the herbivore and parasitoid community (Vrieling et al. 1991). Therefore it is important to study the patterns of associations between direct and indirect plant defence in natural systems under different biotic conditions, i.e. combinations of herbivores and parasitoids with different levels of specialisation with the underlying trophic level.

I did preliminary experiments to test the potential trade-off between the IG levels in a plant and its terpenoid volatile production, but these experiments are not part of this thesis. Further analyses and additional experiments, focused on the attraction of the parasitoids and their effectiveness as indirect defence, need to be done.

Host range and plant defence

Herbivores

Most insect herbivores are specialised feeders: 70% of the species are restricted to one plant family (Bernays and Chapman 1994) and 90% to three or fewer plant families (Bernays and Graham 1988). One factor that contributes to the diversity of herbivores is the defensive chemistry of the host plant. Each species of plant has evolved a unique set of defensive metabolites, which deter attack from most herbivores, except for a few species that have

broken through the defences by evolving counter adaptations. The ability of herbivores to adapt to plant allelochemicals varies between species that differ in their dietary range. Oligophagous species (specialists) are restricted in their dietary breadth, e.g., they feed on several plant species within one genus or one family. Alternatively, polyphagous species (generalists) may feed on many plant species in a range of plant families (Bowers and Puttick 1988, Agrawal 2000b). The specialists are generally able to deal more effectively with most of the defences of a particular plant species or group of plant species. Herbivores use the plant defensive system to which they are adapted as host-finding cues and feeding stimulants (van Loon and Schoonhoven 1999). This way they can avoid plant species containing defensive systems to which they are not adapted (Rhoades 1985). A number of specialist herbivores may even sequester these defence substances into their bodies and use them for their own protection (Bowers 1981). Generalist herbivores, on the other hand, are predicted to be repelled or poisoned by the high levels of chemical defences in plants (Feeny 1976).

Natural enemies of herbivores

Bernays and Graham (1988) argued that generalist natural enemies of herbivorous insects provide a major selection pressure for restricted host plant range. If generalist predators, via avoiding exposure to harmful plant chemicals, act as a selective force, it is expected that 1) they will learn to avoid prey containing deleterious plant chemicals, 2) they will avoid specialist herbivores and attack more generalist herbivores, and 3) they will suffer a reduction in fitness when they eat prey containing harmful plant chemicals. If generalist natural enemies selectively kill herbivores that consume plant material with lower concentrations of allelochemicals in such a way that there is a fitness benefit for the plant, then it is expected that there will be selection for plant individuals with lower defence concentrations (van der Meijden 1996).

Specialist parasitoids that attack one or only a few species of hosts may act as selective agents for host plant shifts that result in an increase in diet breadth of the host insects (Bernays and Graham 1988). Since most parasitoids are more specialised than insect predators in habitat and/or host use, their host insects, at least those that feed on exposed parts of the plant, are more likely to escape their parasitoids by broadening their dietary range of host plant than by narrowing it (Stamp 2001). The idea that insect herbivores may escape generalist enemies by narrowing host plant range and escape specialist enemies by broadening host plant range is a subset of the enemy-free space hypothesis, which is defined as ways of living that reduce or eliminate a species vulnerability to one or more species of natural enemies (Jeffries and Lawton 1984).

Study system

Choice of the system

In this study I use the system of *Plantago lanceolata* L. (Plantaginaceae) and its herbivore-parasitoid complex. This system was chosen for several reasons. First, direct chemical defence in this plant species by a group of monoterpene derivates, the iridoid glycosides (IGs), has been extensively studied by the groups of M. D. Bowers and N. E. Stamp at the University of Colorado, Boulder, USA, and by H. B. Marak and A. Biere at the NIOO-CTE, Heteren, the Netherlands. There is already a lot of knowledge about the effects of IGs on herbivores and pathogens. Secondly, the group of I. Hanski (University of Helsinki, Finland) has extensively studied metapopulation structure and dynamics of one of the specialist herbivores of *P. lanceolata*, *Melitaea cinxia*, and its parasitoids, *Cotesia melitaearum* and

Hyposoter horticola, in natural populations (Åland, Finland). Part of this study is therefore conducted on Åland, because here I have access to the specialist herbivore *M. cinxia* and its parasitoid *C. melitaearum*.

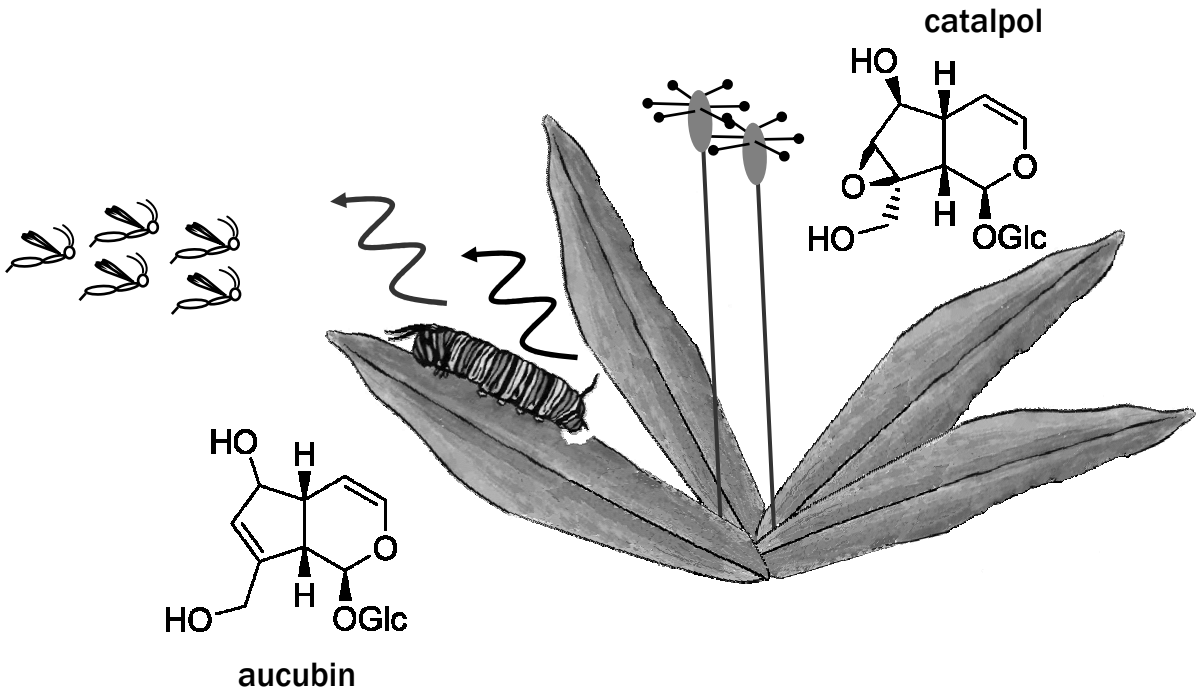


Figure 1.1 Direct and indirect defences in *Plantago lanceolata*.

Iridoid glycosides

The iridoid glycosides are a group of monoterpene derivates found in a large number of plant families (Jensen 1991) that are produced via the isoprenoid biosynthetic pathway (McGarvey and Croteau 1995). They generally deter feeding by generalist insect herbivores but can be used as feeding or oviposition stimulants by some specialists (Bowers 1991).

Among the secondary plant compounds produced by *P. lanceolata* are the two IGs, aucubin and catalpol (Fig. 1.1; Duff et al. 1965). Aucubin is the biosynthetic precursor of catalpol (Damtoft et al. 1983). In natural populations, IG levels in *P. lanceolata* range from undetectable to ca. 9% of its dry weight (Bowers 1991). Iridoid concentrations are partly under genetic control in *P. lanceolata*; significant heritability for leaf IG concentration has been observed in this species (Marak et al. 2000). IG concentrations vary both among populations and among individuals within populations. The concentrations also vary with the developmental state of the plant, including leaf and plant age, and attributes of the environment such as time of the day, weather, soil nutrient conditions and presence of mycorrhizal fungi (Bowers 1991, Bowers and Stamp 1992, 1993, Stamp and Bowers 1994, Adler et al. 1995, Darrow and Bowers 1997).

The role of iridoid glycosides in oviposition choice and larval performance

The specialist *M. cinxia* lays eggs more frequently on *P. lanceolata* with high aucubin concentrations than on individuals with low concentrations (Nieminen et al. 2003). Females of the specialist butterfly *Junonia coenia* are known to select for plants with high aucubin and catalpol concentrations for oviposition (Pereyra and Bowers 1988). The specialist *J. coenia* prefers and shows enhanced growth and survival on diets containing IGs (Bowers 1991), however eating plants that have a high concentrations of IGs comes wit a cost to growth. Non-adapted herbivores such as *Lymantria dispar* show reduced performance on diets containing IGs (Bowers 1991). Also the specialist *M. cinxia* shows a shorter development time when reared on plants with high IG levels, in contrast to the generalist herbivore *Spodoptera exigua* which has a lower pupal weight when reared on *P. lanceolata* plants with high IG levels (Harvey et al. 2005).

Of the two IGs, catalpol is especially important to the chemical defence of larvae, because it is more toxic to generalist herbivores than aucubin (Bowers and Puttick 1988, Bowers 1991).

The role of iridoid glycosides in parasitism

Plantago lanceolata plants selected for oviposition by *M. cinxia* females appear to have higher iridoid concentrations than could be expected by just a random use of plant individuals (Nieminen et al. 2003). Moreover, larvae effectively sequester especially catalpol from *P. lanceolata*, which can relate to their defence against natural enemies (Suomi et al. 2001b). *Cotesia melitaearum,* a specialist parasitoid of *M. cinxia* on Åland, successfully parasitizes larval groups feeding on *Veronica spicata* more often than larval groups which feed on *P. lanceolata* (van Nouhuys and Hanski 1999). One of the explanations for the difference in successful parasitation rate is a difference in plant chemistry between the two species, either in secondary metabolites or in olfactory cues used to locate host larvae. Iridoids are used both in plant and herbivore defence against their respective natural enemies. High concentrations in larvae may function as deterrent to ovipositing *C. melitaearum*, or may cause mortality of parasitoids developing within the relatively well defended hosts (Nieminen et al. 2003).

Predation by ubiquitous generalists would probably be higher if the host and host food plants were not chemically defended, but specialist parasitoids appear to tolerate, overcome, or avoid iridoids sequestered by their hosts (Bowers 1981, Stamp 1982, Stamp 1992a). Harvey et al. (2005) found that the rate of development and the size of *C. melitaearum* parasitizing *M. cinxia* larvae did not vary with the iridoid content of the plants eaten by the host larvae in the laboratory. They used selected lines of *P. lanceolata* with high and low iridoid concentrations. On the other hand, in the field (Åland), where parasitoids may choose among hosts feeding on plants containing different iridoid concentrations, parasitism appears to be associated with low levels of the IG catalpol in the host plant (Nieminen et al. 2003).

The plant

Ribwort plantain (*P. lanceolata*) is a short-lived, perennial herb, with a world-wide distribution and a large ecological amplitude (Sagar and Harper 1964). In the Netherlands the species is typically found in roadsides, hayfields and dry grassland (Haeck 1992). The plant forms a rosette that can survive during the winter. Flowers are produced in spikes on elongated stalks. They are self-incompatible and each flower contains two ovules. It produces relatively large, smooth, oblong seeds about 3mm long weighing about 2mg each.

Herbivores

Specialists

Melitaea cinxia (Linnaeus, 1758) (Lepidoptera: Nymphalidae) - The checkerspot butterfly, Glanville fritillary, has a size of 30-40mm. They are found throughout Europe, northern Africa and in the east from Russia to West Asia. The numbers have declined and the distribution has become fragmented in northern Europe in the last decades. In the Netherlands it is a rare red list species, therefore the field experiments were carried out in Finland. *Melitaea cinxia* disappeared from the Finnish mainland in the late 1970's, but still lives in metapopulations on dry meadows on Åland.

On Åland the Glanville fritillary flies in June and lays eggs in large batches (150-250 eggs per batch on average) on two host plant species, *P. lanceolata* and *Veronica spicata* (Scrophulariaceae). The gregarious larvae hatch in July and spin a communal web inside which they feed on their host plant (Fig. 1.2B). In late August the fourth-instar larvae spin a much denser web in which they diapause over winter. These webs usually contain a group of full-sib larvae. In early April the post-diapause larvae continue to feed gregariously. They spend much time in the sun in compact groups to increase their body temperature and thereby to increase their rate of development. The larvae remain in groups until their fifth moult, after which they disperse and pupate in the vegetation close to the ground in the beginning of May. The caterpillars are attacked by two species of specialist parasitoids, which are the major sources of mortality, apart from variable weather conditions.

Melitaea athalia (Rottemburg, 1775) (Lepidoptera: Nymphalidae) - The heath fritillary is a common species in southern Finland (Marttila et al. 1990), but has declined severely in most of Europe (Warren et al. 1984, Schwarzwälder et al. 1997). It prefers edges of woodland and open woodland but can sometimes be found on flowery meadows. *Melitaea athalia* is part of a group of butterflies restricted to host plants that produce IGs. Its known host plants are Common Cow-wheat (Melampyrum), Figwort (Scrophulariacaea) and Plantains (Plantaginaceae). The adults fly from the end of May till the beginning of August. They lay egg clutches of 60-100 eggs. After hatching the caterpillars eat first gregariously on the oviposition plant (Warren 1987b, Wahlberg 1997). After the first or second molt in July or August they disperse and live solitary. They diapause as larvae and pupate (Fig. 1.2A) the following spring.

Junonia coenia Hübner, 1822 (Lepidoptera: Nymphalidae) - The common buckeye is widely distributed across the North American continent from south-eastern Canada throughout all the U.S. except the northwest. It prefers habitats that are generally open and sunny with low vegetation and bare grounds e.g., beaches and fields. Females lay eggs singly on leaf buds or on the upper side of the leaves of various hosts, including plants from the plantain family (*Plantago* spp), the snapdragon family (*Antirrhinum* spp.), as well as *Mimulus guttatus, Orthocarpus purpurascens, Veronica americana* and *Lippia nodiflora*. Iridoid glycosides serve as both larval feeding stimulants (Bowers 1984) and adult oviposition stimulants (Pereyra and Bowers 1988) for buckeyes. The caterpillars (Fig. 1.2 C) are solitary and eat leaves. Caterpillars and adults overwinter only in the south (Glassberg 2001).

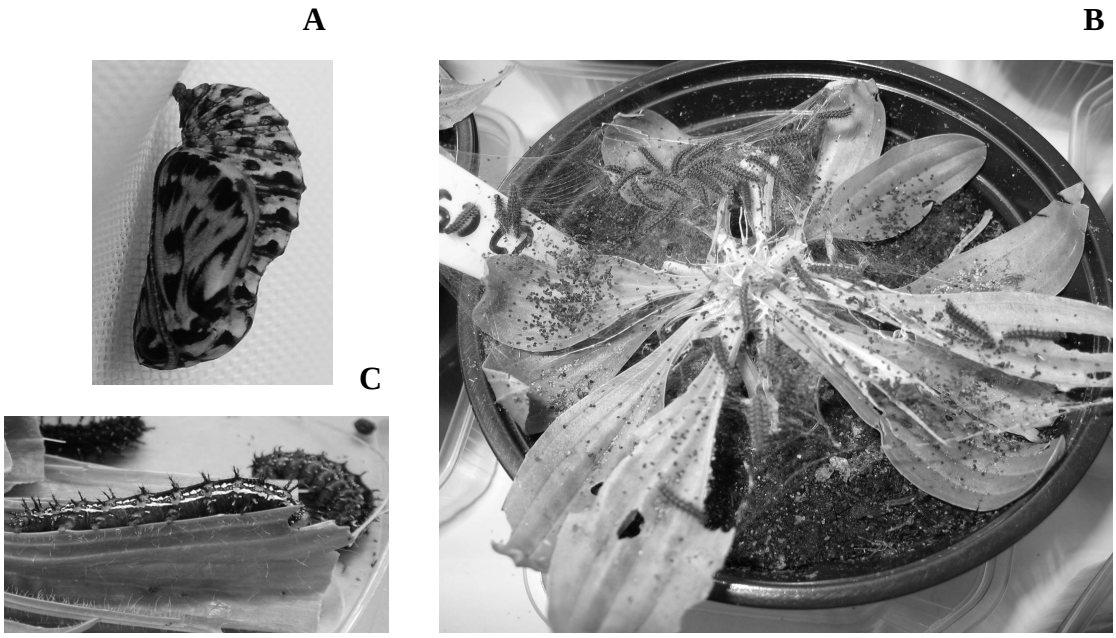


Figure 1.2 Specialist herbivores: A) pupae of *Melitaea athalia;* B) feeding nest of gregarious *Melitaea cinxia* on *Plantago lanceolata*; C) *Junonia coenia* larva feeding on *P. lanceolata*.

Generalists

Spodoptera exigua (Hübner, 1808) (Lepidoptera: Noctuidae) - The beet armyworm originated in southeast Asia. Their seasonal activity varies considerably according to the climate. The life cycle can be completed in as few as 24 days (Wilson 1934). The beet armyworm has a wide host range, occurring as a serious pest of vegetable, field and flower crops. The eggs are laid in clusters of 50-150 eggs per mass. They are usually deposited on the lower surface of the leaf. Eggs hatch in two to three days during warm weather. The larvae normally have five instars during a minimum of 16 days in summer months. Larvae feed on both foliage and fruit. Young larvae feed gregariously. As they mature, the larvae become solitary.Pupation occurs in the soil. The duration of the pupal stage is six to seven days during warm weather. Mating occurs soon after emergence of the moths, and oviposition begins within two to three days. Oviposition extends over a three to seven day period, and the moths usually die within nine to ten days after emergence (Heppner 1998).

Chrysodeixis chalcites (Esper, 1789) (Lepidoptera: Noctuidae) – The golden twinspot originates from southern Europe, the Canary Islands, Africa, Mauritius and the Cape Verde Islands (Zhang 1994). It lays it eggs one at the time or in small groups on a wide range of substrates (Harakly and Farag 1975). Early instar larvae are leaf skeletonizers. Later instars eat the entire leaf, at most leaving the midrib. They are very general feeders on many weeds and crops. Pupae are green with a brown dorsum, or totally brown, and are usually attached to the underside of leaves or any suitable substrate in a silken cocoon.

Parasitoids

The Glanville fritillary butterfly supports a community of eleven parasitoid species on Åland, southwestern Finland (Lei et al. 1997). There are two hymenopteran specialist parasitoids that interact most strongly with the Glanville fritillary butterfly on Åland (Fig. 1.3). These wasps differ greatly in phenology and dispersal behaviour, and consequently affect their host populations differently.

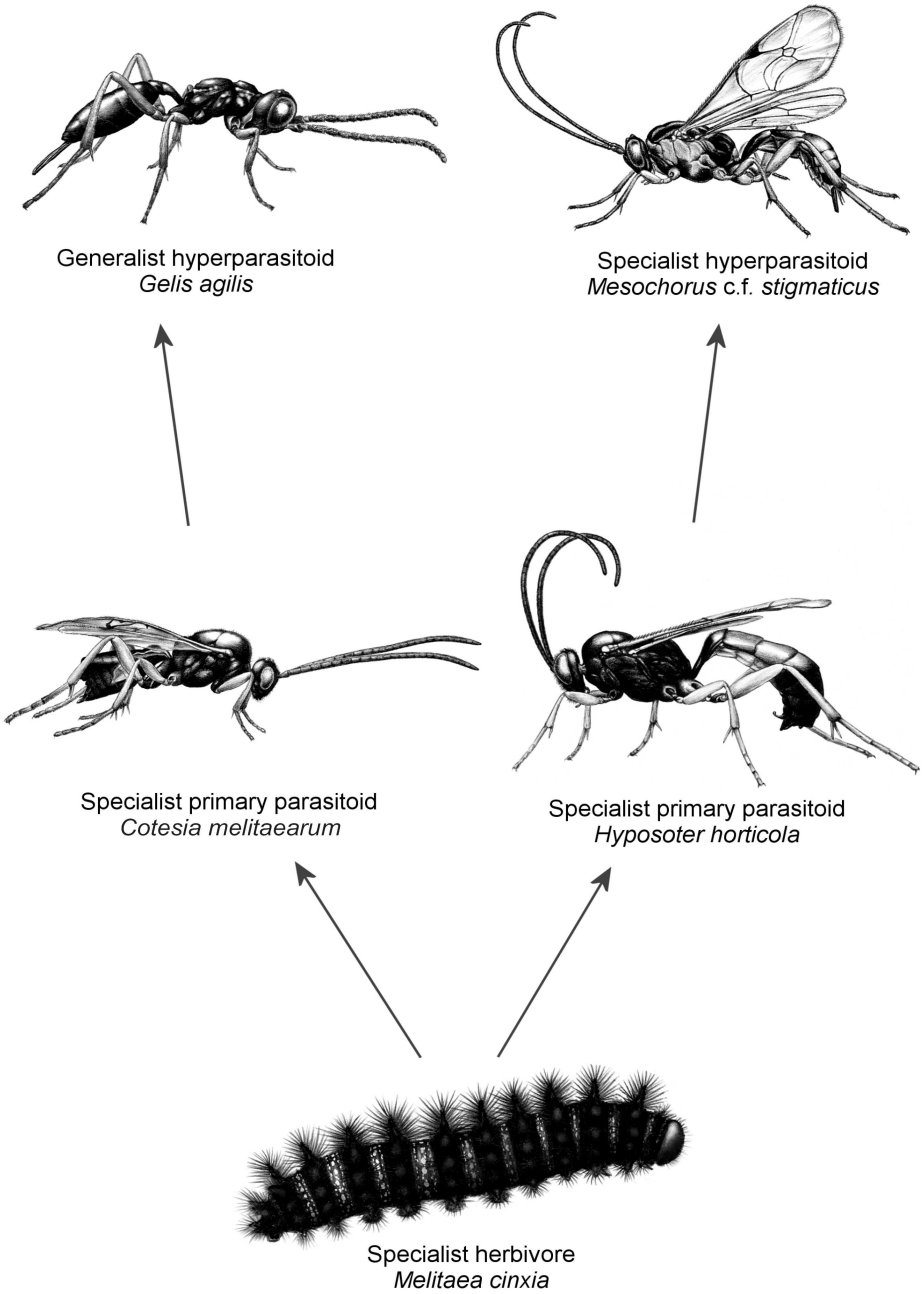


Figure 1.3 The parasitoid food web associated with *Melitaea cinxia* on Åland, involving two primary parasitoids (*Cotesia melitaearum* and *Hyposoter horticola*) and two hyperparasitoids (*Gelis agilis* and *Mesochurus* sp. cf. *stigmaticus*). Drawings by Zdravko Kolev.

Specialists

Cotesia melitaearum (Wilkinson, 1937) (Braconidae: Microgastrinae) – The wasp is a parasitoid of several species of checkerspot butterflies in Europe and Asia. This specialist gregarious koinobiont endoparasitoid has a high intrinsic rate of increase because it can lay many eggs in a single host and has three generations per host generation (year). However, it has a low dispersal ability and many natural enemies of its own. Most of the time the wasp is relatively rare on Åland and lives in small populations, because the habitat is fragmented and the host populations are ephemeral (van Nouhuys and Tay 2001). *Cotesia melitaearum* nevertheless plays an important role in the population dynamics of the Glanville fritillary. When host populations become large in tightly clustered habitat patch networks, the wasp population grows and can increase the risk of host population extinction (Lei and Hanski 1997).

Figure 1.4 A) The specialist parasitoid *Cotesia melitaearum* and B) *Hyposoter horticola* parasitizing *Melitaea cinxia* eggs.

Hyposoter horticola (Gravenhorst, 1829) (Ichneumonidae: Campopleginae) - The other parasitoid wasp, *H. horticola*, is a large and long-lived solitary koinobiont endoparasitoid of checkerspot butterflies in Europe and Asia, though its only certain host is *M. cinxia*. It can disperse extremely well, even to isolated and newly colonised local host populations. The population size of *H. horticola* is limited by the extremely short time that the female wasp can parasitize. She can only lay her eggs in fully formed larvae, which have not yet hatched from the eggs. These larvae are only available for a few hours. *Hyposoter horticola* influences its host population dynamics indirectly by uniformly reducing the host population size by about a third, thus leaving the butterfly exposed to extinction by other means.

Generalists

Cotesia marginiventris (Cresson, 1865) (Braconidae: Microgastrinae) - This wasp is a generalist solitary koinobiont endoparasitoid of noctuid moths. This parasitoid responds vigorously to contact kariomones present in the by-products of the host, such as silk, saliva, and exuviae, but response is strongest to host faeces and to the feeding damage caused by the host larvae (Loke and Ashley 1984, Dmoch et al. 1985). The wasp attacks mostly very young larvae (first to second instar). A single egg is usually laid in each host, and the cocoon hatches in seven to ten days. The host dies shortly after the parasitoid emerges. The exit hole in the side of the larva is only a superficial sign of the actual damage that occurred to the host. Practically all organs inside are consumed by the parasitoid.

Hyposoter didymator (Thunberg, 1822) (Ichneumonidae: Campopleginae) – This is a solitary koinobiont endoparasitoid which attacks noctuid larvae. This parasitoid is considered the most common attacker of species in the genera *Spodoptera, Heliothis* and *Helicoverpa* (Bar et al. 1979, Ingram 1981, Figueiredo et al. 2000). In a field study on maize in Turkey *H. didymator* was the most common and effective attacker of *S. exigua* (Sertkaya et al. 2004). The wasp larvae are tissue feeders and feed on the entire host to complete the development from the embryo to the third larval instar, when they emerge from the dead host and spin a silken cocoon in the vicinity.

Outline of the thesis

The efficiency of indirect defences depends on the effect of the natural enemy on the herbivore. Parasitoids are potential indirect defences of plants. They often have an optimal host instar in which they have the best performance. In **chapter 2** I study this optimal host size for a generalist parasitoid, *H. didymator*, and two of its natural hosts, *S. exigua* and *C. chalcites.*

In **chapter 3** I study the effects of the IGs aucubin and catalpol on the performance of the two generalist herbivores and parasitoids, and on two specialist herbivores, *J. coenia* and *M. cinxia* and one of their parasitoids *C. melitaearum*. Furthermore I look at the sequestration of the IGs in *M. cinxia* and its (hyper)parasitoid complex.

To study whether the preference of the adult *M. cinxia* female is in agreement with the performance of her offspring, I conduct oviposition choice experiments using *P. lanceolata* genotypes with different levels of secondary compounds, which are presented in **chapter 4.**

I also study the host plant use by the heath fritillary butterfly, *M. athalia* in **chapter 5**. Especially, I investigate the habitat preference, what host plant species it prefers for oviposition and the impact of the chemistry of these species. Additionally I conducte a larval performance study using known and potential host plants of this specialist.

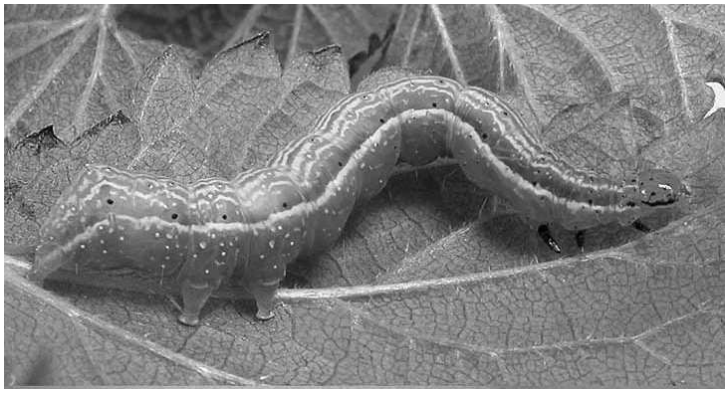
Because the production of constitutive defence chemicals such as IGs in the absence of herbivores will probably bring along allocation costs, in **chapter 6** I investigate the regrowth capability of *P. lanceolata* plants with different levels of secondary compounds. I test if having higher levels of IGs will reduce the capability of regrowth after clipping.

Finally, in **chapter 7** I discuss effects of IGs on herbivores and parasitoids and whether the observed patterns differ among generalist/specialist herbivore-parasitoid combinations. I speculate on compatibility or potential conflict between direct and indirect defences and evaluate the consequences of genotypic differences in direct defence for the oviposition choice of specialist herbivores and parasitoids and for plant fitness.

Chapter 2

Optimum and maximum host size at parasitism for the endoparasitoid *Hyposoter didymator* (Hymenoptera: Ichneumonidae) differ greatly between two host species

J. H. Reudler Talsma, J. A. Elzinga, J. A. Harvey and A. Biere



Abstract - Host size is considered a reliable indicator of host quality and an important determinant of parasitoid fitness. Koinobiont parasitoids attack hosts that continue feeding and growing during parasitism. In contrast with hemolymph-feeding koinobionts, tissue-feeding koinobionts face not only a minimum host size for successful development, but also a maximum host size, since consumption of the entire host is often necessary for successful egression. Here we study interactions between a generalist tissue-feeding larval endoparasitoid, *Hyposoter didymator* (Hymenoptera: Ichneumonidae), and two of its natural hosts, *Spodoptera exigua* and *Chrysodeixis chalcites* (Lepidoptera: Noctuidae). Larvae of *C. chalcites* are up to three times larger than corresponding instars of *S. exigua*, and also attain much higher terminal masses prior to pupation. We hypothesized that the range of host instars suitable for successful parasitism by *H. didymator* would be much more restricted in the large host *C. chalcites* than in the smaller *S. exigua*. To test this hypothesis we monitored development of *H. didymator* in all instars of both host species and measured survival, larval development time and adult body mass of the parasitoid. In contrast with our predictions, *C. chalcites* was qualitatively superior to *S. exigua* in terms of the survival of parasitized hosts, the proportion of parasitoids able to complete development and adult parasitoid size. However, in both hosts the proportion of mature parasitoid larvae that successfully developed into adults was low at the largest host sizes. Our results suggest that qualitative, as well as quantitative, factors are important in the success of tissue-feeding parasitoids.

Introduction

Parasitoid wasps have featured prominently in studies examining the evolution of life-history and development strategies in arthropods (Godfray 1994). Host size is often considered to be the most important parameter influencing parasitoid fitness because it is usually correlated with the total amount of resources available for the developing parasitoid offspring (Charnov 1982, King 1989, Mackauer and Sequeira 1993). This is especially true for idiobiont parasitoids that arrest host development immediately after parasitism (Askew and Shaw 1986). Host size at oviposition thus reflects the total amount of resources available to the offspring of such parasitoids (Mackauer and Sequeira 1993).

A separate group of parasitoids, collectively called koinobionts, attack hosts that continue feeding and growing after parasitism (Askew and Shaw 1986). For many koinobiont species, the size of the host at parasitism may be unrelated to its size when it is eventually killed by the immature parasitoid. The eggs or larvae of many koinobiont parasitoid species remain quiescent or develop only slowly until the host matures and either enters its final stage or pupates (Harvey et al. 1994, Harvey et al. 2000). Thus, although koinobionts may parasitize hosts that are much smaller than themselves, adult parasitoid size is often correlated with the final (terminal) size of the host (Sequiera and Mackauer 1993, Harvey et al. 1994, Harvey et al. 2004). In these species development time is consequently longer in small hosts and adult size is much less affected by variation in host size at parasitism (Vinson and Iwantsch 1980, Elzinga et al. 2003).

When oviposited into small hosts (e.g., early instars), the parasitoid larva will not be able to complete its development until the host grows large enough to provide enough resources for vital metabolic functions. Host species differing in growth rate and potential may reach this critical size for parasitoid maturation at a different moment during their development. In large or fast growing hosts this point may occur earlier than in small hosts or those that grow more slowly. Alternatively, some hosts may also grow too large to successfully support parasitoid development (Beckage and Templeton 1985, Harvey 1996). Large hosts often possess stronger immune defenses than smaller conspecifics (Strand and Pech 1995), or the parasitoid larvae are unable to consume excess host resources and perish trapped within the confines of the host integument (Beckage and Templeton 1985, Harvey 1996). Moreover, most insects are unable to complete their development in a wet environment (Strand 2000) and must seek drier conditions in order to pupate.

However, a more recently evolved trait for parasitoid larvae is to feed primarily on host hemolymph and fat body during their development and thus to consume only a fraction of available host resources. When they are mature, the larvae of hemolymph feeders emerge by perforating the cuticle with their mandibles (Nakamatsu et al. 2006). This enables these parasitoids to consume only the amount of resources that are necessary to complete their development, and reduces the selection pressure for a fixed maternal response for host-size at oviposition. This way they can develop in a very broad range of host sizes and instars (Harvey and Strand 2002). Although this strategy is still rare amongst parasitoid clades, it dominates the ichneumonid subfamilies Microgastrinae and Cheloninae (Gauld and Bolton 1988).

This study examines development of the solitary generalist koinobiont endoparasitoid *Hyposoter didymator* (Thunberg, 1822) (Hymenoptera: Ichneumonidae) in a range of instars in two of its hosts, *Spodoptera exigua* (Hübner, 1808) and *Chrysodeixis chalcites* (Esper, 1789) (Lepidoptera: Noctuidae: Plusiinae). Like its close relatives in the superfamily Campopleginae, such as *H. exiguae* and *V. canescens*, larvae of *H. didymator* must consume the entire host prior to pupation. Later instars of *S. exigua* and *C. chalcites* grow to sizes that are expected to constrain the development of *H. didymator*. Moreover, various instars of *C.*

chalcites are much larger than the corresponding instars of *S. exigua,* and larvae of the former species also attain significantly larger maximal masses just prior to pupation. Consequently, *C. chalcites* was expected to have a stronger limiting effect on the development of *H. didymator* than the smaller *S. exigua.* We specifically addressed the following questions; 1) What host instar/size is optimal for parasitoid development? 2) Is the optimum host instar/size similar in the two host species?

Materials and Methods

Herbivores

For the experiment we used two polyphagous hosts. The beet armyworm, *S. exigua*, originates in Southeast Asia, but has been introduced over many parts of the world. They normally have five larval instars, and mature larvae move to the soil where they pupate. The entire life cycle is about 4-5 weeks with several generations per year.

Chrysodeixis chalcites is native to central and southern Europe, the Canary Islands and Africa. It is an immigrant species in the Netherlands and it can only survive the winter in greenhouses where it can become a pest. *Chrysodeixis chalcites* larvae normally complete six instars before pupation. After feeding, the larvae spin a white cocoon in which they pupate (Goodey 1991). For both herbivore species continuous rearing in the laboratory is possible.

Parasitoid

Hyposoter didymator is native to the Palearctic realm and parasitizes caterpillars of many species of Noctuidae. In a field study on maize in Turkey *H. didymator* was the most common and effective attacker of *S. exigua* (Sertkaya et al. 2004). Parasitoid females attack all larval stages of *S. exigua* and *C. chalcites* when they are available. The wasp larvae feed on the entire host, usually only leaving the skin of the caterpillar. The parasitoid larva then spins a cocoon and pupates next to the carcass.

Rearing

Cultures of *S. exigua* were established from eggs originating from a laboratory culture maintained at the Department of Virology at Wageningen University, the Netherlands. *Chrysodeixis chalcites* cultures were reared from individuals collected from a garden in Nijmegen, the Netherlands. All cultures were kept in plastic Petri dishes on artificial diet (Elzinga 2002) at 25ºC and L:D 16:8. When caterpillars were in their final instar they were placed in plastic containers (15 x 10 x 10cm) with a small amount of diet and for *S. exigua,* with vermiculite in which they pupated. Adult moths were placed in a cage (40 x 50 x 65cm) in a climate room at 25ºC with 55% RH and L:D 16:8h with honey water (1:1) and one plant of *Plantago lanceolata*, that both noctuid species readily accept for oviposition. Newly hatched caterpillars were collected from this cage and placed individually in Petri dishes (8cm ø) on artificial diet.

Hyposoter didymator adults used for this experiment were obtained from a laboratory colony that was started in 1993 from wasps collected from parasitized *S. littoralis* in the south of Spain (Cordoba). Since 2001, they have been reared on *S. frugiperda* in the Laboratoire de Pathologie Comparée at INRA UMII, Saint-Christol-lèz-Aléz, France. Cultures of this parasitoid were kept at 10ºC and L:D 16:8h.

Experimental protocol

To initiate mating, a cage containing 10 males and 5 females of *H. didymator* was exposed to natural light conditions in front of a window. Parasitoid pairs that mated were removed from the cage and placed into a small vial with honey and maintained at 25ºC. Approximately 50 *S. exigua* caterpillars in each of the host instars (L1 to L5) were individually presented to single *H. didymator* females, and were allowed to be parasitized once. Parasitized hosts were placed singly into vials containing artificial diet. This process was repeated in L1-L6 instars of *C. chalcites*. Individual female parasitoids were allowed to parasitize up to 10 hosts randomly chosen from the two different species and different instars. After larval parasitoid emergence, the artificial diet was removed. In addition, the development of a separate cohort of unparasitized larvae (= control) was monitored in both *S. exigua* (n=125) and *C. chalcites* (n=150).

Comparing parasitoid development

In *H. didymator*, several fitness correlates were measured: larval and pupal survival, development time and adult weight. Larval survival was based on precocious host mortality during the experiment. If parasitized caterpillars died before egression, the parasitoid larvae were recorded as 'dead'. Larval development time was measured as the time between parasitism and egression, and pupal development time was defined as the number of days between larval egression and eclosion of the adult parasitoid. Adult body mass was measured on a microbalance (Mettler Toledo, Columbus, USA) after anesthetizing the wasp with CO_2. The number of caterpillars that pupated despite observed parasitism was also recorded.

Data analysis

Larval and pupal development time and adult parasitoid mass were analyzed using Generalized Linear Models (SAS v. 8.2, Procedure GENMOD, SAS Institute, Cary, N.C.) with a normal error distribution and identity link function and with host species, parasitized instar, and their interaction as factors in the model. For illustrative purposes, post-hoc contrasts between instars were calculated using Tukey's HSD test. Effects of host species and instar and their interactive effects on the probability that parasitized hosts survived, produced a successfully egressed parasitoid, or produced an adult parasitoid, were analyzed using GLM with a binomial error distribution and a logit link function (SAS v. 8.2, Procedure GENMOD). Weight variables were ln-transformed prior to analysis to meet assumptions of normality.

Results

Growth and development of unparasitized host species

The larval mass of the two host species was significantly different for each instar (Fig. 2.1; $F = 165.2$; df=1, 312; $P < 0.001$). First instar mass and pupal mass of *C. chalcites* were 4.2 and 3.6 times higher than those of *S. exigua*, respectively; the former species attained a mean pupal mass of approximately 250mg, whereas mean pupal mass in the latter species was approximately 70mg. However, the shape of the growth curves of the two species was quite different (Fig. 2.1). Whereas *C. chalcites* has a fairly constant relative growth rate, *S. exigua* has a high initial relative growth rate (e.g., in instars 1-3), but an earlier decay of growth (e.g., in instars 4-5). As a consequence, host mass showed a significant interaction between host species and instar ($F = 67.0$; df=5, 312; $P < 0.001$) and the mass of intermediate instars (L3 and L4) was actually higher for *S. exigua* than for *C. chalcites*. Despite the extra instar in *C.*

chalcites, the two host species had similar larval development times (mean ± s.e. for *C. chalcites:* 16.7 ± 0.6 days, *S. exigua*: 17.1 ± 0.2 days; F = 1.09; df=1, 116; P = 0.30). Development time from pupa to adult was, however, shorter in *C. chalcites* (8.1 ± 0.2 days) than in *S. exigua* (9.9 ± 0.1 days; F = 93.9; df=1, 105; P < 0.001).

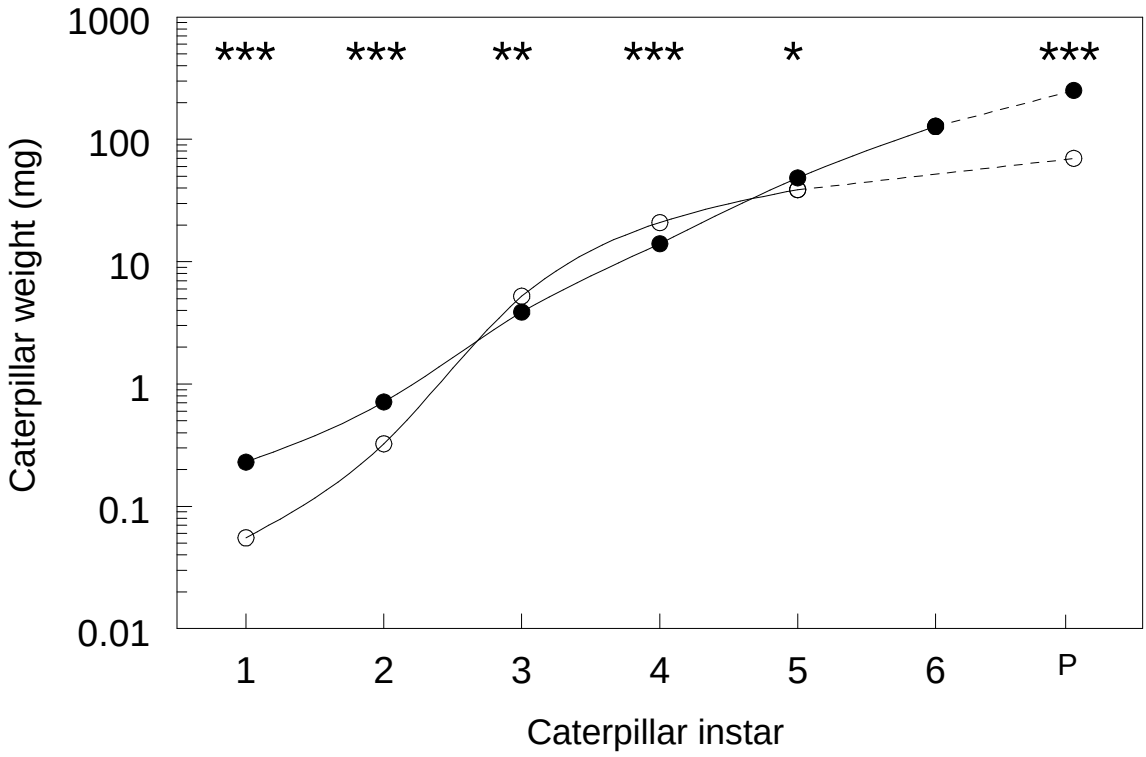


Figure 2.1 Growth trajectories of two host species, *Chrysodeixis chalcites* (closed symbols) and *Spodoptera exigua* (open symbols) across 6 and 5 larval instars, respectively, and pupal weight (P). Bars indicating ± 1 s.e. of the mean all are too small to extend beyond the symbols. Asterisks (top) indicate significance of differences in weight between host species at each larval stage (* P < 0.05; ** P < 0.01; *** P < 0.001).

Survival of parasitized host larvae

Survival of parasitized host larvae ranged from 7% to 85% between the different host species and instars (Figs. 2.2A and B). Survival was significantly affected by the host instar that was parasitized (F = 27.14; df= 5, 529; P < 0.001). Survival of parasitized *C. chalcites* was on average twice as high (56%) as survival of parasitized *S. exigua* (28%; F = 47.50; df= 1, 529; P < 0.001) but the magnitude of the difference depended on the instar that was parasitized (interaction host species x instar: F = 47.20; df= 4, 529; P < 0.001), being most pronounced in L3 (Figs. 2.2A and B).

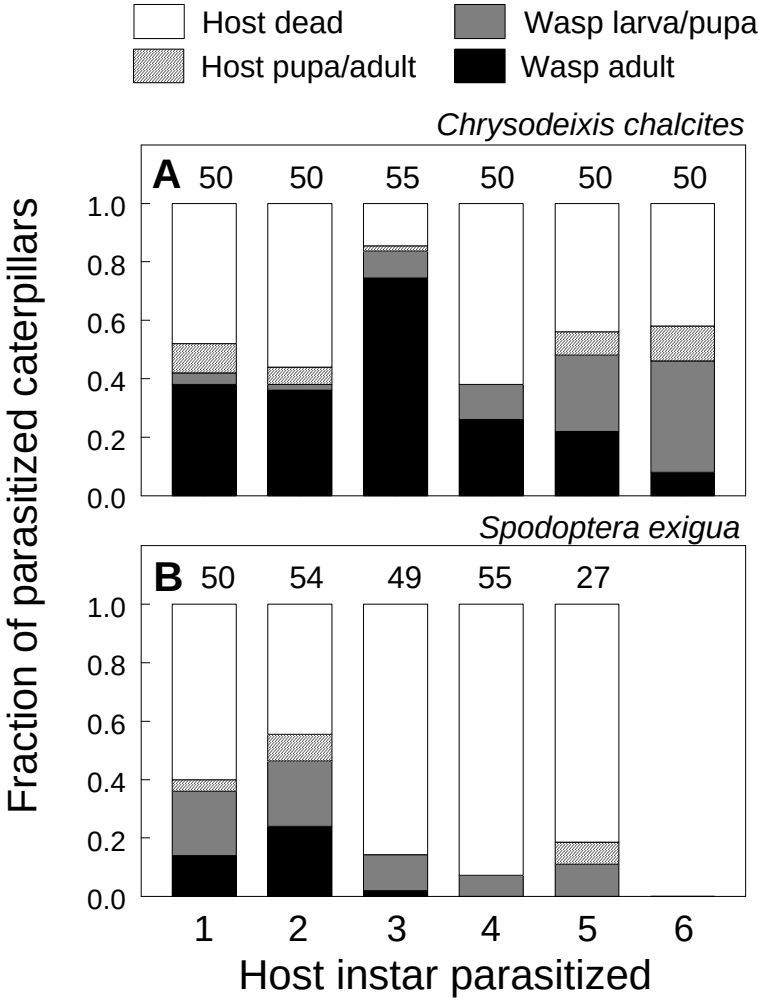


Figure 2.2 Fate of caterpillars of *Chrysodeixis chalcites* (A) and *Spodoptera exigua* (B) parasitized at different larval stages (*S. exigua* only has five instars). White: proportion of caterpillars that died. Striped: proportion of caterpillars that pupated. Grey: caterpillars successfully parasitized (parasitoid larva egresses) but parasitoids fail to develop further than larval or cocoon stage. Black: caterpillars successfully parasitized and emerging parasitoids complete development to adult stage. Sample sizes are indicated at the top.

Effects of host species and parasitized instar on parasitism rate

The proportion of surviving host larvae that were successfully parasitized by *H. didymator* (i.e., in which wasp larvae successfully eggressed) was slightly higher in hosts that were parasitized as L3-4 than in hosts that were parasitized as earlier or later instars ($F = 3.44$; df=5, 226; $P < 0.004$). However, this proportion did not differ between the two host species ($F = 0.28$; df=1, 228; $P > 0.5$). On the other hand, the percentage of wasp larvae that successfully completed their development, and produced adult wasps (Figs. 2.2A and B), was almost twice as high in *C. chalcites* (69.7%) as in *S. exigua* (36.8%; $F = 40.41$; df=1, 198; $P < 0.001$). This percentage was also much higher in hosts that were parasitized as early instars than in hosts parasitized as later instars ($F = 10.23$; df=5, 198; $P < 0.001$), independent of host species (no interaction host species x instar: $F = 0.80$; df=4, 198; $P = 0.41$). In *S. exigua*, host larvae parasitized as L4 and L5 both failed to produce adult wasps. However, *H. didymator* could successfully develop into the adult stage, in all instars of *C. chalcites*, although it left significantly more host tissue behind when parasitoids developed in larger hosts (L5 and L6) than in smaller hosts.

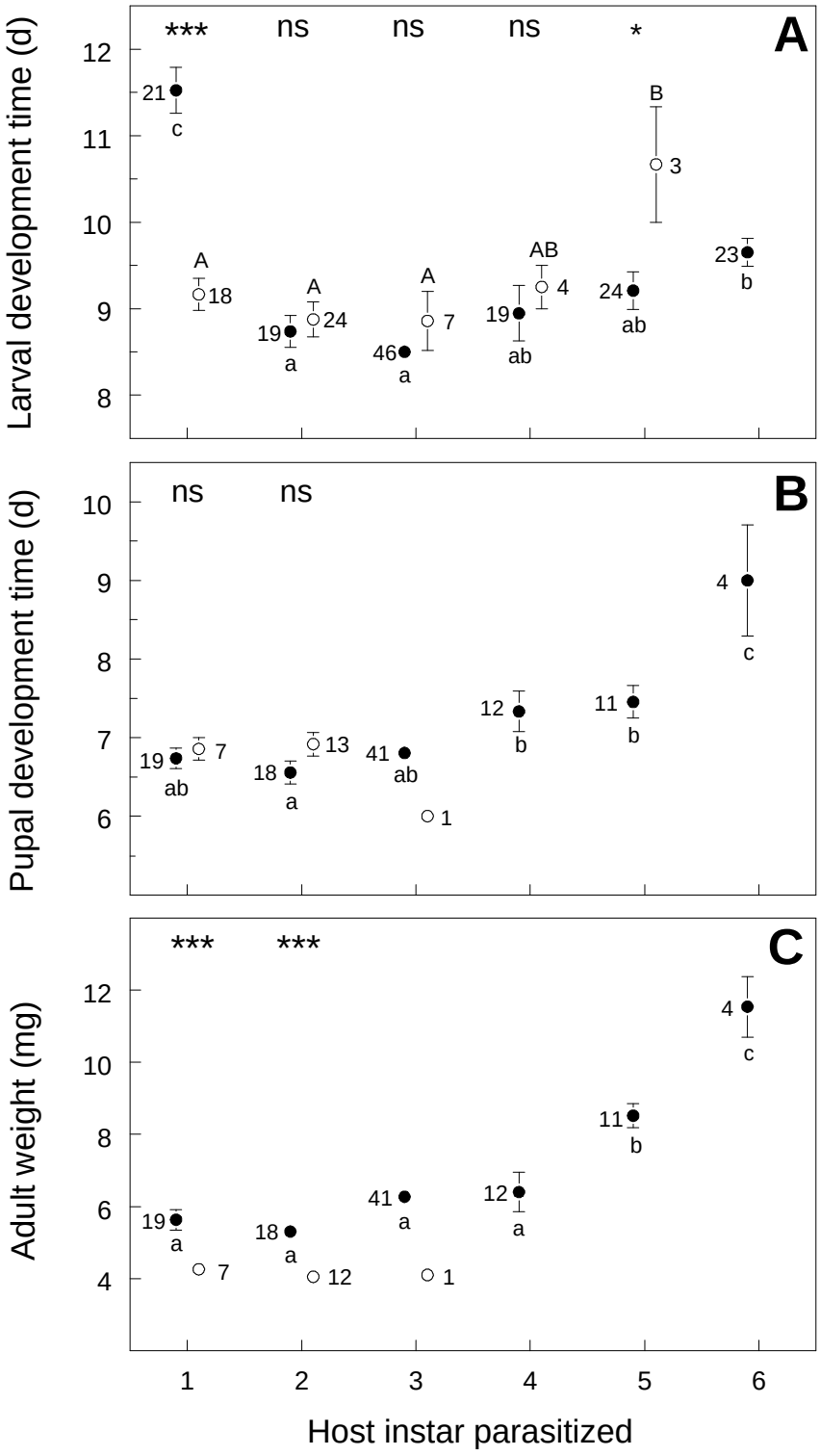


Figure 2.3 Development of *Hyposoter didymator* in the host species *Chrysodeixis chalcites* (closed symbols) and *Spodoptera exigua* (open symbols) parasitized at different larval stages. A) Time from parasitism to larval egression; B) Time from larval egression to eclosion of adult wasp; C) Weight of adult wasps. Bars indicate ± 1 s.e. of the mean. Significance of differences between host species are displayed at the top for each instar separately (ns: not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Sample sizes and differences between instars within host species (non-shared letters) are given to the left and below (*C. chalcites*) or to the right and on top of symbols (*S. exigua*), respectively.

Effects of host species and parasitized instar on parasitoid development time and size

Larval development time of *H. didymator* varied significantly with host instar at parasitism (Fig. 2.3A; $F = 6.51$; df= 5, 226; $P < 0.001$). In *C. chalcites*, mean larval development time exhibited a 'u-shape' across the different host stages. For *S. exigua* larval development time was longest at the later instars. There were, however, marked host-interspecific differences in larval parasitoid development time (interaction host species x instar: $F = 4.67$; df= 4, 226; $P = 0.001$; Fig. 2.3A). When parasitizing the first host instar, *H. didymator* progeny had a significantly longer larval development time on *C. chalcites* ($P < 0.001$), whereas in the fifth instar development time was much longer in *S. exigua* ($P < 0.05$).

Parasitoid pupal development time did not differ between the two host species (Fig. 2.3B; $F = 0.19$; df= 1, 118; $P > 0.5$). In *S. exigua*, where wasps were only able to complete their development to adult when L1-L3 instars were parasitized, development time was independent of host instar. By contrast, in *C. chalcites* parasitoid pupal development time was affected by host instar ($F = 47.84$; df= 1, 100; $P < 0.001$) with the duration of the pupal period extended in hosts parasitized as later instars (Fig. 2.3B).

Adult body mass of *H. didymator* was larger for wasps reared from *C. chalcites* than from *S. exigua* (Fig. 2.3C; $F = 20.4$; df= 1, 117; $P < 0.001$). The magnitude of the difference between host species was independent of the instar that was parasitized (interaction host species x instar: $F = 0.35$; df= 2, 117; $P > 0.5$). In *C. chalcites*, parasitoid adult mass was significantly affected by instar ($F = 47.43$; df= 5, 99; $P < 0.001$); wasps developing in later instars (L5, 6) were larger than conspecifics developing in early instars (Fig. 2.3C).

Discussion

This study has revealed that the development of *H. didymator* varied significantly with the host species on which it was reared, and that the host *C. chalcites* was both qualitatively and quantitatively superior to *S. exigua*. Although pupal mass of *C. chalcites* was more than three times greater than that of *S. exigua*, larval masses of L3 and L4 instar *S. exigua* were significantly greater than those of corresponding instars of *C. chalcites*. However, *H. didymator* was rarely able to develop in those or later instars of *S. exigua*. Parasitoid survival was typically much lower in *S. exigua* than in *C. chalcites*, even in the three instars of *S. exigua* that were suitable for the development of *H. didymator*, and even when *S. exigua* was larger, i.e., in the 3rd instar. By contrast, *H. didymator* was able to complete development to the adult stage in all parasitized instars of *C. chalcites*. Furthermore, adult parasitoids developing in all instars of *C. chalcites*, were much larger than parasitoids developing in the same stages of *S. exigua*. It is possible that the terminal mass of *C. chalcites* larvae when they were destroyed by *H. didymator* was greater than in *S. exigua* larvae, although this was not measured here.

The hypothesis that larvae of tissue feeding parasitoids such as *H. didymator* are constrained when developing in later instars of large hosts, such as *C. chalcites*, as opposed to smaller hosts, such as *S. exigua*, was not supported by our data. Many studies have reported that the growth and development of both tissue and hemolymph-feeding koinobiont parasitoids is affected by the size, age or stage of the parasitized host (Harvey et al. 1994, Harvey et al. 2004, Elzinga et al. 2005). Until recently, maximum host size was thought to be the main constraint on parasitoid fitness, because parasitoid size is often correlated with this parameter (Charnov 1982, King 1989, Mackauer and Sequeira 1993, Godfray 1994). However, Harvey et al. (2004) recently challenged this assertion by arguing that the benefits

of increased size are often more than offset by a concomitant increase in larval parasitoid developmental mortality in large (compared to small) hosts.

Harvey and Strand (2002) compared host-size related constraints on the development of a hemolymph feeding parasitoid, *Microplitis croceipes,* and of a tissue-feeding parasitoid, *Campoletis sonorensis.* Both species are of similar mass as adults (~ 7mg) and have overlapping host ranges. However, whereas *M. croceipes* progeny successfully completed their development in hosts ranging in size from 0.100mg to 350mg at oviposition, *C. campoletis* progeny perished in host weighing over 70mg at the time they were parasitized. Adult *C. campoletis* females readily oviposited into these host caterpillars, but their progeny was unable to consume excess host resources and eventually perished. By contrast, mature larvae of *M. croceipes* experienced no problem in emerging from these very large final instar larvae of their host, *Heliothis virescens*.

In contrast with the results obtained with *C. sonorensis* and other closely related tissue feeders in the Campopleginae of similar adult size (e.g., *H. exiguae, V. canescens*) *H. didymator,* was capable of successfully disposing of resources in even very large *C. chalcites* caterpillars. This includes larvae that exceeded 130mg at parasitism, which is approximately twice as heavy as hosts that can support the development of the similarly sized species *V. canescens* (Harvey 1996) and *C. sonorensis* (Harvey and Strand 2002). Also, failure of *H. didymator* to successfully complete their development in larger larvae of (L4-L5) *S. exigua* that weighed only between 20 and 40mg therefore cannot be explained in terms of a maximum host size constraint. Although we do not know the exact natural host range of *H. didymator,* it is likely that sympatrically occurring native noctuid herbivores in Eurasia, such as *C. chalcites*, are parasitized in the field. Some of the hosts known to be successfully parasitized by *H. didymator* in nature, *Spodoptera litura* and *S. litoralis*, even grow larger then *C. chalcites* in their final instars (Perveen 2000, Morales et al. 2007). However, it is not known if these late instars are parasitized in nature and if *H. didymator* can successfully develop in them. Furthermore, given resource-related constraints on related species, it is likely that *H. didymator* would be similarly constrained in disposing of excess host tissues in hosts over 130mg. We observed that significantly more host tissues were left behind by the mature parasitoid larva after feeding than was the case when the parasitoid developed in small hosts, as has been observed in other Campopleginae (Beckage and Templeton 1985, Harvey 1996, Harvey and Strand 2002). However, for *H. didymator*, the cuticle of even large hosts was obviously thin enough for the mature parasitoid larva to split via peristaltic movements, enabling them to initiate pupation. With the exception of L3 larvae, where success was higher, the egression percentage was approximately 40% in all other instars. Following egression, however, the ability of parasitoid larvae to successfully spin cocoons was less in older than in younger hosts. This suggests that the parasitoid may still be forced to overeat in large hosts and that this factor reduces their ability to pupate. There seems to be a fitness trade-off for *H. didymator* when developing in larger instars of *C. chalcites*; adult size, which in many parasitoid species is correlated with more offspring (Visser 1994, Harvey et al. 2001), is greatest in the largest (L5-6) host instars, whereas parasitoid survival is lowest in these host instars.

In summary, we have shown that the development of *H. didymator* varies significantly with the host species on which it was reared. In contrast to what was expected, *C. chalcites* was both a qualitatively and quantitatively superior host to *S. exigua*. The optimal instar for parasitoid development differed between the two host species: in *S. exigua,* L2 hosts were of the highest quality whereas in *C. chalcites* parasitoids performed best on L3 hosts. Most importantly, the results of this and related studies reveal that the nutritional ecology of host-

parasitoid interactions are exceedingly complex, and that parasitoid phenotypes and fitness parameters are affected by host characteristics in an association-specific manner.

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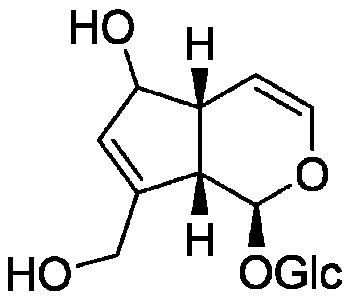
Chapter 3

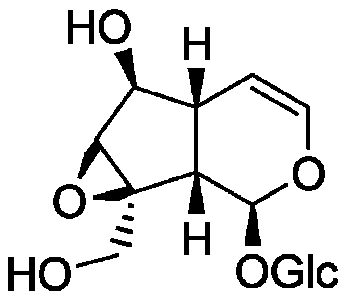
Performance of specialist and generalist herbivores using chemically defended *Plantago lanceolata*: the sequestration of iridoid glycosides up to the hyperparasitoids

J. H. Reudler Talsma, A. Biere, S. van Nouhuys and J. A. Harvey

aucubin



catalpol



Abstract - One of the defence mechanisms of plants against herbivores is the production of allelochemicals. However, the effects of these defence compounds is not necessarily restricted to herbivores because they are also experienced by higher trophic levels, including predators and parasitoids. In this study we examined the effects of two defence chemicals of *Plantago lanceolata*, the iridoid glycosides aucubin and catalpol, on the performance of two generalist and two specialist herbivores and their endoparasitoids. Furthermore, we studied the sequestration of these chemical compounds in the herbivore-parasitoid complex of the specialist herbivore *Melitaea cinxia*. In general, the performance of generalist herbivores was negatively correlated with the levels of iridoid glycosides but effects on their parasitoids were less apparent. On the other hand, effects on specialist herbivores differed between *M. cinxia* and the other specialist herbivore, *Junonia coenia*. Larval development time of *J. coenia* was longer and pupal mass lower when feeding on plants containing high iridoid glycoside levels, whereas these traits were reversed in *M. cinxia.* Similarly, development of *Cotesia melitaearum*, a gregarious endoparasitoid of *M. cinxia* caterpillars, benefited when the wasps developed in larvae reared on high level *P. lanceolata* genotypes. Iridoid glycosides were detected in all tissues and larval, pupal and adult stages of the specialist herbivore *M. cinxia*, in its endoparasitoids and in two hyperparasitoids. This is the first study to report that allelochemicals may be sequestered up to the fourth trophic level. The fraction of catalpol, the more toxic of the two iridoid glycosides, in pupae and adults increased with trophic level.

Introduction

Plants have evolved a wide array of direct defence strategies that enable them to survive in potentially hostile habitats. Direct defences aimed at herbivores include morphological traits, e.g., the production of hairs, spines and trichomes or waxy layers on the leaves (Cooper and Owen-Smith 1986) and chemical toxins, repellents or digestibility reducers (Fraenkel 1959, Ehrlich and Raven 1964). Direct defences may repel or deter herbivorous insects prior to or during ingestion, exert post-ingestive toxic effects or exhibit a combination of both (Berenbaum and Zangerl 1988).

Direct defences may have positive and negative effects on natural enemies of herbivores (e.g., parasitoids). Positive effects may occur if natural enemies can exploit herbivore populations more effectively when the herbivores have a reduced growth rate as a result of ingesting toxic allelochemicals (the "slow growth-high mortality hypothesis", Clancy and Price 1987, Damman 1987).

Plant allelochemicals may also impede the optimal functioning of the herbivore's immune system. One of the mechanisms employed by herbivores to defend themselves against parasitoids is by encapsulating parasitoid eggs or larvae through the action of haemocytes and granulocytes (Nappi 1975, Lackie 1988, Strand and Pech 1995). The success of the encapsulation reaction is often influenced by the health of the herbivore, and this can be affected by a range of physiological stresses including those that are induced by the host plant as a result of poor nutritional quality, starvation, or allelochemicals. A plant with toxins or low nutritional quality may suppress the insect's immune system benefiting the parasitoids (Kraaijeveld and Godfray 1997, Ojala et al. 2005). This can lead to stronger pest suppression.

Negative effects of allelochemicals on natural enemies of herbivores may occur if phytotoxins that are consumed by the herbivore have deleterious effects on the growth, development, or survival of predators and parasitoids (Campbell and Duffey 1981, Barbosa et al. 1986, Gunasena et al. 1990, Barbosa et al. 1991, Paradise and Stamp 1993, Harvey et al. 2003, Harvey et al. 2005). Although many cryptically-coloured herbivores detoxify or excrete plant allelochemicals (Brattsten 1988), other species actively sequester them in their hemolymph where they function as a chemical defence against their natural enemies (Bowers 1980, 1981, Camara 1997b, Hunter 2000, Nishida 2002). Endoparasitoids are particularly susceptible to sequestered allelochemicals, because in these insects the alimentary tract is not externally connected until after the parasitoid larva egresses from the host (Quicke 1997). Consequently, plant toxins accumulate in the body of parasitoid larva and have been recovered from parasitoid by-products including cocoon silk and meconium (Barbosa et al. 1986, Bowers 2003).

When direct defence chemicals are sequestered by herbivores and have a harmful effect on their natural enemies, there is thus a potential conflict between the direct and indirect defence systems of the plant. It is in the plant's interest to produce substances that have a negative impact on the herbivore, but without impeding the natural enemies of the herbivore. In cases where plants have a higher fitness when herbivores are parasitized, it can be expected that natural selection has favoured individuals that employ chemical defences, which have a minimal negative impact on the natural enemies of herbivores (Turlings and Benrey 1998).

Defence mechanisms often function differently between generalist and specialist herbivores. Whereas generalist herbivores are often repelled or poisoned by the direct defence chemicals of plants (Feeny 1976), specialists usually deal more or less effectively with the defence of a particular plant species or group of plant species (Renwick et al. 2001).

Herbivores may also use the plant defensive system to which they are adapted as host-finding cues and feeding stimulants (Bowers 1981, Rhoades 1985). A number of specialist herbivores even sequesters these substances in their own body as a putative defence against their own natural enemies (Bowers 1981). For sequestration a herbivore probably would need physiological or morphological adaptations for the prevention of autotoxity.

The same distinction between generalists and specialists also applies to parasitoids. Some specialised parasitoids are restricted to attacking only one or a few related host species that feed on plants in the same family (Godfray 1994, Quicke 1997). These parasitoids are exposed to a limited set of allelochemicals in the host diet to which they may be adapted (Harvey et al. 2005). By contrast, generalist parasitoids may attack a wide range of host species that in turn feed on plants producing very different kinds of allelochemicals. These parasitoids may suffer during development if challenged by novel chemical compounds in hosts to which they are not adapted (Barbosa 1988, Barbosa et al. 1991, Harvey et al. 2005). Consequently, specialist parasitoids can often deal with herbivores that have consumed plant material containing high concentrations of chemical defences (van der Meijden 1996, Stamp 2001). By contrast, generalist parasitoids may be more efficient at exploiting hosts feeding on less-well defended plants.

In this study we use genotypes of ribwort plantain (*Plantago lanceolata*) that differ in their levels of two main defence chemicals, the iridoid glycosides (IGs) aucubin and catalpol, to study effects of allelochemical variation in the host plant on its specialist and generalist herbivore-parasitoid associations. IGs are monoterpenoids, and some, including aucubin and catalpol, are toxic or deterrent to generalists (Bernays and DeLuca 1981, Bowers and Puttick 1988, Puttick and Bowers 1988, Bowers and Puttick 1989). At the same time these compounds serve as feeding and oviposition stimulants for some species of specialist insects (Bowers 1984, Pereyra and Bowers 1988, Nieminen et al. 2003, Chapter 4). In the biosyntheses of IGs in plants, aucubin is the precursor of catalpol (Damtoft et al. 1983) which is the more toxic of the two (Bowers 1991).

Since sequestration of allelochemicals may profoundly affect the performance of higher trophic levels (Barbosa 1988, Barbosa et al. 1991), we additionally studied qualitative and quantitative aspects of sequestration of these compounds in various developmental stages/tissues of herbivores, parasitoids and hyperparasitoids in one of the specialist associations. It is known from previous studies that the larvae and adults of the specialist butterfly *Melitaea cinxia* sequester aucubin and catalpol at high concentrations (Suomi et al. 2001b, Suomi et al. 2003). However the effect of sequestration of these defence compounds on higher trophic levels has not been studied. The higher trophic levels can accumulate the iriroid glycosides from the lower trophic level they are eating, which can function as a defence against there own enemies. However, the sequestration of IGs in herbivores could also have consequences for the performance of the trophic level directly above. In this study we address the following three questions:

1. Do plant genotypes selected for high levels of direct chemical defence have a stronger negative effect on the performance of herbivores and on their parasitoids than plant genotypes selected for low levels of direct defence?
2. Do observed patterns in the effect of allelochemicals on insect performance differ among generalist/specialist herbivore-parasitoid combinations?
3. Are allelochemicals of *P. lanceolata* sequestered in a specialist herbivore, and can these IGs be detected in its parasitoids and hyperparasitoids?

Materials and methods

Plants

Plantago lanceolata L. (ribwort plantain) (Plantaginaceae) is a rosette-forming, self-incompatible, perennial plant with a worldwide distribution and large ecological amplitude (Sagar and Harper 1964). Among the secondary plant compounds produced by *P. lanceolata* are the two iridoid glycosides (IGs) aucubin and catalpol (Duff et al. 1965, Bobbitt and Segebarth 1969). In natural populations IG levels range from undetectable to ca. 12% of its dry weight (Bowers et al. 1992b, Bowers and Stamp 1992, Fajer et al. 1992). The variation in the constitutive IG amount in *P. lanceolata* is partially genetically determined (Bowers and Stamp 1992, 1993, Adler et al. 1995, Marak et al. 2000), and also varies with the developmental state of the plant, including leaf and plant age, and attributes of the environment such as time of day, weather, soil nutrient conditions and presence of arbuscular mycorrhizal fungi (Teramura 1983, Bowers 1991, Bowers et al. 1992b, Bowers and Stamp 1992, Fajer et al. 1992, Bowers and Stamp 1993, Darrow and Bowers 1997, Chapter 6). The plants used for the performance experiments were derived from an artificial selection experiment, in which plants were selected on the basis of high and low concentrations of total leaf IGs for four generations (Marak et al. 2000). Seven plants, each derived from a different half-sib family from plants selected for low concentrations, and six plants, each derived from a different half-sib family from plants selected for high concentraions, were clonally propagated following a root-cloning method (Wu and Antonovics 1975). This resulted in a maximum of 13 different genotypes available for the experiments. The IG levels in these genotypes varied from 0.20 to 12.8% of the dry weight. The level of aucubin was higher than the level of catalpol in all genotypes.

For the experiments with *M. cinxia* and *Cotesia melitaearum* and for the sequestration experiment we additionaly used *P. lanceolata* plants that were collected from Åland, Finland. In previous studies with plants from Åland IG levels varied from 0 to 4.5% of the dry weight (Nieminen et al. 2003, Saastamoinen et al. 2007). Furthermore, catalpol concentrations in these plants were higher than aucubin concentrations (Nieminen et al. 2003, Saastamoinen et al. 2007), which is the opposite in plants from the artificial selection line (Marak et al. 2000).

In total, we tested the effects of feeding on these different plant genotypes for seven insect species: two generalist herbivores, two generalist endoparasitoids raised on each of these generalist herbivores, two specialist herbivores and one specialist endoparasitoid raised on one of the specialist herbivores. The actual number of plant genotypes used in these experiments varied, depending on availability of sufficient leaf biomass per genotype.

Herbivores in the feeding study

Generalists - The beet armyworm, *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae), originates from southeast Asia, but has been introduced over many parts of the world. The caterpillar is polyphagous and feeds on many herbaceous plants and is a serious pest of crops. Female moths lay several mass clusters of 50-100 eggs, which hatch in 2-3 days. They normally have five larval instars, and mature larvae move to the soil where they pupate. Adult moths emerge about 1 week later, mating occurs soon after emergence, and oviposition begins within two to three days. The entire life cycle is about 4-5 weeks with several generations per year. In the laboratory continuous rearing is possible (Wilson 1934, Tingle and Mitchell 1977).

The golden twinspot, *Chrysodeixis chalcites* (Esper, 1789) (Lepidoptera: Noctuidae) is native to central and southern Europe, the Canary Islands and Africa. Like *S. exigua*, it is a

highly polyphagous crop pest. Eggs are laid singly or in small groups on a wide range of substrates. One female can lay several hundred eggs in her lifetime. Eggs hatch in 4-9 days, and *C. chalcites* larvae normally complete six instars before pupation. After feeding, the larvae spin a white cocoon in which they pupate. The adult moths emerge in 6-15 days, with the entire life cycle being completed in about 5-7 weeks (Goodey 1991).

Specialists - The buckeye butterfly, *Junonia coenia* Hübner, 1822 (Lepidoptera: Nymphalidae), is a common partially migratory species that occurs over most of the United States and southern Canada (Scott 1975). It is a chemically specialized herbivore that is restricted to feeding on four plant families, all of which contain IGs (Bowers 1984). Larvae sequester two similar iridoid compounds, aucubin and catalpol, in their hemolymph (Bowers and Puttick 1986, Bowers and Collinge 1992). These compounds can be a deterrent to both invertebrate (Stamp 1992b, de la Fuente et al. 1994, Camara 1997b) and vertebrate (Bowers 1980, 1981, Bowers and Farley 1990) predators. The adults, however contain no sequestered iridoids (Bowers and Collinge 1992). In northern California, buckeye larvae feed primarily on the introduced ribwort plantain (Scott 1972, Shapiro 1978).

The Glanville fritillary, *Melitaea cinxia* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) inhabits open grassland throughout Europe and temperate Asia. Severe population declines are reported in many European countries (Ehrlich and Hanski 2004). Throughout most of their range they have one generation per year. In her lifetime, a female lays several large egg clusters (150-200 eggs) underneath the leaves of host plants in the genera *Plantago* and *Veronica* (Kuussaari et al. 2004). Larvae hatch after two to four weeks and the larvae spin a communal web on the host plant and feed gregariously throughout most of their development. Iridoid glycosides are known to increase their rate of development (Harvey et al. 2005, Saastamoinen et al. 2007), are sequestered by the larvae (Suomi et al. 2001b, Suomi et al. 2003), and are positively associated with oviposition (Nieminen et al. 2003, Chapter 4). The *M. cinxia* in this study are from Åland, where there is a well-established metapopulation, feeding primarily on *P. lanceolata* (Kuussaari et al. 2004, Nieminen et al. 2004).

Parasitoids in the feeding study

Generalists - *Hyposoter didymator* (Thunberg, 1822) (Hymenoptera: Ichneumonidae) is a solitary endoparasitoid that parasitizes caterpillars of many species in the large family Noctuidae. These include species in the genera *Spodoptera*, *Heliothis* and *Helicoverpa* in several European countries (Bar et al. 1979, Ingram 1981, Vinson 1990, Figueiredo et al. 2000, Schneider et al. 2003). In a field study on maize in Turkey *H. didymator* was the most common and effective attacker of *S. exigua* larvae (Sertkaya et al. 2004). Parasitoid females attack all larval stages of *S. exigua* and *C. chalcites* when they are available. The wasp larvae are tissue feeders and feed on the entire host, usually only leaving the skin of the caterpillar. Mature larvae spin a cocoon and pupate next to the caterpillar carcass.

Cotesia marginiventris (Cresson, 1865) (Hymenoptera: Braconidae) is a fairly generalized solitary endoparasitoid of noctuid moths (Krombein et al. 1979). Females possess short ovipositors and preferentially attack young (first and second instar) larvae or even mature embryos in eggs just prior to hatching. A single egg is usually laid in each host and the larva hatches approximately two days after oviposition. The parasitoid larva feeds primarily on host hemolymph and fat body during its development. After 7-10 days the mature larva then egresses from the host and spins a cocoon, and the adult wasp emerges about another week later. The host dies shortly after the parasitoid emerges. In the laboratory, adult *C. marginiventris* can live up to several weeks and the female is ready to oviposit within 1-2 days of emergence (Snajder and Harvey 2003).

Specialist - *Cotesia melitaearum* (Wilkinson, 1937) is a primary gregarious parasitoid of *M. cinxia* (Nixon 1974). The adult female parasitoid lays broods of 1-40 eggs (depending on host size) in all instars of *M. cinxia* larvae. The *C. melitaearum* used in this study are from Åland. On Åland the wasp has two or sometimes three generations per year, and spends the winter as a larva inside of the host larva. The *C. melitaearum* that parasitizes *M. cinxia* on Åland has a metapopulation structure. (Lei et al. 1997, van Nouhuys and Hanski 2002a).

Insects used for sequestration data

For the study of the sequestration of aucubin and catalpol we used the herbivore-parasitoid complex of the specialist herbivore *M. cinxia* (see above).

Parasitoids - Besides the specialist parasitoids *C. melitaearum* (see above), we also used *Hyposoter horticola* (Gravenhorst, 1829) (Ichneumonidae: Campopleginae). This is a solitary endoparasitoid with a single generation per host generation. *Hyposoter horticola* is restricted to a few Melitaeini, and *M. cinxia* is its only host on Åland (Lei et al. 1997, van Nouhuys and Hanski 2002b). Although it is a larval parasitoid, it lays eggs in the host larva while it is still inside the eggshell (van Nouhuys and Ehrnsten 2004). The wasp develops within the host larva and pupates inside the host integument the next spring.

Hyperparasitoids - *Hyposoter horticola* is parasitized by the solitary ichneumonid hyperparasitoid *Mesochorus* sp. cf. *stigmaticus* Brischke, 1880 (Ichneumonidae: Mesochorinae), which, like its host, is univoltine and very mobile (van Nouhuys and Hanski 2002b). *Mesochorus* species are usually closely affiliated with the host of their primary parasitoid and are likely to have narrow host ranges. *Cotesia melitaearum* is host to the pseudo-hyperparasitoid *Gelis agilis* (Fabricius, 1775) (Ichneumonidae: Cryptinae). It is an abundant, wingless, generalist, ectoparasitic parasitoid which attacks hosts in silken cocoons, inclusindg ichneumonids (Schwarz and Shaw 1999). *Gelis agilis* can greatly reduce the population size of *C. melitaearum*, probably even causing local extinctions (Lei and Hanski 1997, van Nouhuys and Tay 2001).

Rearing

Caterpillars of all species were reared, and when parasitoids where available parasitized in the laboratory with the exception of the parasitoid *C. melitaearum* that was allowed to naturally parasitize laboratory reared caterpillars on experimental plants in the field.

Generalists - Cultures of *S. exigua* were established from eggs originating from a laboratory culture maintained on artificial diet at the Department of Virology at Wageningen University, the Netherlands. *Chrysodeixis chalcites* cultures were reared from individuals collected from a garden in Nijmegen, the Netherlands. All cultures were kept in plastic Petri dishes on artificial diet (Elzinga 2002) at 25ºC and L:D 16:8h. When caterpillars were in their final instar they were placed in plastic containers (15 x 10 x 10cm) with a small amount of artificial diet and with vermiculite into which they would eventually pupate. In order to get larvae for the experiment, adult moths were placed in a cage (40 x 50 x 65cm) in a climate room at 25ºC, 55% RH and L:D 16:8h with honey water (1:1) and one plant of *P. lanceolata* that both noctuid species readily accept for oviposition. Newly hatched caterpillars were collected from this cage and reared in Petri dishes (8cm ø) on artificial diet until they reached instar 3 (L3).

Hyposoter didymator adults were obtained from a laboratory colony that was started in 1993 from wasps collected from parasitized *S. littoralis* in the south of Spain (Cordoba). Since 2001, they have been reared on *S. frugiperda* in the Laboratoire de Pathologie

Comparée at INRA UMII, Saint-Christol-lèz-Aléz, France. In order to extend longevity of the adult wasps, they were kept at 10ºC and L:D 16:8h.

Cotesia marginiventris was obtained from a colony from Nijmegen University, the Netherlands, where they were reared on *S. exigua.* We kept the adults at10ºC and L:D 16:8h.

Specialists - Junonia coenia was obtained from a rearing of the Department of Ecology and Evolutionary Biology of the University of Colorado, Boulder, USA. The first generation was fed artificial diet and the second generation was used in the experiment.

Melitaea cinxia caterpillars used for these experiments were the offspring of laboratory reared butterflies from Åland, SW Finland. The caterpillars were fed field collected *P. lanceolata* leaves until third instar when they were used for the experiment.

Effect of iridoid glycosides on generalist herbivores and their parasitoids

The two generalist herbivores (*S. exigua* and *C. chalcites*) were parasitized by *C. marginiventris* and *H. didymator* at L3. Parasitism was observed as a single insertion and removal of the ovipositor. The parasitized caterpillars and a control group (unparasitized) were reared on freshly excised leaves of *P. lanceolata*. For the parasitoid *C. marginiventris* seven genotypes of *P. lanceolata* with low IG levels and six genotypes with high IG levels were used. On each genotype 20 parasitized and 20 unparasitized (control) caterpillars were reared. The same procedure was repeated for hosts parasitized by *H. didymator*, except that plants from only six low-IG genotypes and four high-IG genotypes were used, each containing 16 caterpillars per treatment and control. All the larvae were provided with fresh leaves daily, and were reared individually in Petri dishes (55mm ø) at 25°C, L:D 16:8h; 70% RH.

The following fitness correlates were recorded for the parasitoids: mortality, larval development time (the number of days between parasitism and egression), pupal development time (the number of days between larval egression and adult wasp eclosion), cocoon mass, adult mass and longevity. The same parameters were recorded for the unparasitized caterpillars, except that the larval development time recorded was from first instar until pupation.

Effect of iridoid glycosides on the development of specialist caterpillars and one of their specialist parasitoids

No parasitoids were available for *J. coenia*, thus for this herbivore species only developmental parameters of the butterfly were measured. The *J. coenia* caterpillars were reared on six low-IG and six high-IG *P. lanceolata* genotypes. The remainder of the set up was the same as for the generalist herbivores and their parasitoids.

Experiments with *M. cinxia* were undertaken by placing gregarious larval groups on intact potted plants in the field. Forty L3 caterpillars were placed onto single plants of each of 26 different genotypes of *P. lanceolata* (16 originating from the Netherlands; including the 13 used in the other experiments, and 10 originating from Åland). These plants were placed in the field in plastic pots covered with a mesh that prevented the caterpillars from escaping, but which allowed the *C. melitaearum* wasps to enter and to parasitize the caterpillars. The plants were left in the field for three weeks, and replaced with the same genotype if they were defoliated by the caterpillars. After three weeks all caterpillars were taken from the plants and placed in groups in plastic containers in a root cellar on Åland, to diapause during the winter. The following spring their diapause was broken by putting them at room temperature. The larvae were fed fresh leaves from the same genotype on which they had been growing over the previous summer. The caterpillars were reared until pupation or until larval parasitoid egression. The same parameters were measured as in the previous experiments, except that

larval development time was determined as the number of days between the breaking of diapause and egression.

Because we allowed the hosts to be parasitized naturally, rather than under observation in the laboratory, only a fraction of the larvae was parasitized. This method allowed us to compare the parasitism rate of larvae on the different types of *P. lanceolata* (among the artificially selected genotypes and between those and the clones from Åland).

Measurement of leaf iridoid glycosides

For all experiments above we measured concentrations of IGs in the leaves that were fed to the larvae. Over the duration of the experiments 2-3 samples of leaf material were collected from all used genotypes. Because not all experiments were performed at the same time we sampled several times. Using high performance liquid chromatography (HPLC) analyses, the leaf concentrations of the IGs aucubin and catalpol were determined and averaged per genotype.

All leaves were freeze dried and then ground to a fine powder with a ball mill (Retsch, type MM 301, Retsch GmbH & Co., Haan, Germany). Finely ground dry material from the leaves (25mg) was extracted in 10ml of 70% MeOH and was shaken overnight. The crude extract was filtered on a Whatman #4 filter paper and the filtrate was diluted ten times with Milli-Q water. The concentrations of the IGs aucubin and catalpol were analysed by HPLC using a Bio-Lc (Dionex Corp., Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4 x 50mm) and analytical column (4 x 250mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD). NaOH (1M) and Milli-Q water were used as eluents (10:90%, 1ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Concentrations were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunnyvale, USA).

Measurements of nutrient levels in Plantago lanceolata leaves

Secondary metabolites such as IGs could covary with leaf nutrient concentrations. In order to address this possibility we measured nutrient levels (N, P and K) of leaves of a subset (13) of the *P. lanceolata* genotypes used in the experiments, using the same ground leaf material as for the IG measurements. Extractions were done with H_2SO_4, salicylic acid, H_2O_2 and selenium following the method of Wallinga (1989b). Colorimetric methods were used to determine concentrations of nitrogen (Walinga et al. 1989a), and phosphorus (Walinga et al. 1989d). Leaf concentrations of potassium were determined by flame AES (Walinga et al. 1989c).

Sequestration of iridoid glycosides in Melitaea cinxia, its parasitoids and hyperparasitoids

In order to see whether differences in parasitoid performance in specialist herbivores fed different IG plants could be due to sequestration and to see if (hyper)parasitoids sequestrate IGs to potentially use for their own defence, we measured IG levels of the specialist herbivore *M. cinxia* and its (hyper)parasitoids. Different developmental stages/tissues of the insects were measured (Table 3.1).

All the *H. horticola*, and hyperparasitoid samples were obtained from laboratory reared *M. cinxia* caterpillars from Åland. The *M. cinxia* and *C. melitaearum* samples were partly from the performance experiment and partly from laboratory rearings from Åland (for the number of individuals used per species, see Table 3.1).

Table 3.1 Summary of the amounts of iridoid glycosides (IGs) (µg/mg) and the ratio catalpol to total IGs, in the body tissue of the host *Melitaea cinxia*, its endoparasitoids *Cotesia melitaearum* and *Hyposoter horticola* and their hyperparasitoids *Mesochorus* sp. cf. *stigmaticus* and *Gelis agilis.* Pupa and cocoon are with the insect present inside, pupal skin is the pupa where the adult egressed from, including the meconium (in the case of *Hyposoter horticola* and *Mesochorus* including the larval skin). Larval skin is the skin of the caterpillar after egression of the parasitoid. For the cocoon stage of *H. horticola* and *Mesochorus* it was inpossible to distinguish wich species was inside.

				µg/mg (mean ± s.e.)		
	Species	stage	n	aucubin	catalpol	total iridoids	catalpol/total IG
herbivore	*M. cinxia*	larva	10	0.49 ± 0.17	23.51 ± 3.21	24.00 ± 3.32	0.98 ± 0.15
		pupa	36	4.19 ± 0.98	29.37 ± 3.73	33.56 ± 4.29	0.87 ± 0.11
		adult	28	4.92 ± 0.76	31.44 ± 2.43	36.36 ± 2.59	0.86 ± 0.10
		pupal skin	29	22.15 ± 4.09	135.65 ± 14.05	157.81 ± 15.99	0.86 ± 0.02
		larval skin	3	0.53 ± 0.28	4.70 ± 2.25	5.22 ± 2.43	0.80 ± 0.19
parasitoid	*C. melitaearum*	larva	5	20.86 ± 12.69	65.33 ± 37.78	86.18 ± 37.78	0.81 ± 0.16
		cocoon	4	0.16 ± 0.11	3.82 ± 0.10	3.98 ± 0.16	0.96 ± 0.05
		adult	18	0.03 ± 0.03	1.73 ± 0.37	1.76 ± 0.37	0.99 ± 0.04
		pupal skin	8	0.86 ± 0.33	1.76 ± 0.38	2.63 ± 0.50	0.71 ± 0.30
	H. horticola	larva	8	1.59 ± 0.47	6.69 ± 1.64	8.28 ± 1.64	0.81 ± 0.09
		adult	9	0.01 ± 0.01	1.28 ± 0.30	1.30 ± 0.30	0.99 ± 0.01
		pupal skin	6	0.49 ± 0.35	43.15 ± 16.47	43.64 ± 16.47	0.99 ± 0.01
hyperparasitoid	*Mesochorus* sp. cf. *stigmaticus*	adult	13	0.00 ± 0.00	0.74 ± 0.17	0.74 ± 0.17	1.00 ± 0.00
		pupal skin	13	0.66 ± 0.26	48.37 ± 9.86	49.05 ± 9.86	0.99 ± 0.01
	G. agilis	adult	4	0.00 ± 0.00	2.39 ± 0.58	2.39 ± 0.58	1.00 ± 0.00
		pupal skin	4	0.00 ± 0.00	1.94 ± 0.42	1.94 ± 0.42	1.00 ± 0.00
	Hyposoter or Gelis	cocoon	6	0.95 ± 0.44	72.13 ± 10.00	73.08 ± 10.00	0.99 ± 0.02

To measure the amount of aucubin and catalpol in the herbivore and (hyper)parasitoids the same method was employed as for the leaves except that we ground the insects by hand in an Eppendorf tube and added 0.7ml 7% methanol for the (hyper)parasitoids and 3ml 70% methanol for *M. cinxia* larvae, adults and pupae. The filtration was done with a 0.2μm filter.

Statistical analysis

All statistical analyses were performed using Statistica (STATISTICA version 7.1, StatSoft Inc., Tulsa, UK). Pearson correlations were used to study associations between the level of IGs in the plant genotypes and the performance of herbivores or parasitoids. An independent t-test was used to compare oviposition preferences of *C. melitaearum* in *M. cinxia* larvae eating from artificially selected and Finnish plants, with differing IGs profiles. For the sequestration data we performed ANOVA with species as factor, followed by a post hoc (Tukey) test for all different developmental stages of the herbivore, parasitoids and hyperparasitoids.

Results

Levels of iridoid glycosides in the different genotypes

All the artificially selected plants used in the experiments had significantly higher levels of aucubin then catalpol (Table 3.2). Their levels in total IG ranged from 0.07 to 12.78% of the dry weight. In the Finnish plants there was no significant difference between the levels of aucubin and catapol (t-test, t=1.95; df=56; P>0.05). The total IG levels ranged from 1.01 to 9.35% of the dry weight (Table 3.2).

Table 3.2 The average, minimum and maximum iridoid glycosides (IG) levels (percentage of dry weight) of the artificially selected and of the Finnish *Plantago lanceolata* plants. For each host and/or parasitoid species we measured the levels separately because not all experiments were performed at the same time.

Origin	Species	Compound	Mean ± s.e.	Min.	Max.
artificially selected	*S. exigua* and *C. chalcites*	Aucubin	2.24 ± 0.40	0.57	4.72
		Catalpol	0.68 ± 0.16	0.20	1.80
		Total IG	2.89 ± 0.51	0.54	6.52
	C. marginiventris	Aucubin	2.01 ± 0.37	0.47	4.20
		Catalpol	0.70 ± 0.15	0.20	2.08
		Total IG	2.70 ± 0.46	0.67	5.34
	H. didymator	Aucubin	2.85 ± 0.47	0.67	5.81
		Catalpol	0.91 ± 0.17	0.25	2.30
		Total IG	3.76 ± 0.59	0.99	8.10
	J. coenia	Aucubin	2.21 ± 0.26	0.52	6.20
		Catalpol	1.03 ± 0.14	0.24	3.90
		Total IG	3.24 ± 0.35	0.87	9.03
	M. cinxia and *C. melitaearum*	Aucubin	3.35 ± 0.30	0.07	11.05
		Catalpol	1.13 ± 0.09	0.11	3.67
		Total IG	4.49 ± 0.36	0.20	12.78
Finnish	*M. cinxia* and *C. melitaearum*	Aucubin	2.74 ± 0.28	0.79	5.88
		Catalpol	2.04 ± 0.23	0.21	4.46
		Total IG	4.79 ± 0.45	1.01	9.35

Effect of iridoid glycosides on generalist herbivores and their parasitoids

Herbivores - The larval development time of *S. exigua* was not significantly affected by concentrations of IGs in the leaves of *P. lanceolata,* but pupal periods in *S. exigua* prolonged with increasing levels of aucubin, catalpol and total IG (Table 3.3). For *C. chalcites* a reverse pattern was found; the duration of the pupal period was not affected by IGs in the diet but larval development time tended to be prolonged when caterpillars had fed on plants with higher levels of aucubin and total IG (Table 3.3). In both generalist herbivore species, pupal mass was negatively correlated with concentrations of aucubin, catalpol and total IG levels in leaves of *P. lanceolata* (Table 3.3; Fig. 3.1). The adult weight of *S. exigua* was not affected by the IGs in its larval diet whereas adult weight of *C. chalcites* decreased with increasing IG levels (Table 3.3).

Table 3.3 Correlations between the iridoid glycoside levels (aucubin, catalpol and total IG) and the performance measurements (larval development time (days), pupal development time (days), pupal weight (mg) and adult weight (mg)) of the generalist herbivores *Chrysodeixis chalcites* (C. c) and *Spodoptera exigua* (S. e). Positive correlations are indicated with + and negative correlation with -, significant levels are indicated after the r-value: +P<0.10,* P<0.05,** P<0.01 and ***P<0.001.

	Species	n	Aucubin	Catalpol	Total IG
Larval development	*C. c*	100	+0.17 $^{+}$	+0.10	+0.18 $^{+}$
Pupal development	*S. e*	55	+0.34**	+0.28*	+0.38***
Pupal weight	*C. c*	99	-0.41***	-0.25*	-0.42***
	S. e	117	-0.22*	-0.23*	-0.26**
Adult weight	*C. c*	83	-0.39***	-0.27**	-0.41***

Table 3.4 Correlations between the iridoid glycoside levels (aucubin, catalpol and total IG) and the performance measurements (larval development time (days), pupal development time (days), pupal weight (mg) and adult weight (mg)) of the generalist parasitoids *Cotesia marginiventris* (C. m) and *Hyposoter didymator* (H. d) on the two generalist herbivores *Chrysodeixis chalcites* (C. c) and *Spodoptera exigua* (S. e). Positive correlations are indicated with + and negative correlation with -, significant levels are indicated after the r-value: +P<0.10,* P<0.05,** P<0.01 and ***P<0.001.

	Species	n	Aucubin	Catalpol	Total IG
Larval development	*C. m* on *C. c*	19	-0.41 $^{+}$	-0.36	-0.42 $^{+}$
Pupal development	*C. m* on *S. e*	93	-0.26*	-0.11	-0.24*
Pupal weight	*C. m* on *C. c*	19	+0.46*	+0.18	+0.41 $^{+}$
	H. d on *C. c*	96	-0.003	+0.31**	+0.01
Adult weight	*H. d* on *C. c*	87	-0.13	+0.20 $^{+}$	-0.04

Table 3.5 Correlations between the iridoid glycoside levels (aucubin, catalpol and total IG) and the performance measurements (larval development time (days), pupal development time (days) and pupal weight (mg)) of the specialist herbivores *Junonia coenia* (J. c) and *Melitaea cinxia* (M. c) and its parasitoid *Cotesia melitaearum* (C. m). Positive correlations are indicated with + and negative correlation with -, significant levels are indicated after the r-value: +P<0.10,* P<0.05, ** P<0.01 and ***P<0.001.

	Species	n	Aucubin	Catalpol	Total IG
Larval development	*J .c*	101	+0.45***	+0.31**	+0.43 ***
	M. c	57	+0.17	-0.27*	+0.07
Pupal development	*M. c*	21	-0.22	-0.40 $^{+}$	-0.27
	C. m	12	-0.53 $^{+}$	-0.36	-0.57 $^{+}$
Pupal weight	*J. c*	101	-0.40***	-0.38***	-0.43 ***

Parasitoids – Mirroring effects of IGs on herbivore development, higher concentrations of aucubin and total IG in the food plants resulted in slower pupal development of *C. marginiventris* reared on *S. exigua* (Table 3.4) and also tended to slow down larval development of *C. marginiventris* reared on *C. chalcites* (Table 3.4). Surprisingly, pupal weight (cocoon mass) of *C. marginiventris* reared on *C. chalcites* increased with increasing levels of aucubin in the caterpillar's food plants and pupal weight of the parasitoid *H. didymator* reared on *C. chalcites* increased with the level of catalpol in the caterpillar's food plants (Table 3.4). The higher pupal weight of *H. didymator* on *C. chalcites* when fed on plants with higher levels of catalpol also tended to result in bigger wasps emerging from these cocoons (Table 3.4).

Effects of iridoid glycosides on specialist herbivores and one of their parasitoids

Herbivores - The two specialist herbivores were differently affected by the IGs in plants on which they were reared. Larval development time of *J. coenia* was prolonged (Fig. 3.2) and pupal mass reduced when fed on plants with higher levels of aucubin, catalpol and total IG (Table 3.5). By contrast, larval development time of *M. cinxia* was shorter when fed *P. lanceolata* genotypes with higher levels of catalpol (Fig. 3.2) and pupal development showed the same trend. Pupal mass of *M. cinxia* did not significantly vary with IG levels (Table 3.5).
Parasitoid – On 15 of the 26 plant genotypes at least one *M. cinxia* caterpillar was parasitized by *C. melitaearum*. On these 15 plants, on average 5% of caterpillars were parasitized, with on average 2.1 parasitoids per parasitized caterpillar. Larval development time of *C. melitaearum* was not significantly affected by concentrations of IG in the diet of *M. cinxia* but the pupal development time tended to be shorter among wasps coming from caterpillars feeding on plant genotypes with high concentration of aucubin and total IG (Table 3.5). There was no significant effect of IGs on the other fitness correlates. The proportion of caterpillars that was naturally parasitized by *C. melitaearum* also did not significantly vary with the level of IGs in the food plants of the caterpillars. However, there was a significant difference in the number of caterpillars parasitized by *C. melitaearum* between the artificially selected and Finnish plants; a larger fraction of caterpillars feeding on the artificially selected genotypes was parasitized (t-test, $t=2.59$; $df=24$; $P<0.05$). A significantly larger number of parasitoid larva and cocoons emerged from the artificially selected plants, compared to the Finnish plants (t-test, $t=2.48$; $df=24$; $P<0.05$). The only significant difference in allelochemistry between Finnish and artificially selected genotype plants was the level of catalpol, which was higher in Finnish plants (t-test, $t=-3.05$; $df=24$; $P<0.01$; Table 3.2). However, the correlation between the number of larvae and cocoons and the catalpol concentration was not significant ($r=-0.29$; $n=26$; $P=0.15$).

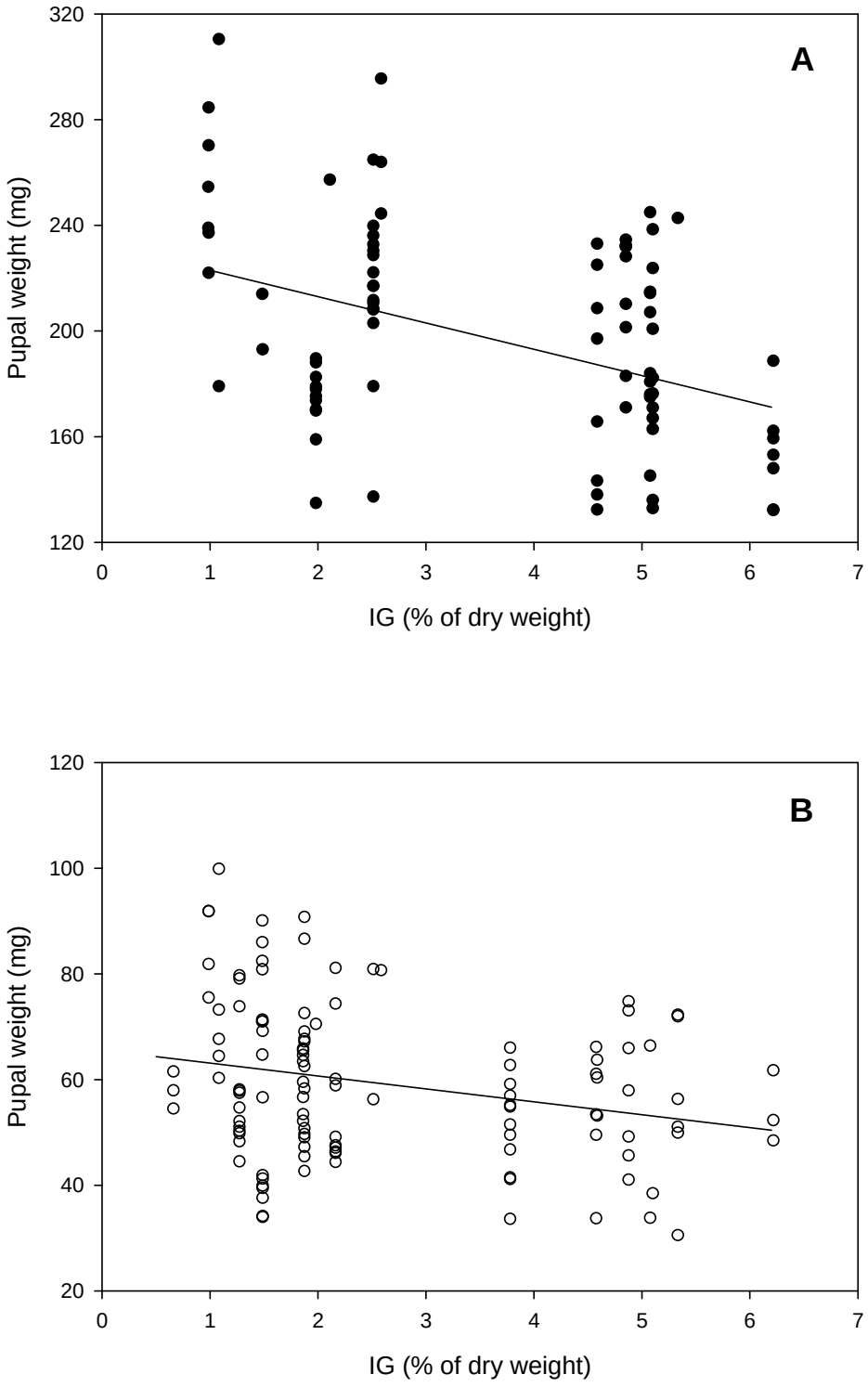


Figure 3.1 pupal weights of the generalist herbivores *Chrysodeixis chalcites* (A) and *Spodoptera exigua* (B) plotted against the per-centage of iridoid glyco-sides (IG) in the larval diet.

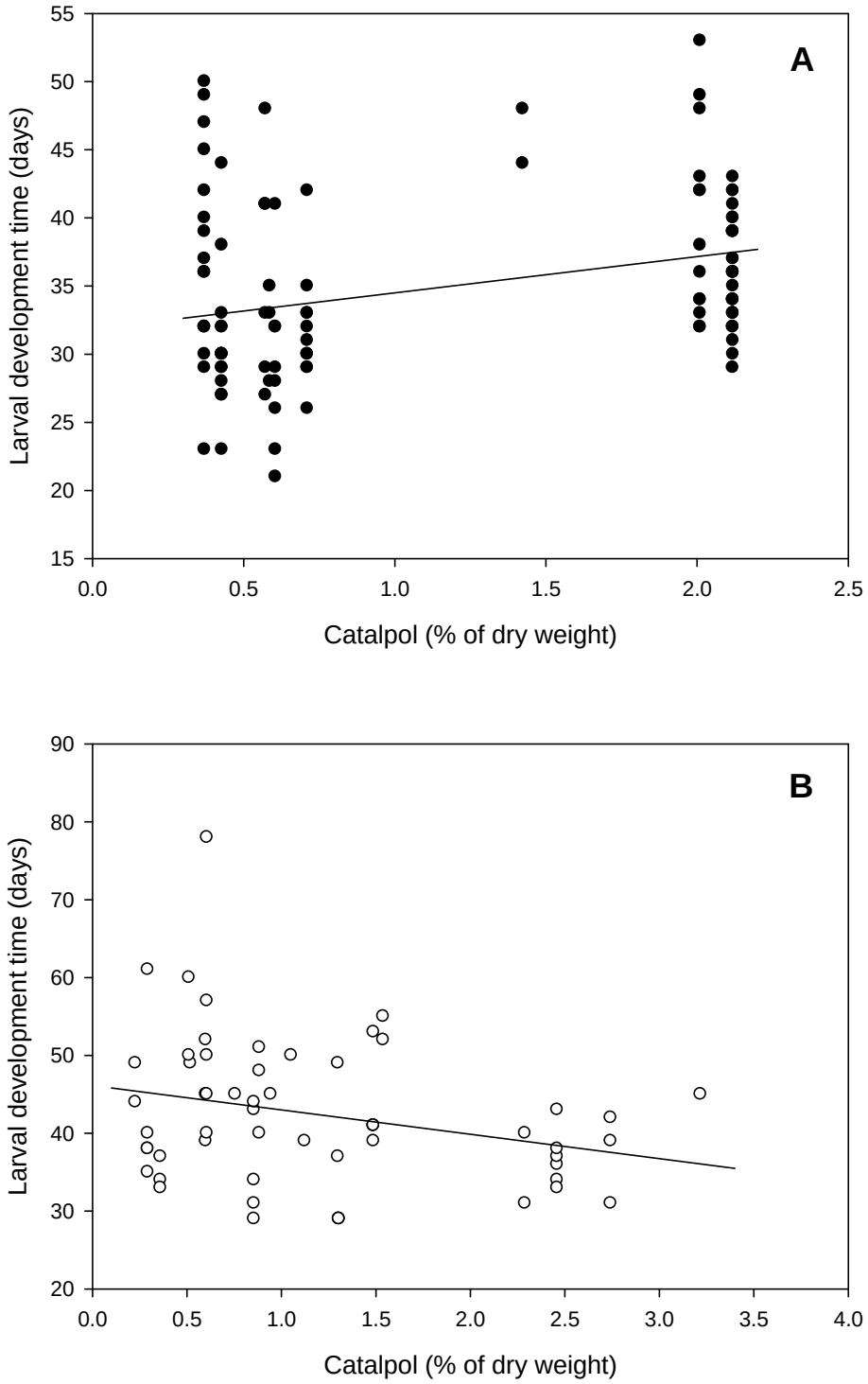


Figure 3.2 Larval development time of the specialist herbivores *Junonia coenia* (A) and *Melitaea cinxia* (B) plotted against the percentage of catalpol in the larval diet.

Leaf nutrient levels of the different Plantago lanceolata genotypes

There were no significant correlations between the levels of IGs in the different artificially selected genotypes of *P. lanceolata* and the nutrient components measured in these leaves (N: r=0.31, n=13, P=0.31; P: r=0.26, n=13, P=0.39; K: r=0.24, n=13, P=0.27; Fig. 3.3).

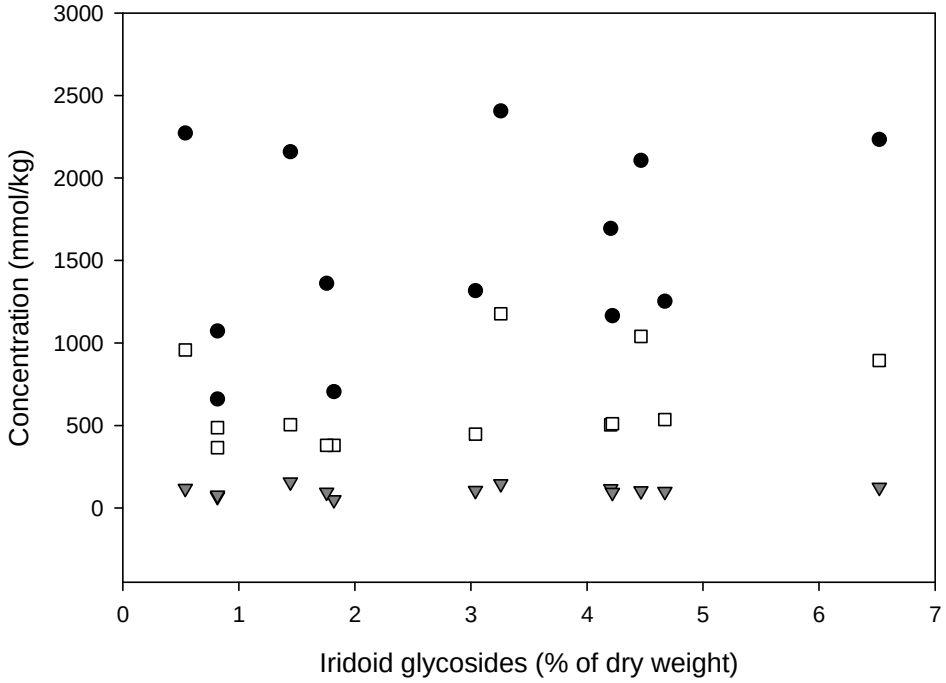


Figure 3.3 The different nutrient concentrations, Nitrogen (black dots), Phosphorus (grey triangles) and Potassium (white squares), plotted against the iridoid glycosides concentrations in *Plantago lanceolata* leaves.

Sequestration of iridoid glycosides in Melitaea cinxia its parasitoids and hyperparasitoids

Significant levels of IG were detected in all developmental stages of both the specialist herbivore *M. cinxia* and its associated parasitoids and hyperparasitoids. The absolute amount of IGs was highest in the pupal skins of *M. cinxia* and differed significantly from the other species (ANOVA, $F_{4, 55}$ =14.42, P<0.00001; Fig. 3.4D). For the herbivore the levels of IGs went up with the developmental stages and were highest in the adult butterfly (Table 3.1). However, for the parasitoids the amounts of IGs went down with the developmental stages, and were lowest in the adult stage (Table 3.1; Fig. 3.4C). There were significant differences between the different species in the amounts of IGs in their body tissues in all their developmental stages (ANOVA, larval: $F_{2, 20}$ =3.81, P<0.04; Fig. 3.4A; pupal: $F_{2, 43}$ =10.32, P<0.001; Fig. 3.4B; adult: $F_{4, 67}$ =68.18, P<0.00001; Fig. 3.4C). The catalpol/total IG ratio significantly differed between the herbivores and their parasitoids and hyperparasitoids. In the larval stage (Fig. 3.5A) *M. cinxia* caterpillars contained a significantly higher ratio than the parasitoids *C. melitaearum* and *H. horticola* (ANOVA, $F_{2, 20}$ = 10.26, P<0.001). However, in the pupae (Fig. 3.5B) an opposite trend was observed (ANOVA, $F_{2, 43}$ = 3.08, P<0.06). Finally, adult *M. cinxia* butterflies had significantly lower proportions of catalpol stored in their tissues than both the parasitoids and the hyperparasitoids, whereas this proportion did not vary between the different parasitoids and hyperparasitoids (ANOVA, $F_{4, 64}$ = 16.27, P<0.000001; Fig. 3.5C).

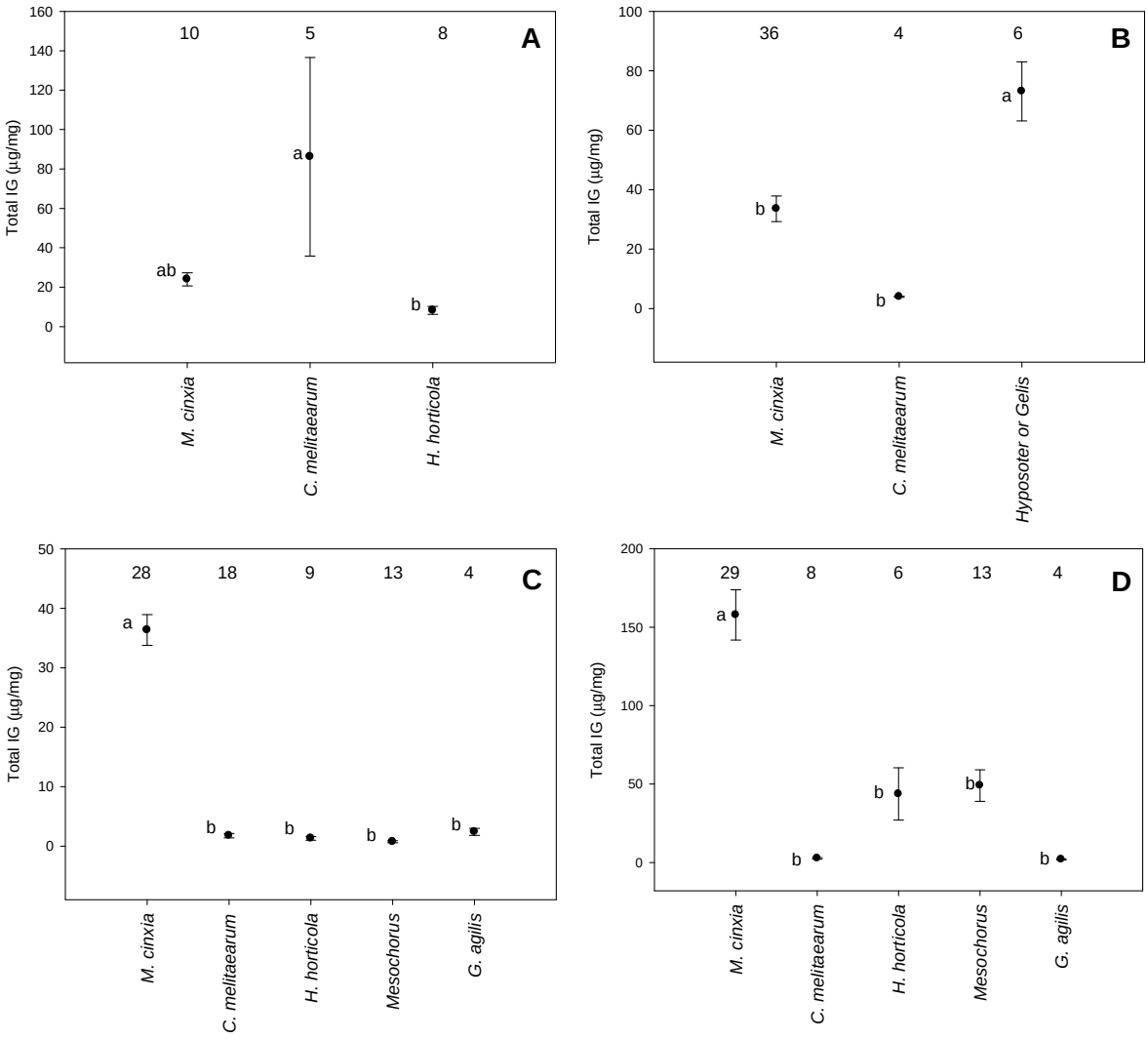


Figure 3.4 The total amount of iridoid glycosides (mean ± s.e.) in the insect larvae (A), pupae (B) adults (C) and pupal skins (D). The figures above the dots represent the total number of individuals used. The numbers above the dots are the total numbers of individuals used. The letters adjacent to the circles indicate which species are significantly different from each other using a Tukey post hoc test.

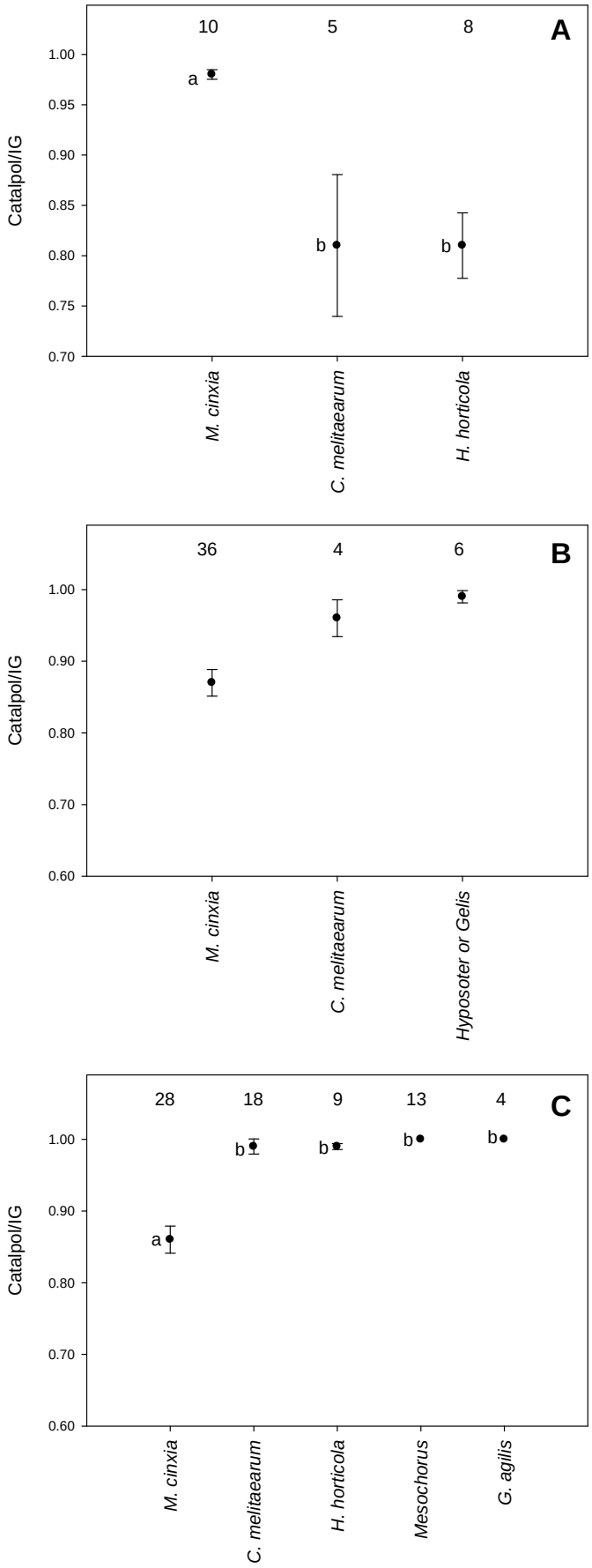


Figure 3.5 The ratio of catalpol to total iridoid glycoside level (mean ± s.e.) in the larvae (A), pupae (B) and adults (C). On the y-axis is the ratio of catalpol to total iridoid level and on the x-axis the different trophic levels. The numbers above the dots are the total numbers of individuals used. The letters adjacent to the circles indicate which species are significantly different from each other using a Tukey post hoc test.

Discussion

The results of this study support the notion that development of generalist and specialist insect herbivores and their parasitoids vary with secondary host plant chemistry (Harvey et al. 2005). The development of both species of generalist herbivores suffered on plants containing higher levels of IGs, but in somewhat different ways. Both herbivores took longer to complete their development on plants with higher levels of aucubin and total IG, and in *S. exigua* catalpol also appeared to exert significant negative effects on larval development. However, whereas in *C. chalcites* larval development time was extended on high IG diets, *S. exigua* pupal (but not larval) development time increased. The results for both species support the slow-growth high-mortality hypothesis (Clancy and Price 1987, Damman 1987) whereby *C. chalcites* would be more vulnerable for larval parasitoids and predators and *S. exigua* for pupal parasitoids and predators when eating a diet with high levels of IGs. Moreover, both species would be at a disadvantage on high iridoid plants if the growing season were time limited due to weather or other extrinsic factors.

Pupal weight was strongly negatively correlated with IG concentrations in the diets of both generalist herbivores. In many herbivorous insects size, such as pupal or adult weight, are strongly correlated with potential fecundity (Leather 1988, Klingenberg and Spence 1997, Saastamoinen et al. 2007), thus high levels of IGs in the diet can potentially lead to a reduction in adult fitness.

In the parasitoids of the generalist herbivores there were well-defined differences between the effect of aucubin and catalpol on the different species. Surprisingly, development of the parasitoid *C. marginiventris* was positively correlated (e.g., shorter total development time) with aucubin levels in host diet. As with the herbivores, different phases of the pre-adult development in *C. marginiventris* also varied when reared on the two host species. When reared on *C. chalcites,* larval development time was shorter on high-aucubin diets, whereas when reared on *S. exigua* pupal development time was reduced on high-aucubin diets. Furthermore, parasitoids reared from both host species on high aucubin diets had larger pupal masses than wasps reared on low aucubin diets. The IG levels in the host diet had no effect on the development time of *H. didymator*. However, when *H. didymator* was reared on *C. chalcites* the cocoon and adult mass was positively correlated with the amount of catalpol in the host diet. There was no effect of the host diet when *H. didymator* developed in the host *S. exigua*. Many studies have reported that the development of koinobiont endoparasitoids and their hosts exhibit strong physiological integration, which is often based on the detection of age-specific changes in the host's internal biochemical environment by the immature parasitoids (Beckage and Templeton 1985, Lawrence 1990, Vinson 1990, Harvey 2005). Under these conditions, it is expected that any delay or negative effect of the development of the host will be similarly reflected on parasitoid development (Sequiera and Mackauer 1992, Harvey et al. 1994, Harvey et al. 2004).

Herbivore performance and thus host quality can be negatively affected by the ingestion and expression of toxic plant allelochemicals that are then vertically transferred to higher trophic levels (Barbosa et al. 1986, Barbosa et al. 1991, Bowers 2003, Harvey et al. 2007). However, in this study parasitoid development was actually found to benefit from hosts fed on high IG diets. One possible explanation for this apparent conflict is that higher levels of allelochemicals in host diet weaken its immune system. This would benefit the developing parasitoids by enabling them to reallocate metabolic energy from immunosuppression or avoidance to growth and development (Kraaijeveld and Godfray 1997, Ojala et al. 2005). This hypothesis remains to be confirmed for our species.

In contrast to the generalist herbivores, the development of the two specialists exhibited patterns that were quite different from each other. The development of *J. coenia* was negatively correlated with IGs. The larval development time was strongly positively correlated with IG concentrations, concomitant with lower pupal masses. Previous studies have reported that *J. coenia* sequesters IGs in body tissues during larval feeding (Bowers and Puttick 1986, Bowers and Collinge 1992, Camara 1997b, 1997a). Sequestration may require costly physiological or morphological adaptations for the prevention of autotoxicity to the insect. Even if the metabolic costs are high, sequestration may still be adaptive for *J. coenia* if the costs are lower then the benefits from a reduction in predation in the field (de la Fuente et al. 1994, Dyer and Bowers 1996).

For *M. cinxia* it was found, as in previous studies (Harvey et al. 2005, Saastamoinen et al. 2007) that higher levels of IGs actually benefit the insects. For instance, higher catalpol levels were correlated with shorter larval and pupal development times, although there was no apparent effect on pupal or adult weight. These data are consistent with the results of a previous study (Harvey et al. 2005) but those authors only examined the total IG levels in *P. lanceolata* on *M. cinxia* performance. Here, we see that the effect is only correlated with the amount of catalpol (and not total IG level).

In *C. melitaearum*, a specialist parasitoid of *M. cinxia,* the only fitness parameter that was correlated with IG levels was pupal development time, and it tended to be lower when the parasitoids were associated with plants containing higher concentrations of aucubin and total IG. In the field, rate of parasitism by *C. melitaearum* has been found to be lower in groups of *M. cinxia* larvae reared on plants containing higher levels of catalpol (Nieminen et al. 2003). This could mean that the parasitoids are avoiding larvae feeding on more toxic plant genotypes. This results are supported by a previous study by Nieminen et al. (2003) who also found that parasitism by *C. melitaearum* occurred most frequently in larval groups that were feeding on plants with low concentration of catalpol.

Because the IG levels of the different genotypes of *P. lanceolata* were not correlated with the nutrient content of these genotypes, it is highly unlikely that the differences in performance on the different genotypes is caused by correlated differences in nutritional quality of these plants.

IGs can only exert direct effects on higher trophic levels if these higher trophic levels are actually exposed to the allelochemicals in their diet e.g., the trophic level immediately below. Thus, if IGs are sequestered by the herbivores they can directly influence primary parasitoids and if the primary parasitoids sequester these compounds the same is true for secondary parasitoids. An analysis of body tissues in larval, pupal and adult stages of *M. cinxia* showed that high concentrations of IGs were sequestered from the food plants. In all stages, catalpol was present in higher levels than aucubin whereas in most tested plants the level of aucubin was higher than that of catalpol. This could be due to the fact that aucubin is a precursor of catalpol (Damtoft et al. 1983) and could be further processed into catalpol by the insects. However, Bowers and Collinge (1992) found that when *J. coenia* larvae are fed on artificial diets exclusively containing aucubin, only aucubin is detected in tissue of the caterpillars, so aucubin is not metabolized into catalpol. If the same is true for *M. cinxia,* it is likely that they sequester catalpol more efficiently than aucubin from the plant leaves, which has also been found for *Euphydryas phaeton* (Nymphalidae) (Belofsky et al. 1989) and *J. coenia* (Bowers et al. 1992a). It is known that catalpol is the more toxic component, at least for generalist predators (Bowers and Puttick 1986, Stermitz et al. 1986, Belofsky et al. 1989, Bowers 1992).

Although it is well known that plant allelochemicals that are sequestered by herbivores can affect the survival, development, morphology and size of their parasitoids (Campbell and

Duffey 1979, Duffey et al. 1986, Gunasena 1988, Barbosa et al. 1991, Harvey et al. 2005, Harvey et al. 2007), only two studies have thus far examined whether parasitoids themselves store these compounds (Barbosa et al. 1986, Bowers 2003). In both these studies only trace amounts of plant allelochemicals were recovered from the adult parasitoids of *Cotesia congregata*. This parasitoid presumably does not utilize allelochemicals in a defensive capacity but deals with the accumulated concentrations of plant allelochemicals by shunting them into cocoon silk and meconium (waste products remaining in the cocoon after adult emergence) (Barbosa et al. 1986). In our study we recovered much higher amounts of plant allelochemicals from the adult body tissue of *C. melitaearum* and *H. horticola*. However, for *H. horticola* the highest amounts of IGs were found in the pupal exuviae, probably because these samples included the host larval skin and meconium. The difference between the *C. congregata* and *C. melitaerum* might reflect differences in the level of specialization of the parasitoids. *Cotesia congregata* attacks a wide range of sphingid larvae that feed on plants with very different types of allelochemicals: catalpol in Catalpa trees fed on by its host, *Ceratomia catalpae*, and nicotine in tobacco plants fed upon by another host, *Manduca sexta* (Barbosa et al. 1986, Bowers 2003). The parasitoid may not be optimally adapted to sequester these quite different compounds. *Cotesia melitaearum* is a specialist on *M. cinxia*, which feeds on two closely related plant genera in the family Plantaginaceae, which both contain IGs. This parasitoid, and probably the other closely related *Cotesia* species using irioid glycoside feeding hosts (Kankare and Shaw 2004) have the capacity to sequester IGs defensively, like their host, although this has not yet been tested.

In summary, this study reports that the effect of direct defence on insect development is species-specific. This is also the first study, as far as we know, to report actual sequestration of allelochemicals in a parasitoid and potentially in its primary hyperparasitoid. For the plant, the most efficient means of defence depends on the identity of the attacker. Direct defence compounds are probably most effective against generalist herbivores, but often not against specialized herbivores. In this case, indirect defences, such as releasing a volatile blend of odours aimed at attracting natural enemies of the herbivore, are probably more effective (Vet and Dicke 1992, Dicke 1999b). A possible conflict between these two defence strategies may arise if the plants that are most attractive to natural enemies also posses stronger direct chemical defences that exhibit clear negative effects on the performance of predators and parasitoids. Future studies should experimentally integrate the two defence strategies in plants in order to better understand how this conflict is resolved.

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Chapter 4

Oviposition cues for a specialist butterfly: plant chemistry and size

J. H. Reudler Talsma, A. Biere, J.A. Harvey and S. van Nouhuys

Abstract - The oviposition behaviour of a butterfly is based on a complex set of stimuli and responses. In this study we looked at the effect of plant secondary chemistry (the iridoid glycosides aucubin and catalpol) and aspects of plant size (mainly the number of leaves) of the host plant *Plantago lanceolata*, on the oviposition behaviour of its specialist lepidopteran herbivore *Melitaea cinxia*. Iridoid glycosides are known to deter feeding or decrease the growth rate of generalist insect herbivores, but can be oviposition cues for specialist butterflies and feeding stimulants for their larvae. Previous studies of the same species showed that oviposition was associated with high levels of aucubin in the field, but could not distinguish whether the higher levels of aucubin were the cause (active choice) or consequence of oviposition (induction in plant). We conducted a set of dual- and multiple-choice experiments in cages and in the field. In the cages we found a positive correlation between the pre-oviposition level of aucubin and the number of ovipositions, indicating that the association reflects an active oviposition decision rather than plant induction following oviposition. The results also suggest a threshold concentration below which females do not distinguish among levels of iridoid glycosides. In the field, the size of the plant appeared to be a more important stimulus than iridoid glycoside concentrations, with bigger plants receiving more ovipositions than the smaller ones regardless of their secondary chemistry. The variation in the use of predominant cues for oviposition may be dependent on environmental conditions.

Introduction

Finding and choosing a host plant for oviposition is a challenging task for female herbivorous insects, and the decision made by the female may have far-reaching consequences for her fitness and that of her offspring. Under natural conditions, ovipositing insects experience many external stimuli (e.g., visual and olfactory cues), their own internal physiological stimuli and a series of environmental constraints (e.g., availability of host plants) (Visser 1986, Bernays and Chapman 1994, Badenes et al. 2004). Oviposition site selection is crucial for the successful development of larvae (Singer 1986, Mayhew 1997). Optimal oviposition theory (Jaenike 1978) predicts that oviposition preference should correlate with host plant suitability for offspring development (Awmack and Leather 2002), although experimental studies do not appear to unequivocally support such a positive correlation between preference and performance (Thompson and Pellmyr 1991, Mayhew 2001, Scheirs and De Bruyn 2002). This hypothesis was elaborated by Price (1991) when he developed the 'plant vigour hypothesis', predicting that vigorous plants that grow faster and ultimately reach a larger than average size should be preferred by the herbivore. Both plant biomass and nutritional quality are supposed to be higher in vigorous plants (Heisswolf et al. 2005, Lastra et al. 2006).

The range of host plants accepted for oviposition is often very narrow, which has at least partly been explained by the co-evolutionary process of adaptation and counter-adaptation of herbivorous insects to the defence chemicals of their hosts, resulting in a predominance of specialized herbivores with narrow host ranges (Ehrlich and Raven 1964, Mitter et al. 1988, Jaenike 1990). Plant secondary chemistry is seen as the major constraining force behind the patterns we see today in the use of host plants by insects (Brower and Brower 1964, Feeny 1991, Futuyma 1991, Becerra 1997, Nylin and Janz 1999).

In this chapter, we study the role of plant secondary chemistry and size of the host plant *Plantago lanceolata* L. (Plantaginaceae) on the oviposition behaviour of its specialist herbivore, the checkerspot butterfly *Melitaea cinxia* (Linnaeus, 1758) (Lepidoptera: Nymphalidae). Most lepidopteran species, 90-95% (Stamp 1980, Hebert 1983), leave an individual egg on a plant and move on before laying another. By contrast, all checkerspot butterflies such as *M. cinxia* lay their eggs in clusters (Singer 2004). Butterflies that lay eggs singly must often find on the order of 20-50 oviposition sites per day and may be more time limited than egg limited (Courtney 1982). For butterflies that lay their eggs in clusters the cost of prolonged search or host assessment is less. A highly discriminating female that delays her oviposition by rejecting most hosts can simply lay a larger cluster when she eventually does oviposit (Singer 2004).

In general, butterflies are attracted to alight on plants by mixtures of visual and olfactory stimuli (Rausher 1978, Feeny et al. 1989). A positive response to visual stimuli is to alight and taste a plant. In order to assess whether a plant is chemically acceptable, a female checkerspot butterfly scratches the surface of a leaf with the first pair of tarsal claws. If the plant is then accepted for oviposition, the butterfly curls her abdomen to the underside of a leaf and oviposits. In checkerspot butterflies plant acceptance depends on physical features of the oviposition site such as the size, shape and orientation of the leaf (Singer 2004).

Most checkerspot species are oligophagous or even monophagous on plant species belonging to 16 different families (Olmstead et al. 1993, Olmstead et al. 2001). All but two of these families are in the single subclass Asteridae, and members of all but two of these families produce iridoid glycosides (IGs) as plant secondary compounds (Jensen et al. 1975, Higgins 1981, Jensen 1991, Tolman and Lewington 1997, Olmstead et al. 2000, Wahlberg 2001).

In this study we focus on two IGs, aucubin and catalpol, which are found in more than ten families of plants (El-Naggar and Beal 1980), and are the most abundant IGs in *P. lanceolata* (Suomi et al. 2001a, 2002). These IGs are known to function as oviposition cues for the specialist butterfly *Junonia coenia* (Pereyra and Bowers 1988) and as feeding stimulants for at least some checkerspot butterflies (Bowers 1983). *Melitaea cinxia* larvae perform better on diets with higher iridoid levels (Harvey et al. 2005). It is generally thought that specialist herbivores benefit from the defensive chemistry of their host through reduced competition with generalist herbivores and inhibition of generalist natural enemies (Bowers 1980, Dyer and Bowers 1996, Camara 1997b, Theodoratus and Bowers 1999).

Nieminen et al. (2003) examined the pattern of oviposition by *M. cinxia* on their food plant, *P. lanceolata* in the field. The authors compared plants used for oviposition with neighbouring and random plants in the patch. They found that plants used for oviposition contained significantly higher concentrations of aucubin than neighbouring and random plants. Additionally, plants selected for oviposition had higher catalpol concentrations than neighbouring plants, indicating that ovipositing females prefer to oviposit on plants with higher levels of IGs.

An alternative explanation for the positive association between IG levels and oviposition in Nieminen et al. (2003) is that the presence of butterfly eggs on leaf tissues leads to an induction of IGs in the plant (Nieminen et al. 2003). Induction of IGs by *M. cinxia* oviposition has not been investigated so far, but IG production in *P. lanceolata* can be induced both by fungal infection (Marak et al. 2002b) and by herbivory (Darrow and Bowers 1999, Stamp and Bowers 2000). Furthermore, Peñuelas et al. (2006) found that leaves of *Lonicera implexa* (Camprifoliaceae) that bore egg clusters of *Euphydryas aurinia* (a close relative of *M. cinxia*) had 15-fold higher concentrations of IGs than directly opposite leaves on the same plant. Furthermore, other studies have reported that allelochemicals in plants can be induced through oviposition and the presence of eggs on or imbedded in the leaf surface (Blaakmeer et al. 1994, Agrawal 2000a, Colazza et al. 2004, Hilker et al. 2005).

We performed a set of dual- and multiple-choice experiments in cages and in the field to answer the following questions: 1) What is the effect of *P. lanceolata* plant chemistry on the oviposition behaviour of *M. cinxia*? 2) Does oviposition cause induction of IGs in the host plant? 3) What is the effect of plant size (mainly number of leaves) on the oviposition behaviour of *M. cinxia* butterflies?

Methods and Materials

Study species

Melitaea cinxia (Glanville fritillary) butterflies used for these experiments were the offspring of field-caught butterflies from Åland, Finland. On Åland the butterflies fly in June and lay large clusters (150-200 eggs) underneath the leaves of their host plants, *P. lanceolata* and *Veronica spicata* L. (Scrophulariaceae) (Kuussaari et al. 2000). Larvae hatch after two to four weeks depending on the temperature. The larvae spin a communal web on the host plant and feed gregariously during the rest of the summer. Because of their restricted mobility, small larvae depend on the host plant the adult female chose for oviposition (Kuussaari et al. 2000, Kuussaari et al. 2004). The larvae diapause gregariously in a silk winter nest, becoming active again in spring. In the last instar, the larvae disperse and feed individually. They pupate within the vegetation in early May.

Plantago lanceolata (ribwort plantain) is a rosette-forming, self-incompatible, perennial plant with a worldwide distribution and large ecological amplitude (Sagar and Harper 1964). Among the secondary plant compounds produced by *P. lanceolata* are the two iridoid glycosides (IGs) aucubin and catalpol (Duff et al. 1965). In natural populations IG levels range from undetectable to ca. 9% of its dry weight (Bowers 1991). In the field on Åland these levels range between 0.6-2.2% for aucubin and between 0.7-2.0% for catalpol (Nieminen et al. 2003). The variation in the constitutive IG amount in *P. lanceolata* is partially genetically determined (Bowers and Stamp 1992, 1993, Adler et al. 1995). Most of the plants used for the oviposition experiments were derived from an artificial selection experiment, in which plants were selected on the basis of high and low concentrations of total leaf IGs for four generations (Marak et al. 2000). Nine plants, each derived from a different half sib family from the low line, and six plants, each derived from a different half sib family from the high line, were clonally propagated following a root-cloning method (Wu and Antonovics 1975). This resulted in 15 different genotypes used in experiment 1 and 2. In addition, five new crosses were made between pairs of plants of the low line and five crosses between pairs of plants of the high line. From each of these crosses a single offspring was raised and clonally propagated. This resulted in five genotypes with low (L1-L5) and five genotypes with high (H1-H5) IG levels, that were used in experiment 3 and 4. In experiment 1 and 2 we additionally used 15 plants collected from the field on Åland, with initially unknown IG level.

Oviposition experiments

Four experiments were carried out to study the oviposition response of *M. cinxia* to levels of IGs and plant size of *P. lanceolata.* In experiments 1-3, we offered potted plants to butterflies in cages, using dual (experiment 1) or multiple (experiments 2, 3) choice tests. In experiment 4, we transplanted experimental plants and butterflies to a natural field plot. Experiments were carried out in Finland, except for one of the cage experiments (experiment 3) that was carried out in the Netherlands.

Experiment 1: small cages, Finland. Two *P. lanceolata* plants of different genotypes, randomly selected from the nine genotypes of the low and six genotypes of the high selection line were put in a small mesh cage (38 x 38 x 44cm) that was placed outside at Nåtö Biological Station, Åland, in June 2005. Before the plants were put in the cage we counted the number of leaves and the second fully grown leaf was taken from the plant for HPLC analyses. A mated female *M. cinxia* butterfly was added to each cage along with a sponge with honey water (1:3) to provide a source of nutrients for the butterflies. At the end of the day, all plants and butterflies were removed from the cages. The plants were then checked to see if the butterflies had oviposited on the plants. The following day the number of eggs in each cluster was counted. This process was repeated over successive days with plants and butterflies without oviposition experience added to the cages. We used eight small cages each day over the course of 13 days. In total we used 84 different plant pairs and 44 different female butterflies. The cages were put outside when the weather was sunny and warm. The IGs aucubin and catalpol of the plants were analyzed using HPLC.

Experiment 2: large cages, Finland. Twelve *P. lanceolata* plants of different genotypes were selected from the nine genotypes of the low and the six genotypes of the high selection line and put in a large cage (1 x 1 x 1 m) outside at Nåtö Biological Station, Åland, Finland. Before the plants were placed in the cage we counted the number of leaves and the second fully grown leaf was taken from the plant for HPLC analyses. Six mated female *M. cinxia*

butterflies were added to the cage and also provided with access to honey water in a sponge. The experiment was performed as described above. Only one large cage was used each day over the course of nine days.

The butterflies were only replaced if they died or if there were many egg batches laid in a day. In total we used 21 different females. The plants that had no oviposition were put back randomly in the cage the next day. Plants with oviposition were replaced with fresh plants the next day.

To see if there was systemic induction of IGs in the plant after oviposition in experiment 1 and 2, we determined the IG levels in leaves from 28 plants. We compared the IG level of the second fully grown leaf (sampled before oviposition) with that of the leaf opposite the leaf that bore the egg batch (sampled in the evening after oviposition).

Experiment 3: large cages, the Netherlands. Ten cages (1 x 1 x 1 m) were placed outside near the Netherlands Institute of Ecology at Heteren. In each cage we put ten plants, five genotypes with low (L1-L5) and five genotypes with high (H1-H5) levels of leaf iridoid glycosides (IG) (see study species). All the cages represented single replicates. From each of the plants we counted the number of leaves and we collected the sixth fully grown leaf for chemical analyses. Into each cage we released a single male and female of *M. cinxia*. The following days we checked for evidence of oviposition, but we did not remove the plant or the butterflies. We marked the leaves onto which the female butterflies had oviposited, but did not take the eggs away. The experiment was terminated when all of the butterflies had died. In total we used 12 female butterflies from 25th May till 16th June.

Experiment 4: field site, Finland. We used the same 10 genotypes of *P. lanceolata* as in the above experiment, five with low IG (L1-L5) and five (H1-H5) with a high level of leaf IG. In the fall of 2001, we made 40 clonal replicates of each genotype using the root-cloning method from Wu and Antonovics (1975). Plants were maintained over the winter in 11cm pots filled with potting soil in an unheated greenhouse in Heteren, the Netherlands. On May 8, 2002, roots of the c. 25cm tall plants were washed to remove adhering potting soil, plants were put in moist bags, shipped to Finland and stored at 4ºC until transplantation. A small field was selected as transplantation site, in an open, dry rocky area on peat soil with sparse shrubs and trees near Tvärminne Zoological Station, southwest Finland. This site represents a suitable habitat within the distribution range of *P. lanceolata* and *M. cinxia*, but at the time of the experiment neither of these species were observed to occur naturally. On May 11, 2002, the plants were planted in 40 patches of 10 plants, each patch containing one individual of each genotype. The roots were gently placed in small slits in the soil to minimize disturbance of the natural vegetation. Plants within the patches were planted 10cm apart and the patches were at least 2 m apart. They were watered as needed.

Melitaea cinxia larvae were collected from Åland in 2001, overwintered as 5th instar in the lab at Nåtö Biological Station, and pupated in the spring of 2002. Three-hundred adult butterflies were introduced to the Tvärminne site the morning after they emerged. This occured between June 6 and 9, 2002, when the experimental plants had regrown new leaves under the prevailing habitat conditions.

We harvested one or two fully grown leaves of each plant from seven patches, and air-dried them for HPLC analysis of IGs to estimate the average level of IGs for each genotype in the field, based on the plants sampled from these seven patches. Starting when the first butterflies were released on June 6, plants were checked daily for the presence of egg clusters. For each egg cluster we recorded whether eggs hatched, whether caterpillars managed to

produce a winter-nest and the number of larvae per winter-nest. As some caterpillars moved from the oviposition plant to nearby plants, the number of damaged plants exceeded the number of oviposited plants and some winter-nests were produced on non-oviposition plants. On June 15 we measured the number of leaves and the length and width of the longest leaf of each experimental plant. The experiment ended in the autumn when most caterpillars had gone into diapause.

Chemical analyses

For the experiments performed on Åland (1 and 2), the second fully grown leaf was taken from all the *P. lanceolata* plants used for the oviposition experiment. They were air-dried in open envelopes as were the leaves collected in experiment 4 in Tvärmmine. The leaves from the cages in Heteren (experiment 3) were frozen at -80˚C and then freeze-dried. After the leaf drying step, the procedure for chemical analyses was the same for all experiments. Leaves were ground with a Laboratory Vibration Mill (MM 301, Retsch GmbH & Co, Germany). Fine ground dry material of the leaves (25mg) was extracted in 10ml of 70% MeOH and was shaken overnight. The crude extract was filtered on a Whatman #4 filter paper and diluted ten times with Milli-Q water. The concentrations of the IGs aucubin and catalpol were analysed by HPLC using a Bio-Lc (Dionex Corp., Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4 x 50mm) and analytical column (4 x 250mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD). NaOH (1M) and Milli-Q water were used as eluents (10:90, 1ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Concentrations were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunnyvale, USA).

Statistical analyses

For the cage experiments (1, 2 and 3) differences between leaf IG concentrations and the number of leaves from plants with and without oviposition were analysed with paired t-tests. (STATISTICA version 7.1, StatSoft Inc., Tulsa, UK). In experiment 1 the plants with and without oviposition in the same cage were paired, in experiment 2 and 3 the plant with oviposition was paired with the mean level of the plants without oviposition in the same cage. For experiment 1, the number of leaves, the number of eggs and the aucubin level were log10 transformed prior to analyses to meet assumptions of normality and homogeneity of variances. Similarly, for experiment 2 all IG compounds (aucubin, catalpol and the sum of aucubin and catalpol, "total IG") and the number of leaves were log10-transformed. In experiment 3, aucubin was log10-transformed, whereas catalpol and total IG were square-root transformed. For all three cage experiments we used (Pearson) correlations to look for associations between the egg cluster size laid on a host plant and the iridoid level or the number of leaves present on that plant. In experiment 4, differences in size and leaf IG concentrations among genotypes and patches were analysed using generalized linear models (Procedure GENMOD, SAS v. 8.2, SAS Institute, Cary, NC) with a normal error distribution. Leaf length, leaf width, leaf number and the product of these ("plant size index"), as well as the concentrations of aucubin, catalpol and total IG were square-root transformed prior to analyses to meet assumptions of normality and homogeneity of variances. Plant size index was used as a covariate in analyses of IGs to estimate size-independent genotypic differences in secondary chemistry. Effects of plant size, genotype and patch on the number of egg clusters, number of hatched clusters and number of diapausing clusters per plant was analysed using a GLM with a Poisson error distribution and a log link function after (x+1) transformation of the independent variables. Since levels of aucubin and catalpol were only measured on a subset of the plants, yielding insufficient data on phenotypic associations

between secondary metabolites and oviposition, effects of aucubin and catalpol on oviposition were assessed at the genotypic level only by univariate and multiple regression of genotype means for size and secondary metabolite levels on genotype means for oviposition traits.

Results

Oviposition experiments

Experiment 1: small cages, Finland

In total 18 different females laid 21 egg clusters on different plants. Plants onto which *M. cinxia* oviposited had a significant higher level of aucubin (2.56 ± 0.43) than plants without (1.57 ± 0.37) oviposition (paired t-test, t=2.78, df=16, P=0.013; Fig. 4.1A). In 71% of the cases the females chose the plant with the higher aucubin level. By contrast, there were no consistent differences in the level of catalpol between plants selected (2.25 ± 0.35) and ignored (2.59 ± 0.35) for oviposition. In 47% of the oviposition choices the plant had a lower level of catalpol, whereas in 53% of the choices the plants with the higher levels of catalpol were preferred (paired t-test, t=-0.72, df=16, P=0.48). There was no significant difference in the total IG level between plants with (4.81 ± 0.58) and without (4.16 ± 0.55) oviposition (paired t-test, t=0.81, df=16, P=0.43).

The number of leaves on the plants ranged from 6 to 26, but they had no significant effect on oviposition choice (paired t-test, t=0.45, df=15, P=0.66). In 43 % of the cases the plant onto which the butterfly oviposited had fewer leaves than the alternative plant, whereas in 50% of the cases it had more leaves and in 7% of the cases they had the same amount of leaves. There was no correlation between the egg cluster size laid on a host plant and the iridoid level or the number of leaves present on the plant (aucubin: r=-0.085, n=17, P=0.75; catalpol: r=+0.15, n=17, P=0.56; total iridoid level: r=+0.19, n=17, P=0.46; #leaves: r=+0.43, n=16, P=0.10).

Experiment 2: large cage, Finland

In total nine plants received 11 egg batches. In 78% of the oviposition events in the large cage, the female put her eggs on a plant with a higher than average concentration of aucubin then the other plants in the cage (Fig. 4.1B). However, the difference in the level of aucubin between plants that were chosen for oviposition and plants that were ignored was not significant (paired t-test, t=1.14, df=8, P=0.29; Fig. 4.1B). All of the other parameters, such as the level of catalpol, total IG level or the number of leaves were not related to oviposition choice of the *M. cinxia* butterflies in this experiment (paired t-tests, catalpol: t=-0.20, df=8, P=0.85; total IG level: t=1.03, df=8, P=0.33; #leaves: t=1.43, df=8, P=0.19). There was no correlation between the egg cluster size a female laid on a host plant and the IG level or the number of leaves present on the plant (aucubin: r= -0.11, n=9, P=0.78; catalpol: r=-0.22, n=9, P=0.57; total IG level: r=-0.29, n=9, P=0.46; #leaves: r=+ 0.08, n=9, p=0.83).

In total we analysed concentrations of IGs in leaves of 28 plants before and after oviposition in experiment 1 and 2. The before and after measures did not differ significantly, suggesting that there was no induction of IGs by the oviposition event (paired t-test, aucubin: t=-0.15, df=27, P=0.89; catalpol: t=0.22, df=27, P=0.82; total IG level: t=1.7, df=27, P=0.10).

Experiment 3: large cages, the Netherlands

In total 12 plants received 15 egg batches. The iridoid levels of the plants were not significantly related to the oviposition choice by the butterflies in the cage (paired t-tests,

aucubin: t=-0.52, df=11, P=0.61; Fig. 4.1C; catalpol: t=0.43, df=11, P=0.67; total IG level: t=0.45, df=11, P=0.66). However, there were significantly higher rates of oviposition on plants with fewer leaves (t=-2.70, df=12, P<0.05; Fig. 4.1D). The number of leaves on the plant ranged from 6-49 and was negatively correlated with the iridoid level of the plant (aucubin: r=-0.47, n=59, P<0.001; catalpol: r=-0.45, n=59, P<0.001; total IG level: r=-0.48, n=59, P<0.001).

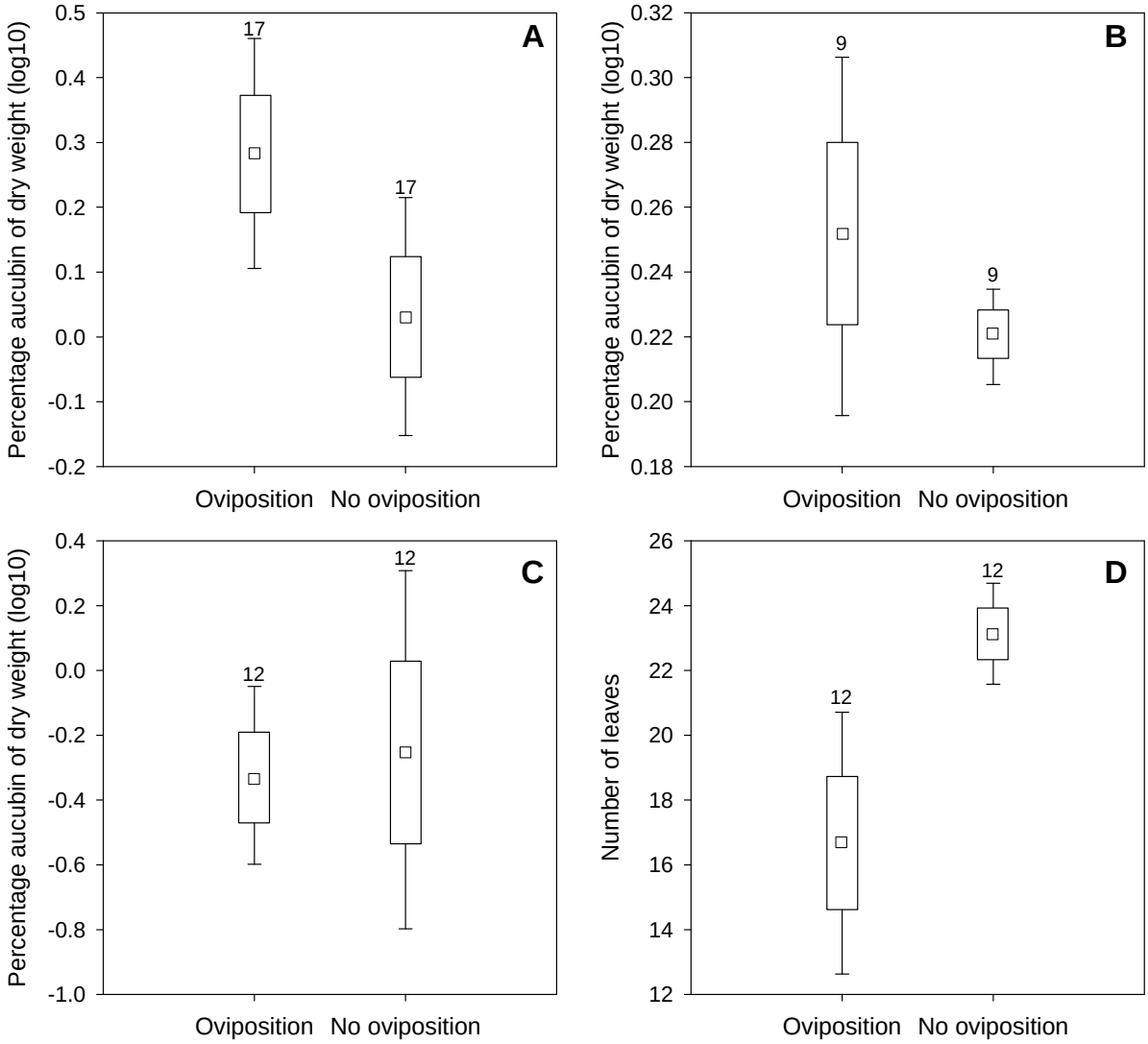

Figure 4.1 The average percentage (log10) of dry weight of aucubin of *Plantago lanceolata* plants with and without oviposition by *Melitaea cinxia* in experiment 1 (A), 2 (B) and 3 (C). (D) The average number of leaves of *P. lanceolata* plants with and without oviposition by *M. cinxia*, in experiment 3. See text for a description of the experiments.

Experiment 4: field site, Finland

Size and chemistry of plant genotypes in the field.

Plant size differed both among genotypes and patches (Table 4.1 and 4.2). Genotypes showed three-fold variation in the index that was calculated to estimate plant size. Genotypes also showed significant, circa four-fold, variation in their average leaf concentration of IGs (Table 4.1). The range of aucubin levels (nine-fold) was larger than the range of catalpol levels (four-fold, Table 4.1). Part of the variation in total levels of IGs and catalpol among plants were associated with differences in plant size (Table 4.2). The total IG level of the plant was negatively correlated with both the number of leaves and the size index of the plants (phenotype level: #leaf: $r=-0.55$, $n=64$, $P<0.001$, size index: $r=-0.43$, $n=64$, $P<0.001$; genotype level: #leaf: $r=-0.77$, $n=10$, $P<0.01$, size index: $r=-0.68$, $n=10$, $P<0.05$). However, the levels of both aucubin and catalpol were independent of the patch where plants were growing (Table 4.2). Genotypes from the high selection line (H1-5) generally had higher levels of total IG than genotypes from the low line (L1-5) (Table 4.1), but levels of the constituent components aucubin and catalpol varied greatly among genotypes within lines.

Table 4.1 Characteristics of 10 *Plantago lanceolata* genotypes selected for low (L1-L5) or high (H1-H5) leaf IG in the Tvärminne field site and occurrence of *Melitaea cinxia* on them. Values for leaf IG concentrations and plant size are back-transformed least square estimates from GLM with block and genotype effects. Values within columns that do not share a common letter have non-overlapping 95% confidence intervals. Occurrence of *M. cinxia* is summed over the 40 replicate plants per genotype: the observed numbers of plants with egg clusters (P), total numbers of egg clusters (C), hatched clusters (H) and diapausing groups in winter-nests (D).

	Leaf IG (% dw)			Plant size (cm)				*M. cinxia*			
	Total	Aucubin	Catalpol	Size index	Leaf number	Leaf length	Leaf width	P	C	H	D
L1	2.15 ^{a}	0.55 ^{a}	1.57 ^{a}	179.0 ^{f}	17.9 ^{c}	10.2 ^{ab}	0.99 ^{bc}	11	20	17	5
L2	5.77 ^{bc}	1.98 ^{cd}	3.77 ^{bc}	89.6 ^{bc}	6.2 ^{a}	13.4 ^{c}	1.03 ^{c}	1	1	1	1
L3	5.79 ^{bc}	2.93 ^{d}	2.80 ^{b}	95.9 ^{bcd}	9.7 ^{b}	11.8 ^{bc}	0.85 ^{ab}	3	3	3	1
L4	5.30 ^{b}	2.62 ^{cd}	2.67 ^{b}	101.1 ^{cde}	7.7 ^{ab}	9.7 ^{a}	1.34 ^{d}	5	5	1	1
L5	6.29 ^{bcd}	0.85 ^{ab}	5.41 ^{d}	64.9 ^{ab}	9.8 ^{b}	9.4 ^{a}	0.70 ^{a}	3	4	4	3
H1	7.27 ^{cde}	1.85 ^{c}	5.39 ^{d}	125.3 ^{de}	9.8 ^{b}	12.0 ^{bc}	1.03 ^{c}	3	3	3	0
H2	8.60 ^{e}	4.93 ^{e}	3.65 ^{bc}	90.1 ^{bcd}	7.0 ^{a}	13.0 ^{c}	0.97 ^{bc}	1	1	1	1
H3	7.91 ^{de}	1.62 ^{bc}	6.20 ^{d}	83.5 ^{abc}	8.0 ^{ab}	10.5 ^{ab}	0.98 ^{bc}	4	10	9	5
H4	6.65 ^{bcde}	2.00 ^{cd}	4.51 ^{cd}	56.0 ^{a}	6.0 ^{a}	9.0 ^{a}	0.93 ^{bc}	7	12	8	2
H5	5.07 ^{b}	1.72 ^{c}	3.32 ^{bc}	138.9 ^{ef}	9.2 ^{b}	13.8 ^{c}	1.07 ^{c}	3	4	1	2

Table 4.2 Effects of patch and genotype on the size and leaf IG concentration of *Plantago lanceolata* in the Tvärminne field site. Size index was used as a covariate in analyses of IGs. Values are quasi-F values from GLM analyses of deviance (* P<0.05; ** P<0.01; *** P<0.001).

Source	df	Size index	Leaf number	Leaf length	Leaf width	df	Total IG	Aucubin	Catalpol
Covariate						1	35.3 ***	3.0	30.9 ***
Patch	39	2.5 ***	2.5 ***	1.9 **	2.5 ***	7	1.3	1.1	1.6
Genotype	9	12.0 ***	22.9 ***	10.9 *	11.5 ***	9	12.9 ***	13.5 ***	10.1 ***
Error	334					49			

Oviposition and performance of M. cinxia on different plant genotypes.

Egg clusters of *M. cinxia* were found on 41 plants, or 10.3% of the plants in the experimental field site. The total number of egg clusters was 63, with a maximum of 7 per plant and 9 per patch. Hatching was observed for 48 (76.2%) of the egg clusters. One-third of the egg clusters eventually produced winter-nests that harboured on average 11.5 diapausing larvae. Of these nests, 13 were on the initial oviposition plant and 8 on non-oviposition plants to which larvae had moved during the season.

The number of egg clusters per plant significantly increased with plant size and differed both among patches and among genotypes (Table 4.3), ranging from a mean number of 0.025 to 0.50 clusters per plant for different genotypes. Similar effects were found for the number of successful clusters, i.e. clusters that hatched and that produced winter nests (Table 4.3). Effects of plant size on the number of egg clusters were mainly due to an increase in oviposition with the number of leaves per plant (Fig.4.2A). The association between maximum leaf length and oviposition was non-linear, plants with leaves of an intermediate length of ca. 8cm were the most often used for oviposition (Fig. 4.2B), whereas leaf width was not associated with the number of egg clusters on a plant (P>0.5). Univariate regressions (Table 4.4) showed that at the genotype level, two factors significantly contributed to genotypic differences in the number of egg clusters per plant: average levels of aucubin and average leaf length (Table 4.4, Fig. 4.2B and C). In a multiple regression, the effect of aucubin disappeared (Table 4.4), indicating that it was partly mediated by genotypic correlations with other factors. In particular, this involved a negative correlation with leaf number (Fig. 4.3) that tended to have a positive effect on oviposition (Fig. 4.2A), and a positive correlation with maximum leaf length, which had a negative effect on oviposition in the range of values for the genotype means (Fig. 4.2B).

Table 4.3 Effects of plant size, patch and genotype of *Plantago lanceolata* in the Tvärminne field site on the number of *Melitaea cinxia* egg clusters per plant that were oviposited, hatched, and produced winter-nests. Values are quasi-F values from GLM analyses of deviance (* P<0.05; ** P<0.01; *** P<0.001).

	df	Egg clusters	Hatched	Diapausing
Plant size	1	45.6 ***	56.5 ***	13.2 ***
Patch	39	2.3 ***	2.2 ***	1.8 **
Genotype	9	5.1 ***	5.4 ***	2.2 *
Error	333			

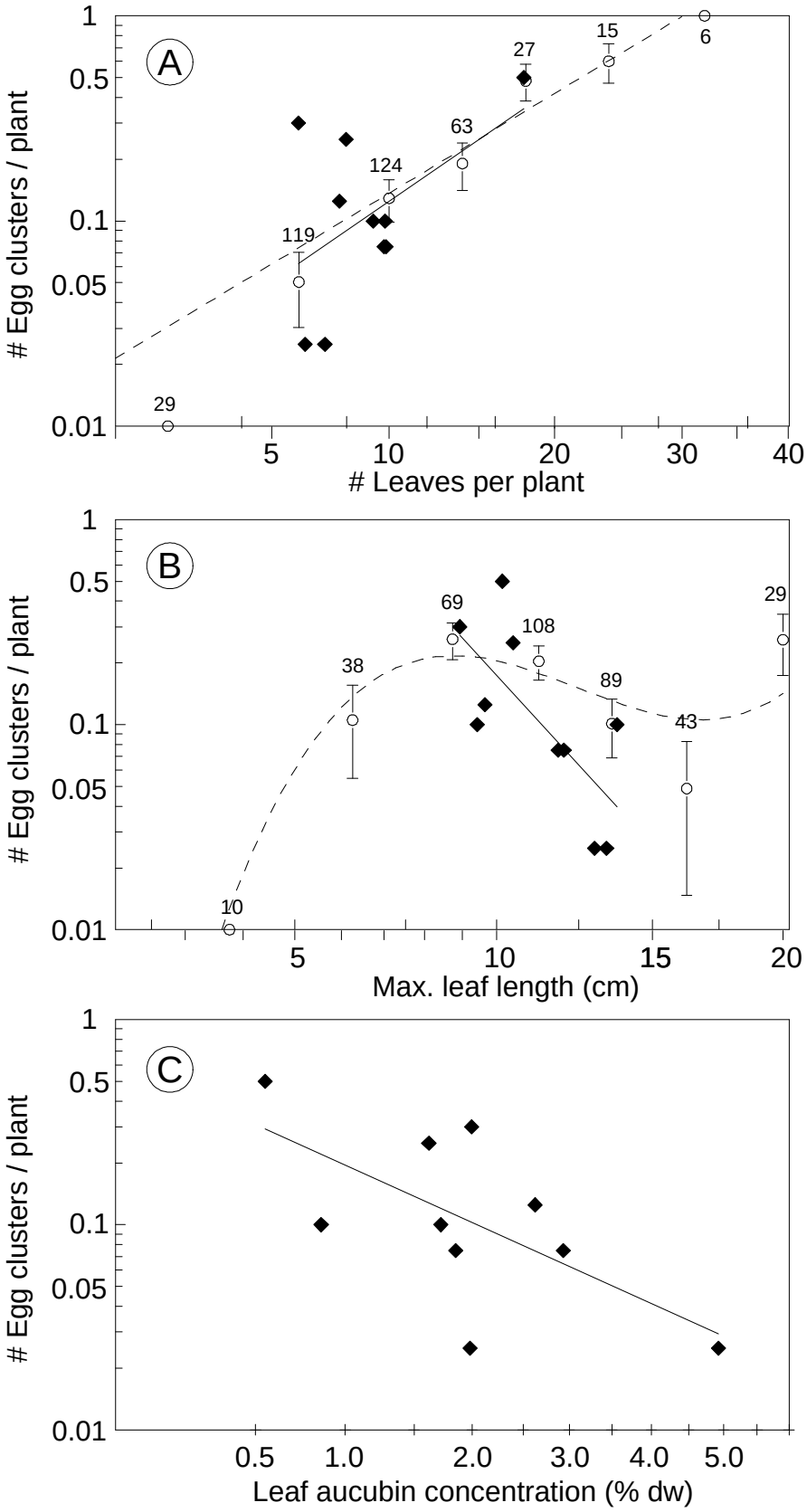

Figure 4.2 Number of *Melitaea cinxia* egg clusters per *Plantago lanceolata* plant as a function of (A) number of leaves per plant, (B) length of the longest leaf per plant, and (C) leaf aucubin concentration. Black diamonds represent genotype mean values with cor-responding solid lines of re-gression. Open circles (sample size on top) represent mean numbers of egg clusters for classes of phenotypic values of leaf number and leaf length ± 1 s.e. (class limits indicated above the X-axis). Dotted lines are corresponding polynomial regression lines based on parameter estimates from Poisson regressions of the phenotypic data. Note the square root scale of axes.

Table 4.4 Univariate and multiple regressions of genotype means for morphological and chemical traits on genotype means for the number of *Melitaea cinxia* egg clusters per plant of *Plantago lanceolata* in the Tvärminne field site. Values are standardized regression coefficients ($^{+}$ P<0.10; * P<0.05; ** P<0.01; *** P<0.001).

	Univariate	Multivariate
Total IG	-0.503	-
- Aucubin	-0.648*	-0.231
- Catalpol	-0.140	-
Plant size index	+0.199	-
- # Leaves per plant	+0.503	+0.278
- Max. leaf length	-0.718*	-0.583^{+}
- Max. leaf width	-0.005	-
Model R^2		0.71*

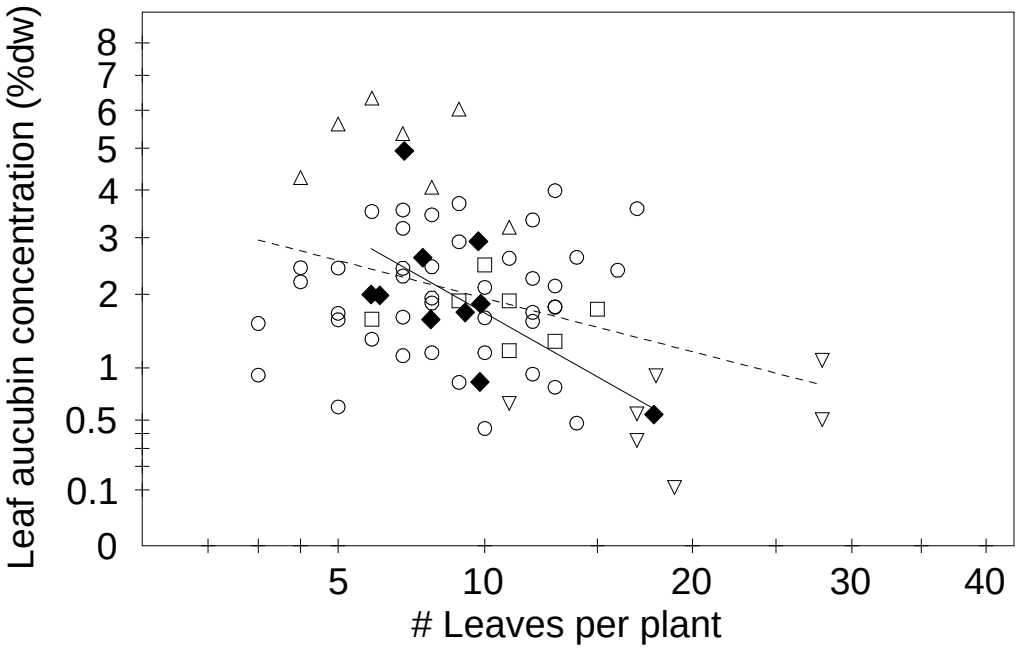

Figure 4.3 Regression of phenotypic values (open symbols, dotted line) and genotype means (solid symbols, solid line) of leaf aucubin concentration on the number of leaves per *Plantago lanceolata* plant. Genotypes with intermediate (H5), high (H2) and low (L1) aucubin levels are indicated by open squares, upward and downward pointing triangles, respectively.

Comparison of plant chemistry between the four experiments

If we compare the IGs from all four experiments, it is clear that the total amount of IGs as well as the ratio of aucubin and catalpol to the total IG differs between the experiments (Fig. 4.4 and Table 4.5). In the small cages and in the field experiment in Finland, most plants had on average a higher level of catalpol than aucubin (62% and 70% of the plants, respectively). In the large cage experiment in Heteren all plants had a higher level of aucubin than catalpol, but in this experiment the level of IGs was on average very low. Finally, in the large cages used on Åland, in 70% of the plants the level of aucubin was higher than the level of catalpol. Especially striking is the huge difference between the plants used for the experiment in Heteren and the field experiment in Finland, because plants used for these two experiments were from the same genotypes. Levels of aucubin were roughly similar in the two experiments, but the amount of catalpol in experiment 3 had a mean of 0.16% of the dry weight whereas in experiment 4 it had a mean of 4.0% (independent t-tests, aucubin: $t=3.64$, $df=18$, $P=0.084$; catalpol: $t=8.27$, $df=18$, $P<0.001$; Table 4.5). Although the absolute levels of IGs were significantly higher in experiment 4, the rank order of the genotypes was consistent across both experiments (Fig. 4.5).

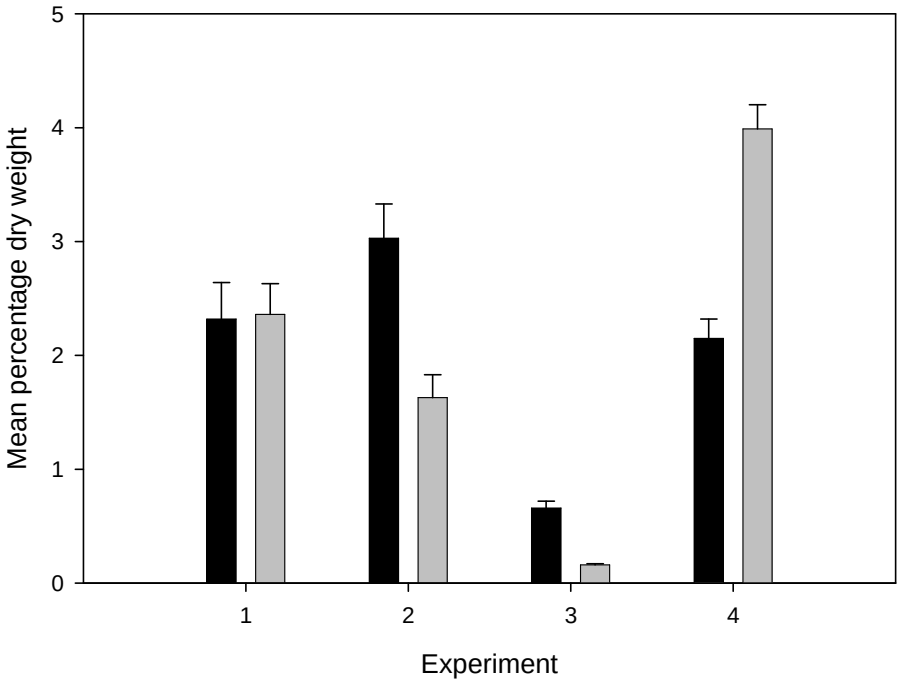

Figure 4.4 Mean aucubin and catalpol level of *Plantago lanceolata* plants used in the four different experiments. Black bars indicate the mean aucubin level; grey bars the mean catalpol level. Experiment 1: small cages, Finland; 2: large cage, Finland; 3: large cage, the Netherlands; 4: field site, Finland. See text for a detailed description of the four experiments.

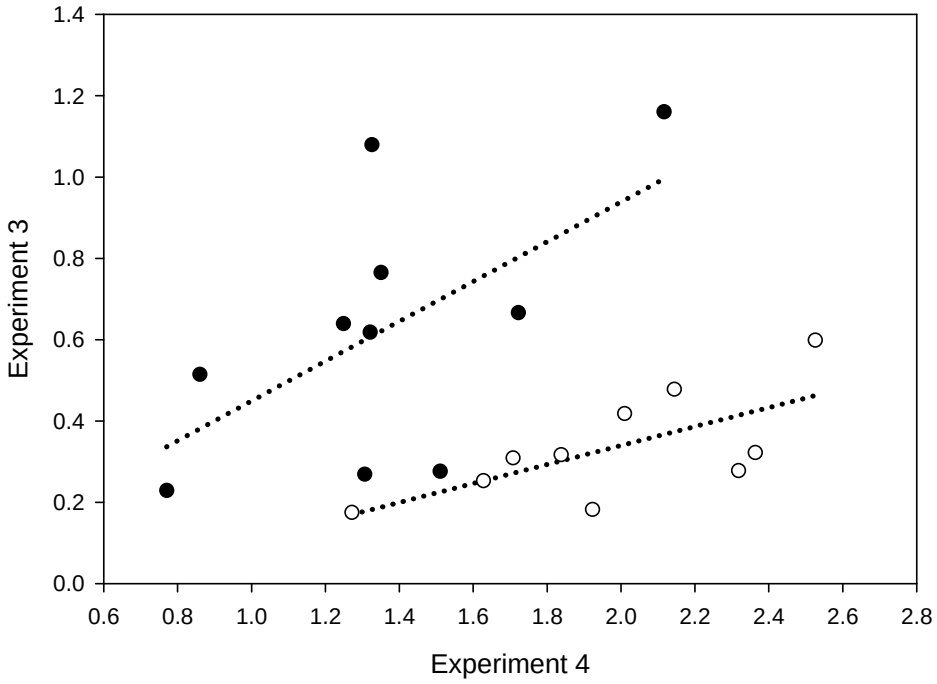

Figure 4.5 The IG-levels of *Plantago lanceolata* plants used in experiment 3 versus the IG levels of the plants used in experiment 4. The levels of aucubin are indicated with black symbols and the levels of catalpol with white symbols. See text for a detailed description of the experiments.

Table 4.5 Descriptive statistics of the iridoid glycoside (IG) levels of the *Plantago lanceolata* plants used in all four experiments.

Experiment	IG	n	Mean	Min.	Max.	S.d.	S.e
1	aucubin	29	2.32	0.35	6.51	1.73	0.32
	catalpol	29	2.36	0.55	5.80	1.46	0.27
	total	29	4.67	1.40	11.57	2.36	0.44
2	aucubin	20	3.03	1.02	5.60	1.34	0.30
	catalpol	20	1.63	0.58	3.98	0.87	0.20
	total	20	4.66	2.38	8.08	1.56	0.35
3	aucubin	149	0.66	0.01	4.19	0.76	0.06
	catalpol	144	0.16	0.00	0.93	0.17	0.01
	total	144	0.83	0.03	4.38	0.88	0.07
4	aucubin	67	2.15	0.11	6.33	1.37	0.17
	catalpol	67	3.99	0.78	8.35	1.73	0.21
	total	67	6.14	0.88	12.06	2.20	0.27

Discussion

Butterflies that lay eggs in clusters, such as checkerspots are expected to spend more time discriminating among hosts than solitary egg laying species (Singer 2004). When a searching *M. cinxia* alights on a host, it typically "tastes" it, rests for a while before moving to another part of the plant or to another plant, tastes again, etc., until she finds the plant chemically acceptable and starts to oviposit. The female clearly decides where to oviposit based on cues she obtains from the plant. The results of the cage experiments on Åland (experiment 1 and 2) both suggest that the oviposition choice of the female is related to the level of aucubin in the plant. In the dual choice tests there was a significant preference for plants with a higher level of aucubin, and in the multiple choice test, the females preferred plants that had higher levels of aucubin than the average level of aucubin of all plants in the cage. These results are in agreement with those of Nieminen (2003) obtained by sampling from natural populations in the field. It is important to note that, in contrast to the field data obtained by Nieminen (2003), we sampled leaves for IG measurements before oviposition occurred, hence we can exclude the possibility that the higher levels of aucubin in the plants selected for oviposition were simply a consequence of the induction of IGs following oviposition. There also was no difference in the IG-levels of leaves before and after oviposition, indicating that the oviposition event itself did not systemically induce the production of IGs. However, since we did not measure IG levels before and after oviposition in the leaf that was actually selected for oviposition, we cannot rule out the possibility that local induction in the leaf selected for oviposition occurs (Peñuelas et al. 2006). Another reason we did not detect induction might be the timing of the induced response. We took the leaves for sampling on the same day as the oviposition, but it is possible that the induction of the IGs took longer than a single day. Unfortunately, in contrast to studies of induction of secondary metabolites following herbivory, timing of induction following oviposition is poorly known. Induction of IGs in *P. lanceolata* following fungal infection has been observed as early as six hours after inoculation (Marak et al. 2002b) but induction after leaf damage by caterpillars of the specialist *Junonia coenia* was not observed until six days after herbivory (Fuchs and Bowers 2004).

The level of aucubin is correlated with the total level of IGs. Despite the fact that specialist herbivores usually pay a cost of dealing with the secondary metabolites in their preferred hosts (Camara 1997a), several experiments have shown that *M. cinxia* larvae have a better performance on plants with a higher level of IGs than on plants with a lower level of IGs. Larvae have a shorter development time, higher larval weight and they tend to have a larger pupal size (Harvey et al. 2005, Saastamoinen et al. 2007, Chapter 3). These factors are usually correlated with a higher fitness (Roff 1992). There are many factors that affect larval fitness of *M. cinxia,* but larval size is strongly correlated with overwintering mortality, which can be very high (Nieminen et al. 2001, van Nouhuys et al. 2003, Kuussaari et al. 2004). The shorter development time may also be an advantage for *M. cinxia* because of the short growing season (Kuussaari et al. 2004) and fast development decreases the period of time the caterpillar will be vulnerable to its natural enemies (van Nouhuys and Lei 2004).

Another advantage of feeding on plants with a high level of IGs may be chemical defence against natural enemies such as predators and parasitoids. Specialized larvae feeding on iridoid-producing plants are able to sequester these iridoids and become distasteful or noxious themselves (Bowers 1980, 1981, Bowers and Puttick 1986, Franke et al. 1987, Gardner and Stermitz 1988, Belofsky et al. 1989, Bowers 1990, L'Empereur and Stermitz 1990, Stermitz et al. 1994, Camara 1997b, Suomi et al. 2001b, Chapter 3).

The fact that we did not observe a difference in oviposition choice between plants with high or low levels in aucubin, catalpol or total IG level in experiment 3 (large cages Heteren)

could be caused by the overall low level of IGs in these plants. Perhaps the female *M. cinxia* is not able to discriminate between such absolute low levels of IGs (on average lower than 1% of the dry weight) or maybe the relative difference between the plants was not big enough to make them distinguishable.

The results of experiment 4, the field experiment, differed from the results of the cage studies. Notably, the size of the plant (mainly the number of leaves per plant) had a positive effect on the oviposition preference of the females, while in the cage experiments there was no association (experiment 1 and 2) or even a significantly negative association (experiment 3). We expected that size of host plants would be an important aspect of their suitability for *M. cinxia* because plants selected for oviposition should be large enough to support the growth of all (usually more than a hundred) larvae that hatch from the egg batch. As soon as the gregarious larvae hatch, they spin a communal web on the host plant and they are usually restricted to the plant they hatch on (Kuussaari et al. 2004). A stronger impact of host size on oviposition preference in the field than in cage experiments can be expected for instance if plants in the field are in a critical range of sizes, whereas plants in the cages are all large enough to support larval development. However, plants in the cage experiments on average did not produce more leaves than plants in the field. A more likely explanation for the difference in importance of host size for oviposition between the field and cage studies is that the plants used in the cages were more similarly sized than the plants used in the field experiment. In the field, the difference in leaf number between the smallest and largest plant was 78; in the cage studies in Finland the mean difference in leaf number between the plants between which *M. cinxia* could choose was only 3.3 in experiment 1 and 7.9 in experiment 2. Because of this smaller variation in plant size in the cage experiments the oviposition choice of the female in the cages could be based to a greater extent on chemical rather than on visual stimuli.

In all cage experiments, IG levels of each plant individual were measured for each individual plant. Associations between oviposition and traits including IGs could therefore be studied at the individual plant level. By contrast, in the field (experiment 4), IG levels were measured for a subset of plants only. Based on these measurements, genotype mean IG levels were used to assess associations between oviposition and IGs. This resulted in a loss of power (10 genotype values) to detect associations and made it impossible to disentangle effects of morphological traits and IGs on oviposition of individual plants. Another difference between field and cage studies was the overall level of IGs. Even though the ranking of genotypes with respect to IG concentrations was comparable between the cages (experiment 3) and the field (experiment 4), the absolute amounts were very different, reflecting environmental and/or developmental effects on overall levels of IGs. This suggests that these plants are highly plastic in their IG levels in different environments but that genotypes show roughly similar responses to environmental conditions.

In summary, our results show that a female *M. cinxia* discriminates between host plants for oviposition. For females in the field, the size of the plant is a positive visual stimulus; in the cage, where plants are more similar in size and probably in visual appearance, chemical stimuli apparently are more important. When we look at the plant chemistry, in general, *M. cinxia* prefers plants with a higher level of aucubin. Since these levels were measured prior to oviposition, we can exclude that the positive association between higher levels of aucubin and oviposition in our experiments resulted from induction by the oviposition event itself.

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Chapter 5

Host plant use by the heath fritillary butterfly, *Melitaea athalia*: plant habitat, species and chemistry

J. H. Reudler Talsma, K. Torri and S. van Nouhuys

Abstract - We present a study of habitat use, oviposition plant choice, and food plant suitability for the checkerspot butterfly *Melitaea athalia* Rottemburg (Lepidoptera: Nymphalidae) on Åland, Finland. We found that on Åland, unlike on the mainland of Finland and most other parts of its range, *M. athalia* flies predominantly in open meadows. The plant species it prefers for oviposition in tis study, *Veronica chamaedrys* L., *V. spicata* L. (Scrophylariaceae) and *Plantago lanceolata* L. (Plantaginaceae) also grow in open meadows. This difference in host plant and habitat use between Åland and other regions may reflect local adaptation to land use practices and geology that maintain clusters of small open meadows. At small scale the butterflies prefer to oviposit on plants in open rather than grassy sites. Preferred plant species were equally attractive for oviposition in mixed species patches as in mono-specific patches. Despite the fact that the presence of IGs is an important trait distinguishing host from non-host species used by *M. athalia*, ovipositing preference within the group of (potential) host species and among individual plants within host species was largely independent of IG concentration. Although the adult butterflies chose specific plant species for oviposition, as larvae they performed well on twelve out of thirteen plant species, both on known host plants as well as on related plants that occur on Åland.

Introduction

Most herbivorous insects are selective in their oviposition choice, both at the level of plant species, and at the level of plant individuals within these species (Ng 1988, Singer and Lee 2000). What criteria are used and how stringent they are defines the host range, as well as the habitats in which an herbivore is found. Most herbivorous insects specialize in a taxonomically or chemically related group of host species that grow in similar habitats. For instance, checkerspot butterflies (the Melitaeini in the family Nymphalidae) are oligophagous or monophagous on plant species belonging to 16 families that inhabit meadows, forest edges and forest clearings (2004). Of these, most of the plants used are in 11 families that share iridoids as plant secondary compounds (Jensen et al. 1975, Jensen 1991, Wahlberg 2001). The most common group of these iridoids are the iridoid glycosides (IGs) (Damtoft et al. 1997, Li et al. 1999, Sturm and Stuppner 2001), which are deterrent to many generalist herbivores (Bowers and Puttick 1986, Stamp 2001), but are tolerated or even sequestered by specialists (Bowers and Puttick 1986, Stermitz et al. 1986, Franke et al. 1987, Belofsky et al. 1989, L'Empereur and Stermitz 1990, Mead et al. 1993, Stermitz et al. 1994, Suomi et al. 2001b, Harvey et al. 2005, Chapter 3). The caterpillars ability to sequestrate these plant secondary chemicals is thought to make them unpalatable or unsuitable for generalist predators and parasitoids (Bowers 1980, Camara 1997b).

In this study we investigate the habitat and host plant use by the oligophagous checkerspot, heath fritillary, *Melitaea athalia* (Rottemburg, 1775) (Lepidoptera: Nymphalidae) on Åland in southwestern Finland. This butterfly is a common species in southern Finland (Marttila et al. 1990), but has declined severely in most of Europe (Warren et al. 1984, Schwarzwälder et al. 1997). The critical factor governing the survival of *M. athalia* is its dependence on the continual creation of specific and very short lived types of habitat (Warren 1987a).

Melitaea athalia is part of an ecologically and evolutionarily well-studied group of butterflies (Ehrlich and Hanski 2004) that is of interest to conservation ecologists (Cowley et al. 1999). The aim of this paper is to describe habitat and host plant use by *M. athalia* where it differs from other regions, and is not in decline. A second motivation for this study is to assess the ecological overlap of *M. athalia* with the well studied Glanville fritillary butterfly, *Melitaea cinxia* (Linnaeus, 1758) (Lepidoptera: Nymphalidae), which co-occurs on Åland.

First, we present the natural habitat use of the adult butterflies on Åland by transect counts during the flight season in forest edges, dry and mesic meadows, herb-rich road verges and field edges, clearings within forest and semi-open forests. Because human land use on Åland is different from that on the mainland of Finland and Britain we expect the habitat use by *M. athalia* also to differ. Second, we present an oviposition choice experiment in a large outdoor cage, in which butterflies could freely fly and oviposit among (potential) host plant species over a two week period. The plant species available to the butterfly included most of the species that are known to be used in Finland and Britain as well as several related species present on Åland.

For the congener *M. cinxia*, the IGs are known to be positively associated with oviposition (Nieminen et al. 2003) and larval development (Harvey et al. 2005, Saastamoinen et al. 2007). To investigate the role of IGs in *M. athalia* host plant choice, both with respect to their discrimination among species and among individuals, we analyzed the content of the main IGs, aucubin and catalpol, of all plants, using HPLC before using them in the oviposition experiment. Finally, we compared the ranking of host species with respect to oviposition preference with their ranking in terms of performance of their offspring (development time, survival and diapause weight) by rearing prediapause larvae on 13

different (potential) food plants. These plants included the species used in the oviposition experiment as well as five related species growing in habitats that could potentially be used by *M. athalia* on Åland.

Material and methods

Natural history and study system

In Britain, where *M. athalia* is endangered, it inhabits *Plantago*-rich grasslands, *Melampyrum*-rich woodland clearings and sheltered heathlands containing scattered *Melampyrum* (Warren 1987c). The larvae have been observed feeding on the Plantaginaceae *Plantago lanceolata* L., *P. major* L. and *Digitalis purpurea* L. and the Scrophulariaceae *Melampyrum pratense* L., *Veronica chamaedrys* L., *V. hederifolia* L. and *V. serpyllifolia* L. (Warren 1987b). Due to natural succession its habitats often remain suitable only for a few years (Warren 1987b, 1987c, Wahlberg et al. 2002). The major factor causing the decline of *M. athalia* in Britain during the last 150-200 years is thought to be the decline of choppings as a major form of woodland management, which causes a stop to the supply of new habitats for *M. athalia* (Warren 1987a). Furthermore, the species is thought to be relatively immobile, and since most habitats are short-lived, in order to survive, colonies often have to move as conditions become unsuitable. A combination of these attributes make the species particular vulnerable (Warren 1987c).

On the mainland of Finland *M. athalia* is known to occur along forest edges and in openings within forests, such as wood clearings and abandoned fields (Selonen 1997, Wahlberg 1997, Wahlberg et al. 2002). Female *M. athalia* butterflies were observed by Wahlberg (1997) to land and tap on *V. chamaedrys* and *Melampyrum sylvaticum* L., before ovipositing on an adjacent plant. Post diapause larvae, which are mobile, have also been recorded on *Melampyrum pratense* in Finland (Wahlberg 2000).

The study was conducted on Åland in southwestern Finland during the summer of 2004 (larval feeding) and the spring and summer of 2005 (habitat use and oviposition by adult butterflies). Eggs and post diapause larvae of *M. athalia* have been found on *Veronica spicata, V. chamaedrys*, and *P. lanceolata* on Åland (S. van Nouhuys, personal observation) but their use of other host plants is unknown. The *M. athalia* butterflies and larvae used in the experiments were laboratory reared individuals. They were the progeny of field collected post diapause larvae from Åland that were reared to adulthood on a mixture of *V. spicata* and *P. lanceolata* leaves. Males and females from the different collection sites were mated and the females were placed in cages with potted *V. chamaedrys, and V. spicata* for oviposition. The eggs from the plants were collected daily and moved to Petri dishes. Upon hatching the larvae to be used as adult butterflies were fed *V. spicata* and *P. lanceolata* leaves throughout their development. The larvae used for the larval feeding experiment were moved to the experimental plant in their first instar, soon after hatching.

Habitat use during adult flying season

To observe what types of habitat the adult *M. athalia* uses on Åland we conducted a transect study using the method of Pollard (1997). The region chosen for the three transects was characterised by high habitat heterogeneity and *M. athalia* was know to be common in this region. The 2.6 to 2.9 km long transects crossed habitats in which *M. athalia* is likely to occur (dry and mesic meadows, herb-rich road verges and field edges, clearings within forest and semi-open forests). The transects were divided into sections according to habitat types and separate counts were made for each section. The occurrence of *M. athalia* was monitored by

one person walking each transect route twice a week for six weeks (week 23-28). One last transect walk occurred in week 30 to get information about the length of the flight period. The transect walks were carried out in warm and bright weather between 11.00 and 17.30.

All *M. athalia* butterflies seen within five meters of the transect walker were counted. They were caught with a net to ensure correct identification and to record their sex. The behaviour of the butterflies prior of being caught was also recorded (feeding, mating, flying or basking).

Oviposition host plant preference

This experiment was conducted in a large cage (26 x 30 x 3 m) covered with a mesh that allowed natural environmental conditions inside. The natural vegetation in the cage included nectar plants that the butterflies could feed upon, but no potential host plants. This cage has previously been used to measure the movement behaviour and life history traits (longevity and fecundity) of the butterfly *M. cinxia* (Hanski et al. 2006).

Plants of eight different plant species were transplanted from natural Åland populations in the spring into 9cm diameter pots. Four species, *P. lanceolata* (Pl), *V. spicata* (Vs), *V. chamaedrys* (Vc), *M. pratense* (Mp), are known host plants of *M. athalia*. Two others, *P. major* (Pm) *and V. officinalis* (Vo) L. were included as potential host plants (Table 5.1). Each species was replicated 25 times for a total of 144 plants. Initially, two other plant species, *Melampyrum sylvaticum* (Ms) L. and *M. nemorosum* (Mn) L., were to be included in the experimental set up, however, the conditions in most of the cage were too dry for them. Ten individuals of both species were still placed in a shady moist corner of the cage to see if *M. athalia* would use these species, but they were not included in the statistical analyses.

The 144 plants were set up in the cage in 12 groups consisting of 12 plants each in a three by four plant rectangle. The plant groups were then placed in a three by four grid (Fig. 5.1). The distance between the pots in each group was 15cm and the distance between the groups was 8 m. Six of the groups were monocultures and the other six groups were mixed, with two plants of each of the six species included.

The vegetation in the cage was not uniform. Plots A, B, C, E, F and K were bare areas. Whereas, plots D, G, H, I, J and L were surrounded by vegetation (Fig. 5.1). To separate the effects of microclimate and neighbouring plant from plant species, the locations of the plants were rearranged every evening. Each of the 12 groups stayed together but the location of the group in the cage as well as the order of the pots within each group was randomized daily.

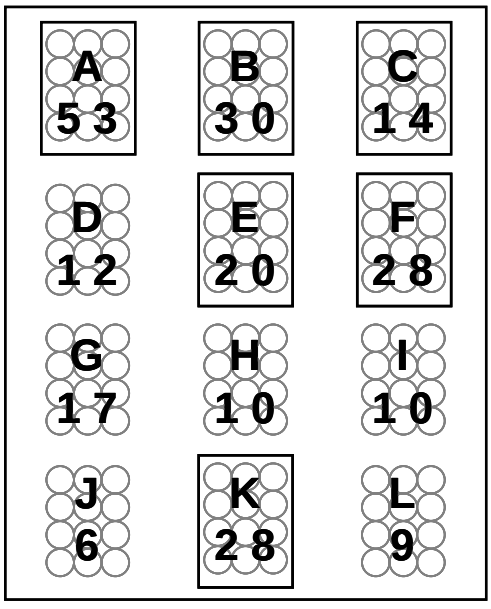

Figure 5.1 Schematic drawing of the locations of plant groups in the cage and the number of *Melitaea athalia* egg clusters laid in each location. Plots A, B, C, E, F and K are surrounded by bare area (outlined plots), plots D, G, H, I, J and L are surrounded by vegetation (no outline). Note that groups of plants were randomized daily so the locations are not associated with a plant species or configuration.

An unrelated experiment was running in the cage at the same time (S. van Nouhuys, in prep.), which meant that there were 42 potted *V. spicata* plants available for oviposition throughout the experiment. These additional plants were subdivided in two groups: "transect" plants and "array" plants. The 20 "transect" plants were placed singly in a four by five grid, wit six meters between the plants. The 22 "array" plants were placed in two lines in the centre of the cage. The two lines where 18 meters apart, but within each line the plants were spaced 5cm apart so that the plants were touching each other. These adjacent *V. spicata* plants were at least 2 meters away from plots A-J.

Newly emerged adult butterflies (93 females and 79 males) were individually marked and released into the cage between 1st of July and the 3rd of July 2005. They were observed to begin feeding and mating soon after release. At the end of each day, between 17:30 and 19:00 all of the plants (including the additional *V. spicata* plants from the "transects" and "arrays") were checked for eggs. All egg clusters were removed from the plants and put in Petri dishes to determine the number of eggs in each cluster. By July 14th (after two weeks) most of the butterflies had died and the experiment ended.

Chemical analyses

To study the association between oviposition choice and plant secondary chemistry, we analysed the IGs aucubin and catalpol as the percentage of leaf dry weight for all the plants used in the oviposition experiment and the additional "array" and "transect" *V. spicata* plants. Before the experiment started, one medium-aged leaf from each plant was taken and air-dried in an open envelope. The leaves were ground to a fine powder with a ball mill (type MM 301, Retsch GmbH & Co., Haan, Germany). The fine ground dry material (25mg/sample) was extracted in 10ml of 70% MeOH and was shaken overnight. The crude extract was filtered on Whatman #4 filter paper and the filtrate was diluted ten times with Milli-Q water. The concentrations of aucubin and catalpol were analysed by HPLC using a Bio-LC (Dionex Corp., Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4 x 50mm) and analytical column (4 x 250mm) (for the *Veronica* species we used a Carbopac PA 20 guard (3 x 30mm); analytical column (3 x 150mm)). For pulsed amperimetric detection (PAD) we used an ED50 electrochemical detector. NaOH (1M) and Milli-Q water were used as eluents (10:90%, 1ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Concentrations were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunnyvale, USA).

Larval performance

Prediapause *M. athalia* larvae were fed 13 different food plant species in Petri dishes in the laboratory. Next to the eight (5 known and 3 potential) food species used in the oviposition experiment we included five extra potential species in the performance experiment; *Veronica longifolia* L., *Rhinanthus minor* L., *R. serotinus* (Schönh), *Odontites littoralis* (Fr.) and *Linaria vulgaris* Miller (Table 5.1). All of these species occur on Åland in habitat that could potentially be used by *M. athalia*. First instar larvae from approximately 40 egg clusters were combined and then separated into 95 groups of 10 larvae (except those feeding on *P. major* and *R. minor* which were started in the second instar, because the host plants where not sooner available). The larvae were kept in groups because the early instars of *M. athalia* are gregarious. The groups were randomly assigned to feeding treatments. The larvae were kept on filter paper in the Petri dishes and given leaves picked daily from naturally occurring plants. There were 10 replicate dishes of the five known food plants as well as for *V. longifolia* and five replicates of the seven potential food plants. We measured three performance parameters: 1) development time as the number of days from second instar until

all of the larvae in a dish had reached diapause (4th instar), 2) the weight of individual larvae at diapause and 3) the number of larvae in each dish surviving to diapause.

Table 5.1 All (potential) host plant species used in the experiments.

	abreviation	Family	Oviposition experiment	Performance experiment
Known host plants				
Melampyrum pratense L.	MP	Scrophulariaceae	X	X
Melampyrum sylvaticum L.	MS	Scrophulariaceae	X[1]	X
Plantago lanceolata L.	PL	Plantaginaceae	X	X
Veronica chamaedrys L.	VC	Scrophulariaceae	X	X
Veronica spicata L.	VS	Scrophulariaceae	X	X
Potential host plants				
Melampyrum nemorosum L.	MN	Scrophulariaceae	X[1]	X
Plantago major L.	PM	Plantaginaceae	X	X
Veronica officinalis L.	VO	Scrophulariaceae	X	X
Veronica longifolia L.	VL	Scrophulariaceae		X
Linaria vulgaris Miller	LV	Scrophulariaceae		X
Odontites littoralis (Fr.)	OL	Scrophulariaceae		X
Rhinanthus minor L.	RM	Scrophulariaceae		X[2]
Rhinanthus serotinus (Schönh)	RS	Scrophulariaceae		X[2]

[1]Plants additionally placed in the oviposition experiment, but not included in the statistical analyses.
[2]The experiments with these plants were started with second instar larvae, therefore they were not included in the analysis of development time.

Statistical analysis

Statistical analyses for the oviposition experiment were performed using the statistical program SPSS v.13.0 (SPSS Inc., Chicago, Illinois). To test for differences in the number of egg clusters laid per plant species or per plot Kruskal-Wallis tests were used. This non-parametric test was used because the data were not normally distributed. Mann-Whitney U-tests were used for further pairwise comparisons of the plant species. Mann-Whitney U-tests were also used to compare the number of egg clusters on plants in the bare vs. the vegetated plots and in the mixed vs. single species groups. To test for differences in egg cluster sizes between the different species we did an analysis of variance, with species as factor. Data on the size of egg clusters were square root transformed before statistical analysis to increase the normality of their distribution. These analyses did not include the "transect" and "array" *V. spicata* plant. To compare the number of egg clusters per *V. spicata* plant in the experimental plots A-J with those in the additional "transect" and "array" plants we used the Fisher's exact test.

For the analyses of the chemical data we used Statistica (STATISTICA version 7.1, StatSoft Inc., Tulsa, UK). We included all of the plants in the cage. We performed an ANOVA on IG content with plant species as factor, with a post hoc test (Tamhane) to see if the plant species differed in their IG content. To compare the IGs of plants with and without oviposition we used a t-test within each species. We calculated Pearson correlations per species to test for associations between the total number of egg clusters laid on a plant and the IG concentration of that plant and between the average number of eggs in a cluster (total number of eggs on a plant divided by the number of clusters on that plant) and the IG concentration.

The effect of food plant species on the performance of *M. athalia* larvae on the 13 plant species was analysed using ANOVA in the statistical program Stata (Statacorp, College station, USA). For the analyses of development rate (number of days from second instar until all the larvae in a dish were in diapause) and of survival (number of larvae surviving to diapause) the experimental unit was Petri dish, and the effect of plant species was tested. For the analysis of weight of individual larvae at diapause, Petri dish was nested within food plant species. *Rhinanthus minor* and *P. major* were not included in this analysis because these treatments were started later, when the larvae were already moulting to third instar. For each of the three analyses (development rate, survival and weight at diapause), the plant species were compared by constructing post-hoc contrasts of the performance on each plant species with the average performance. Interpretations of the statistical differences were made using the Bonferroni adjustment for multiple tests.

Results

Habitat use

Altogether, 141 *M. athalia* butterflies were recorded in the transects during the six weeks of data collection (ten transect walks), 118 of them were males and 23 females. Most of the butterflies were flying (87♂; 7♀) at the moment of observation, the rest were feeding (18♂; 7♀), basking (11♂; 7♀) or mating (2♂; 2♀). The feeding butterflies were resting on or taking nectar from the flowers of the following plants: *Achillea millefolium, Allium schoenoprasum, Filipendula ulmaria, Hieracium umbellatum, Knautia arvensis, Leucanthemum vulgare, Ranunculus spp., Trifolium pratense* and *Trifolium repens*.

The density of *M. athalia* was highest in dry meadows, but they were also found in mesic meadows, herb-rich road verges, semi-open forest habitats and field edges. The species was missing entirely from dense forests and from herb-poor open landscapes (Table 5.2).

Table 5.2 The number of *Melitaea athalia* butterflies in each habitat type, and the amount of each habitat type present in the three transects combined.

Habitat type	Total length (m)	Total number of *M. athalia*	Average number of *M. athalia* per km per survey
Dry meadows	1140	50	4.4
Mesic meadows, herb-rich road verges	2420	66	2.7
Semi-open forest and forest edges	2020	22	1.0
Field edges	570	3	0.5
Forest	730	0	0
Herb-poor road verges	1380	0	0

Four of the known host plants were present in parts of the transects, *V. spicata* (14.5%), *V. chamaedrys* (35.5%), *P. lanceolata* (32%) and *M. pratense* (6.5%). Butterflies were observed most frequently in transect parts where *V. chamaedrys, V. spicata* and *P. lanceolata* grew. In 82%, 100% and 95%, respectively, of the transect parts where these plants were present, the butterfly was present too. In 50% of the transect parts where *M. pratense* occurred, *M. athalia* was also present.

Oviposition host plant preference

During the two weeks of the experiment *M. athalia* butterflies laid in total 455 egg clusters (on average 4.9 per female); 237 of them were on the actual study plants, one on the *M. nemorusum* plants present in the shady corner of the cage, and the other 217 on the *V. spicata* "transect" (55) and "array" (162) plants.

The number of egg clusters received differed significantly among host plant species, with most egg clusters laid on *V. chamaedrys*, *V. spicata* and *P. lanceolata* (Kruskall-Wallis, $P<0.001$; Fig. 5.2).

Each plant species was present in the cage as a group of 12 plants of the same species and as part of six mixed groups which included two individuals of each of the six species. There was no difference between the number of egg clusters laid on plants that were in the mixed or single species groups (Fig. 5.2; Mann-Whitney U-tests, $P>0.1$ for all species).

During the experiment, we observed that some females used more than one host plant species for ovipositing, even during the same day. We observed 12 individuals laying eggs on two different host plant species (eight oviposited on both *V. chamaedrys* and *V. spicata,* two on *P. lanceolata* and *V. spicata,* one on *P. major* and *V. spicata,* and one on *V. spicata* and *V. officinalis*).

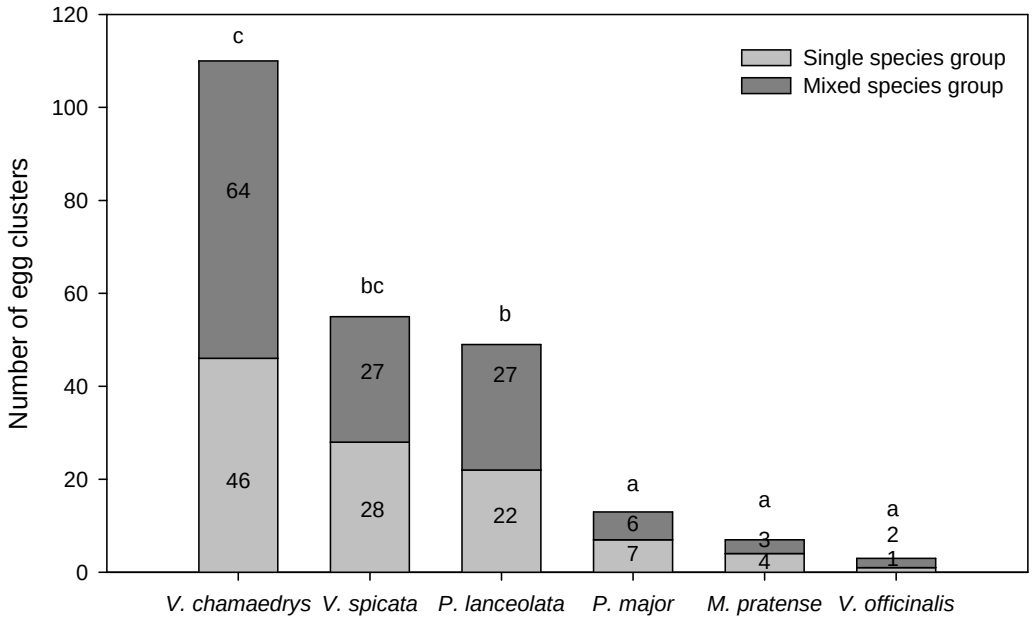

Figure 5.2 The number of egg clusters laid on plants in single species (light grey bar) and mixed groups (dark grey bar). Groups significantly different from each other in number of clusters ($P<0.05$, using a Mann-Whitney U-test) are represented with different letters above the columns.

Some areas in the cage attracted significantly more ovipositing females than the others, regardless of which set of plants were present. The overall difference between the plots is statistically significant (Kruskall-Wallis test, P< 0.005) with bare plots (A, B, C, E, F and K) receiving more egg clusters (on average 28.8 cluster per plot) than vegetated plots (D, G, H, I, J, L; on average 10.7 clusters per plot; Mann-Whitney U-test, P< 0.001).

Veronica spicata plants that were part of the "array" plants received significantly more egg clusters (on average 3.7 per plant) than those in the "transect" (on average 1.4 per plant) or our main experimental *V. spicata* plants (on average 1.6 per plant) (Fisher exact test "array" vs. "transect": P<0.0001; "array" vs. experiment plants: p<0.02).

The mean egg cluster size in the experiment was 43.19 (s.e 1.9) eggs per cluster, with a minimum of three and a maximum of 201 eggs. There were no significant differences in the egg cluster size among the different plants species (ANOVA, $F_{5, 236} = 1.2$, P>0.1; Fig. 5.3).

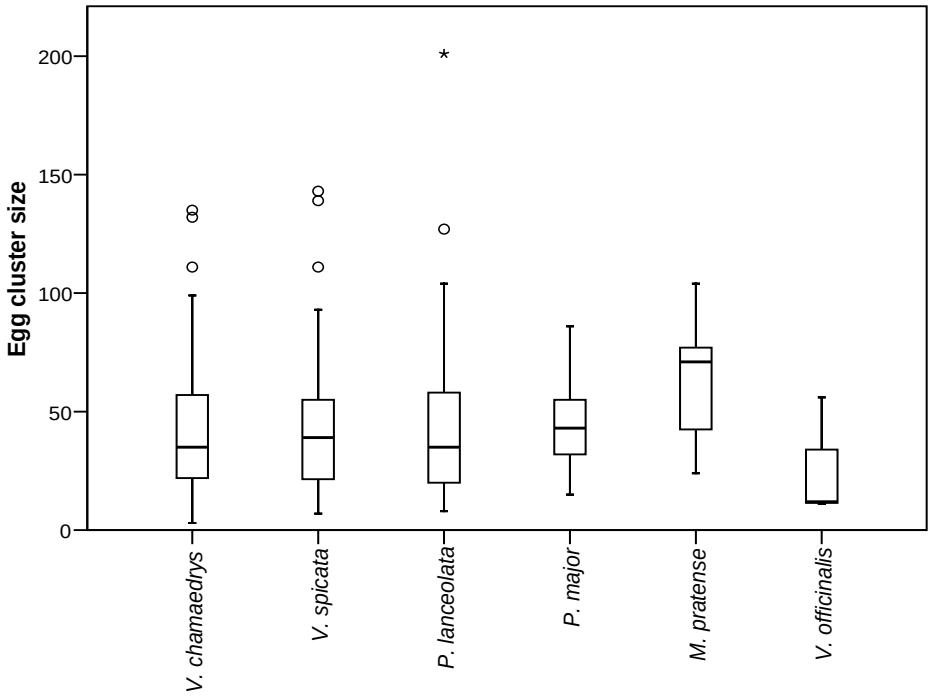

Figure 5.3 Sizes of *Melitaea athalia* egg clusters laid on each plant species. The median is indicated with the horizontal black bar in the box. The box encloses the upper and lower quartile and the error bars indicate the smallest and largest obser-vations that were not outliers. The outliers are indicated with open circles, and the extreme with an asterisk.

Iridoid glycoside content of plants in the oviposition experiment

The plant species differed in IG content (ANOVA, aucubin: $F_{7, 212} = 53.8$, P<0.001; catalpol: $F_{7, 197}$ 103.8, P<0.01; Fig. 5.4) ranging from a mean total content of 0.72 % of the dry weight in *P. major* to 11.53% in *M. pratense*. The species that received most egg clusters (Vc, Vs, and Pl) did not have particularly high or low IG concentrations. The IG concentration also varied among individuals within a plant species. However, there was no correlation between IG concentration in a plant and its probability to receive *M. athalia* eggs. Only in the additional "transect" *V. spicata* plants we did see significantly higher levels of catalpol and total IG in the plants that received egg clusters compared to plants that did not (t-tests, catalpol: t=2.53, df=34, P<0.02; total IG: t=2.21, df=34, P<0.04).

The number of egg clusters per individual plant ranged from zero to 13. For most plant species in the oviposition experiment there was no significant correlation between the number

of egg clusters and the iridoid content of the plants. However, for *P. major* and *V. chamaedrys* high total IG concentrations, and in particular high aucubin (for *P. major*) was correlated with a greater number of egg clusters (total IG Pm: r=0.49, n=24, P<0.01; Vc: r=0.6, n=12, P<0.04; aucubin Pm: r=0.5, n=24, P<0.01).

Egg cluster size was not correlated with IG concentration except for the "transect" *V. spicata* plants. In these plants the number of egg clusters ranged from zero to 12 and the average egg cluster size ranged from 15.5 to 81 eggs and increased with the catalpol concentration (r=0.35, n=36, P<0.04; Fig. 5.5) and showed a similar trend for total IG concentration (r=0.31, n=36, P<0.07).

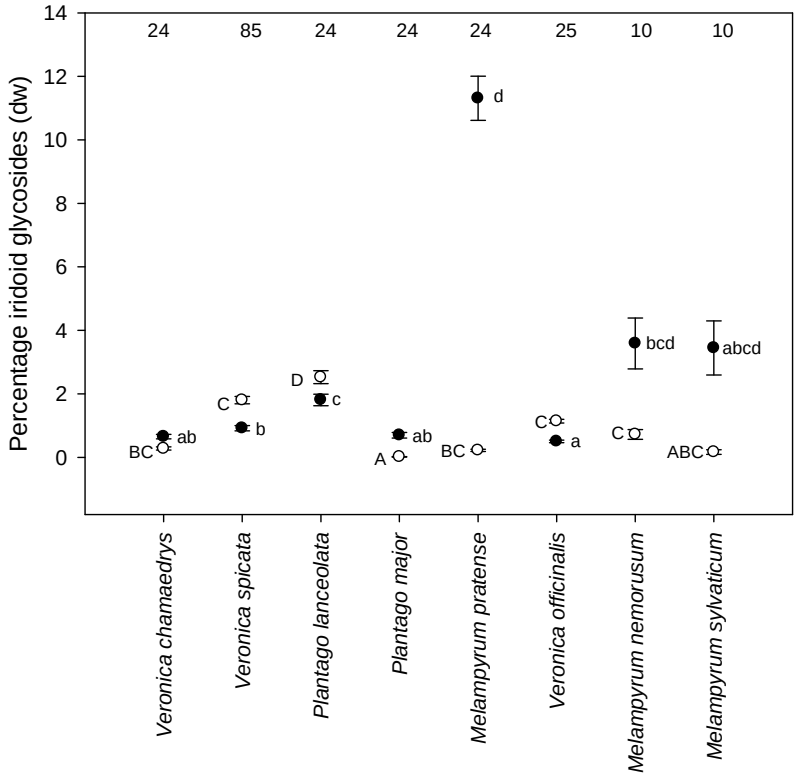

Figure 5.4 Iridoid glycoside content (percentage per mg dry weight) of each plant species used for the oviposition experiment. The circles and error bars are the means and standard errors for aucubin (black) and catalpol (white). The numbers at the top of each column are counts of the number of plants analyzed. The letters adjacent to the circles indicate which plant species significantly differed in aucubin and catalpol content using a Tamhane post hoc test (capitals left for catalpol, letters right for aucubin).

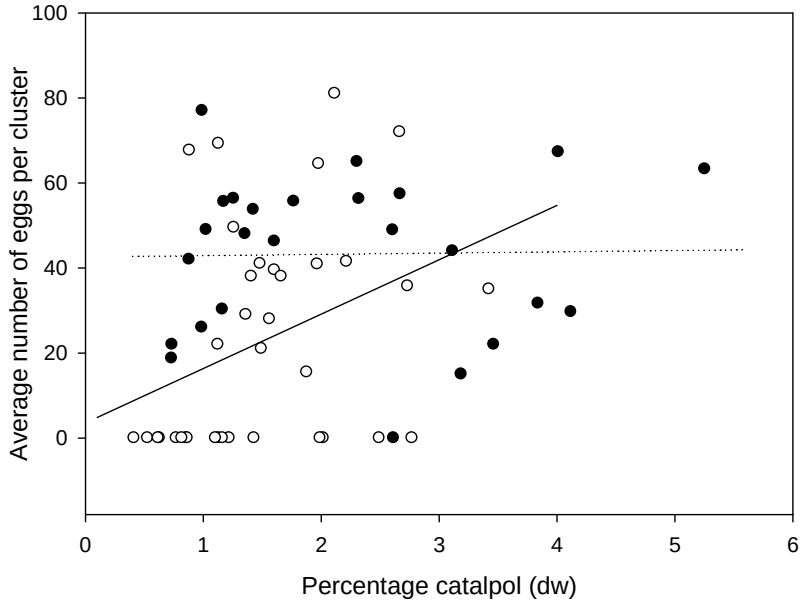

Figure 5.5 The average number of *Melitaea athalia* eggs per cluster per plant as a function of the concentration of catalpol in the additional *V. spicata* plants in the cage (array: black and dotted line; transect: white and solid line).

Larval performance

Larvae performed relatively well on all plant species except on *V. officinalis* (Fig. 5.6). The average number of days it took for all of the larvae in a dish to develop from second instar to diapause varied from 12 to 21. Overall, food plant species was related to rate of development (ANOVA, $F_{10, 79} = 10.99$, $P<0.001$). Larvae developed most quickly on *M. nemorosum*, and developed slowest on *V. officinalis* and *M. sylvaticum* (Fig. 5.6A).

Few larvae died during the experiment, but there was a significant effect of food plant species on the per-Petri dish rate of survival (ANOVA, $F_{12, 82} = 15.8$, $P<0.001$). Survival was particularly low on *V. officinalis* (Fig. 5.6B). At diapause most larvae weighed between 4.5 and 5.5mg. This differed significantly among those feeding on different food plants (ANOVA, $F_{12, 669} = 6.28$, $P<0.001$), but only substantially for the small larvae feeding on *V. officinalis* (Fig. 5.6C).

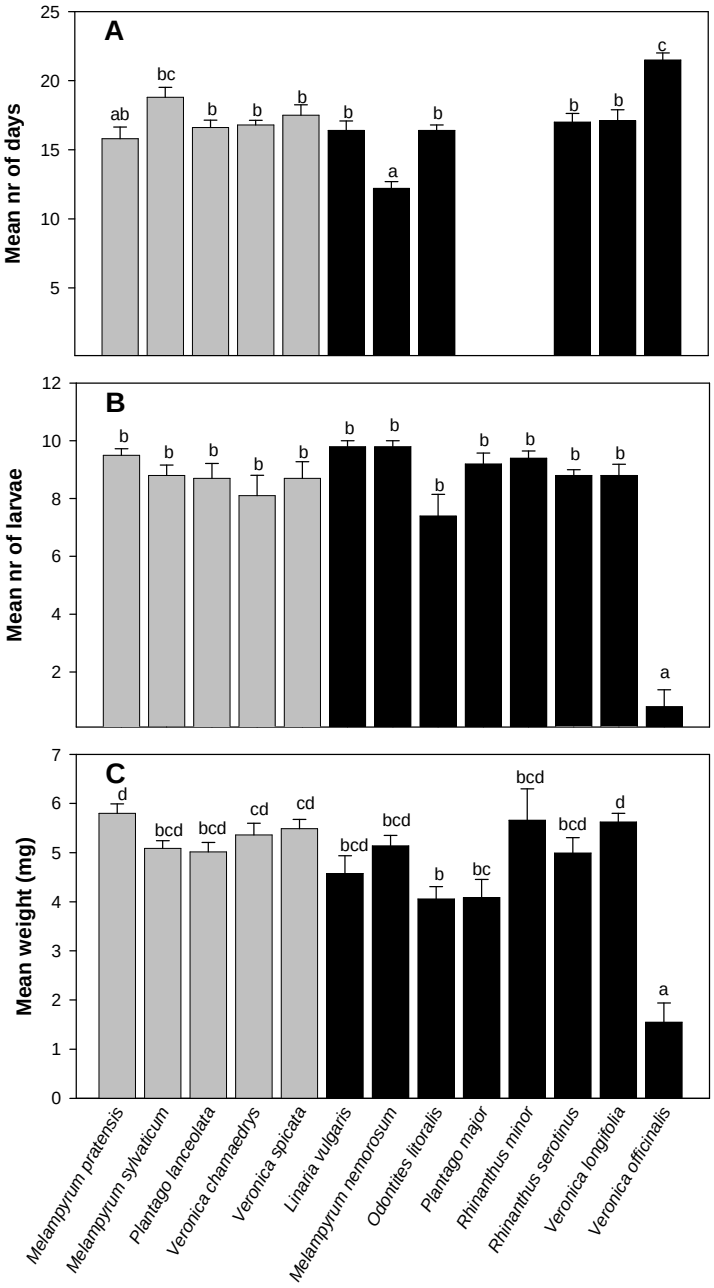

Figure 5.6 The performance of prediapause *Melitaea athalia* larvae fed in the laboratory on five known host plants (grey bars) and eight potential food plants (black bars). A) Rate of development (days from second instar to diapause); B) survival: number of caterpillars surviving to diapuse (out of 10 per dish); C) mean weight at diapuse. The letters on top of the bars indicate which plant species significantly differed from each other ($P< 0.05$; ANOVA with post hoc tests).

Discussion

Habitat use

Melitaea athalia occurs in several habitat types. The main habitat for the species on Åland turned out to be dry meadows, but it also occurred in mesic meadows and semi-open forest habitats. These results are mostly in agreement with a previous study done on Åland in the summer of 2002 in which Schulman et al. (2005) monitored the occurrence of several butterfly species in agricultural areas on randomly chosen sampling plots of one square kilometre. The number of *M. athalia* butterflies per kilometre was similar to our study, although they encountered more butterflies on forest edges. On the mainland of Finland *M. athalia* has been found to use mostly semi-open forest habitats and to be missing from open meadows, perhaps because they are small and isolated (Selonen 1997) and *M. athalia* is relatively sedentary (Wahlberg et al. 2002, Franzen and Ranius 2004). The landscape on Åland is very different from that of the mainland, with dry open meadows being more common and less isolated. In Britain habitat use by *M. athalia* is also known to differ between regions (Warren et al. 1984, Warren 1987b). In south-western England abandoned hay meadows are the main habitat whereas in south-eastern England the species occurs along the margins of cleared plots next to deciduous forest (Warren, 1984). Neither of these habitat types occur on Åland.

Oviposition host plant preference

We found that *M. athalia* from Åland primarily oviposited on *V. chamaedrys, V. spicata* and *P. lanceolata*. The pre-rearing of the caterpillars on *V. spicata* and *P. lanceolata* could have influenced this oviposition preference. However, we predominately see the butterflies flying in places where these favourite plants grow. Furthermore, these are also the plants on which we find eggs naturally on Åland. On the mainland of Finland, *V. chamaedrys* is probably the main host plant for the species at least for oviposition, but the larvae also feed on *Melampyrum* species (Wahlberg 1997). In Britain, *M. pratense* is the sole host plant for the species in heathlands, whereas in grasslands the primary hosts are *P. lanceolata* and *V. chamaedrys* (Warren 1987b). For successful larval development, the host plant needs to grow under appropriate environmental conditions, which in the case of checkerspots often means, a warm dry microclimate (Kuussaari et al. 2004). *Melampyrum* species grow in more shady places than *Veronica* or *Plantago* species, therefore the larval development time is longer so that the larvae probably cannot reach their overwintering instar before the end of the short growing season on Åland. There might have been selection not to use *Melampyrum* species, or the shady habitats in which they occur.

In addition to our main set of 144 oviposition plants, the butterflies also had access to an extra 42 “transect” and “array” *V. spicata* plants. The “array” plants received a disproportionately high number of egg clusters. There are several possible explanations for this. First, the location of the “array” plants on bare ground may have affected the results. Additionally, *M. athalia* females prefer to oviposit near the ground (Warren 1987b) and these “array” *V. spicata* pots were buried in the ground rather than placed on the soil which appeared to make them more accessible.

The main oviposition host plants used by butterflies in our experiment (*V. spicata, V. chamaedrys* and *P. lanceolata*) are common in dry meadows on Åland. The data from the transect counts revealed that dry meadows are the main habitat for *M. athalia* on Åland. The two studies combined show that the butterflies fly in the areas where the main host plants are found, and that dry meadows and plants growing there are important in the ecology of the species.

Egg clusters

There was no association of egg cluster size with host plant species. This finding is a bit surprising, because one might predict that ovipositing females should increase egg cluster size with host quality (Skinner 1985). If preferred plant species are considered as high quality plants, females might be expected to lay larger egg clusters on them.

Host plant frequency

In our experiment *M. athalia* used plant species with the same frequency, whether they were in single or mixed species groups. One might expect that plants in the single species groups of preferred plantspecies would be more attractive than the two individual plants among less preferred hosts. For example Janz et al. (2005) found that females of the butterfly *Polygonia c-album* spent more time and laid more egg clusters in patches with a high frequency of their preferred host. The results from our experiment indicate that *M. athalia* responds to a host plant species at a fine scale, and has no trouble finding the species it prefers even when it is surrounded by other (potential) host species. It may be important that the surrounding plant species all are suitable larval food plants, accessible to the larvae that are mobile enough to move among plants after the second instar.

Surrounding vegetation

There were clear differences in the numbers of ovipositions that occurred in different parts of the cage. Some areas attracted more ovipositing females than others, independent of which set of host plants were present. Plants in bare areas received significantly more egg clusters than plants in vegetated plots. This is in agreement with the results of Warren (1987b) who found *M. athalia* laying eggs close to the ground. In all habitat types, the batches were generally laid among fairly short vegetation (average height < 20cm). In woods, eggs were frequently laid in areas of sparse vegetation with a large percentage of bare ground. In grassland habitats, females laid their egg batches in chiefly open, sunny situations with a particularly warm microclimate (Warren 1987b). Plants that grow in a bare area have a warmer microclimate and they are also more conspicuous then plants in vegetated areas.

Chemical contents of the oviposition plants

All the host plants used for the oviposition experiment contained the two IGs aucubin and catalpol. The concentrations of these compounds differed significantly among plant species. The host range of *M. athalia* is restricted to plants producing IGs. Not only are IGs unlikely to be detrimental to their larvae (Harvey et al. 2005, Saastamoinen et al. 2007, Chapter 3), but they can also be sequestered (Suomi et al. 2002), and confer protection against generalist natural enemies (Bowers 1980, Bowers and Puttick 1986, Camara 1997b). Given this, we might expect plant species with higher amounts of IGs to be preferred host plants. No correlation between the oviposition preference for a host plant species and the average IG content of that species was found. This indicates that the butterflies either cannot discriminate among plants based on IGs or that they can, but make choices based on other factors.

Other checkerspot species are known to distinguish among individuals of the same species (Singer and Lee 2000, Singer et al. 2002) and at least sometimes this discrimination is correlated with IGs. The closely related and co-occurring butterfly species *M. cinxia* prefers to oviposit on plants with high aucubin concentrations. In natural populations Nieminen et al. (2003) found eggs on plants with a higher aucubin level than neighbouring or random plants. A similar pattern is found in the experiments described in Chapter 4, which shows further that the IGs were not induced by oviposition. There also is indication of a threshold concentration below which females do not distinguish between the different levels of IGs (Chapter 4). We

found no such pattern for *M. athalia*. Within the single species group plants that received eggs did not have higher IG content than their neighbouring plants.

Studies of plant-feeding insects have often found that host quality varies with secondary chemistry among species (Bolser and Hay 1996, Hartmann 1996, Julkunen-Tiitto et al. 1996) and among individuals (Bowers and Stamp 1992). Within some plant species we did find a positive correlation between the average number of eggs in a cluster and the IGs that were measured. This pattern was most clear for the "transect" *V. spicata* plants, for which the amount of catalpol was positively correlated with the average number of eggs in a cluster. The fact that we did not generally find such correlations may either indicate that *V. spicata* is an exception, or that effects of individual plant IGs can only be deteced in larger sets of plants, where statistical power is sufficiently high. If this were the case we cannot rule out small effects of IGs on oviposition choice within the other plant species as we find in *P. major* and *V. chamaedrys*, where the total IG level is correlated with the number of egg clusters laid on these plants, but it is clearly not the predominant factor.

Larval performance

Most larvae used in the performance experiment survived, even on plant species that are not known to be used by the butterfly naturally. Their performance in terms of development time, survival and weight was rather similar on all host plants except *V. officinalis* on which they performed poorly. The latter result corresponds well with the results from the oviposition preference experiment in which only three out of the 455 egg cluster were laid on *V. officinalis* plants. Furthermore, Schwarzwälder et al. (1997) found that in grasslands in southern Switzerland, where *V. officinalis* was quite abundant, only very few caterpillars were observed feeding on it. Instead they fed on *V. chamaedrys* and *P. lanceolata*. It is interesting to note that larvae had fastest development and 100% survival when fed *M. nemorosum*. While other species of this genus are known to be host plants of *M. athalia*, this particular species is not known to be used. *M. nemorusum* generally grows in broad-leaved forests, but also in shadowy conditions in fresh meadows. This is a type of habitat in which adult *M. athalia* may occur, but because of the microclimate conditions (under trees and in the shadow) the plants may not be suitable for oviposition.

With the exception of these two plant species (*V. officinalis* and *M. nemorusum*), our analysis of larval performance suggests that the ovipositing butterflies do not benefit in terms of the development of their larvae by choosing one of the host plant species over another. One implication of this is that for larval performance it would hardly matter whether the host plant species that the females selected in our oviposition experiment occured in a mixed-species group or in a single-species group. This is important for the large post-diapause larvae that are mobile and solitary, feeding on plants other than the one that they started on.

The larvae in this experiment were fed in Petri dishes with leaf pieces, so they did not experience whole-plant chemistry, local environmental conditions and natural enemies. However, it is worth noting that for the related butterfly *M. cinxia*, whose distribution overlaps with that of *M. athalia*, no systematic difference in larval performance on two different host plant species were found even in natural field conditions (van Nouhuys et al. 2003).

Overall, we found that on Åland *M. athalia* uses open meadows more than on the mainland of Finland and in Britain. This is probably because land use on Åland results in relatively numerous and well connected open meadows. This means that the distribution of *M. athalia* on Åland overlaps more with the co-occurring *M. cinxia* than would be expected based on the distribution of *M. athalia* on other places. The set of host species preferred by *M. athalia* in

our large cage experiment corresponded well with the set of host species that was frequent in the habitat types where we found adults flying. *Veronica chamaedrys*, *V. spicata* and *P. lanceolata* were used more than the *Melampyrum* which is known to be a preferred hostplant elsewhere, and was present in the study area but grows in more sheltered habitat.

The butterflies choose plants for oviposition in open rather than vegetated areas. Within a suitable area they are attracted to plant species at the level of an individual plant rather than at the level of a plant patch, i.e., they are not strongly affected by the frequency of the plant species. The butterflies also distinguish among species, prefering *V. chamaedrys* most strongly, than *V. spicata* followed by *P. lanceolata*. That these species are used with preference even among mixed groups strengthens the idea that the butterflies make choices at the species level. On the other hand, we know from observing the behaviour of individuals that among the preferred plants an individual butterfly uses more than one species, even in a single day.

Although the presence of IGs is an important trait distinguishing host plant species from non-host plant species for *M. athalia* and other Melitaeini (Wahlberg 2001), plant secondary chemistry played less of a role than might be expected based on work on the congener, *M. cinxia*. An exception to this is *V. spicata*, for which oviposition was positively correlated with catalpol concentration. Perhaps this is not surprising because *M. athalia* occurs in more diverse habitats and has a broader host range than *M. cinxia.*

The ranking of host plant species with respect to oviposition preference only partly corresponded with their ranking in terms of suitability for larval performance. The host species that was a poor food plant, *V. officinalis,* was also the least preferred for oviposition, confirming a preference-performance link. But while *M. athalia* showed distinct oviposition preferences within the remaining group of offered plant species, e.g., preferring *V. chamaedrys* over *P. lanceolata,* larval performance on these species was very similar. While most species were suitable host plants, it is interesting to note that one of the best plants, *M. nemorosum*, was a species that is not known to be used by the butterfly.

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Chapter 6

Trade-offs between chemical defence and regrowth capacity in *Plantago lanceolata*

J. H. Reudler Talsma, S. C. Honders, H. Turin and A. Biere

Abstract - Resistance and tolerance are alternative plant strategies to cope with the impact of herbivores. Since resources available for allocation to defence are limited and resistance and tolerance are likely to serve the same functions for plants, the occurrence of trade-offs between these two strategies has been assumed. In this study we investigated whether there are trade-offs between resistance and one specific aspect of tolerance, the ability to regrow after defoliation. We used offspring of crosses between plants of ribwort plantain (*Plantago lanceolata* L.) that differed in their levels of iridoid glycosides (IGs, allelochemicals that confer resistance to generalist herbivores), to investigate whether high-IG plants 1) suffer allocation costs in terms of shoot and root growth, 2) have a reduced regrowth ability (tolerance) after defoliation and 3) whether such costs are more pronounced under nutrient stress. Total biomass produced by high-IG plants was not lower than that of low-IG plants; hence there was no evidence for an allocation cost in terms of total growth. However, high-IG plants produced fewer inflorescences than low-IG plants (reproductive cost) and allocated less biomass to roots than low-IG plants. Despite their lower relative investment in root mass, high-IG plants did not suffer a reduced capacity to regrow shoot mass after defoliation. However, after regrowth, root mass of high-IG plants grown under nutrient-poor conditions was significantly lower than that of low-IG plants, suggesting that under these conditions shoot regrowth comes at a larger expense of root growth in high- than in low-IG plants. We speculate therefore that if there is repeated defoliation, high-IG plants may eventually fail to maintain shoot regrowth capacity and that trade-offs between resistance and tolerance in this system may not show up until repeated defoliation events occur.

Introduction

Plants have to defend themselves against a multitude of natural enemies. Resistance and tolerance represent two general strategies of plant defence against herbivores, reducing the amount of herbivore damage and the impact of herbivory on plant fitness, respectively (Crawley 1983, Rausher 1992, Stowe et al. 2000). Resistance includes investments of energy and resources in secondary metabolites (Foulds and Grime 1972, Coley 1983) and morphological structures that reduce herbivore performance (antibiosis) or preference (antixenosis). However, such investments may not provide an adequate defence mechanism against all types of damage. Abiotic disturbances such as mowing or trampling can remove part of the primary biomass, regardless of the plants resistance. Also, many specialist herbivores are unaffected by the chemical defence of the food plant to which they have adapted (Crawley 1983, Giamoustaris and Mithen 1995, van Dam et al. 1995, van der Meijden 1996). In these cases tolerance would be an alternative defence mechanism. Tolerance mechanisms include increased photosynthetic activity, compensatory regrowth, utilization of stored resources, phenological changes, and mechanisms related to physiology and morphology at the time of damage (Tiffin 2000). Since resources available for allocation to defence are limited and resistance and tolerance are likely to serve the same functions for plants, the occurrence of trade-offs between these two strategies has been assumed (van der Meijden et al. 1988, Fineblum and Rausher 1995). Trade-offs are expected to occur if plant resources are limited and both defensive strategies have allocation costs.

Within many natural plant populations there is genetic variation in the levels of chemical defence compounds against herbivores and pathogens (Simms 1992, Karban and Baldwin 1997). The maintenance of such genetic variation is often explained by ecological or allocation costs of chemical defence. Ecological costs arise if defence against one organism is accompanied by effects on interactions with other species that result in a decrease of fitness (e.g., deterrence of mutualists, attraction of herbivores or reduced intra- or interspecific competitive ability (Strauss et al. 2002)). Allocation costs imply that the production, transport, storage, self-detoxification, activation and/or turnover of secondary plant compounds used for chemical defence results in lower plant fitness in the absence of natural enemies due to reduced growth and survival or reproduction (Simms and Rausher 1987, Simms 1992, Karban and Baldwin 1997, Strauss et al. 2002). Costs of defence are expected to increase under stress conditions such as low light or nutrient conditions and competition (Bergelson and Purrington 1996). This expectation is based on two assumptions. First, trade-offs between different functions such as growth and defence are more pronounced when resources are more severely limiting (Herms and Mattson 1992). Second, environmental stress can cause increased production of defence chemicals (Gershenzon 1984, Hirata et al. 1993, Dixon and Paiva 1995). However, a review of studies addressing costs of defence in relation to stress did not reveal a general pattern that costs are more pronounced in stressful environments (Bergelson and Purrington 1996, van Dam and Baldwin 2001). Two factors may be responsible for this. First, increased production of secondary metabolites under severe resource limitation may not always have a substantial extra cost. For instance, nutrient shortage may lead to a relative excess of fixed carbon in the plant that can be transferred into carbon-based secondary metabolites at virtually no extra cost (Herms and Mattson 1992). Secondly, competitive stress may not result in enhanced costs of defence, if the production of these defences also provides some benefit in competitive interactions (Siemens et al. 2002).

In this study we address trade-offs between resistance and one specific aspect of tolerance, the ability to regrow after defoliation. We investigate whether plants with higher levels of secondary metabolites (resistance trait) 1) suffer allocation costs in terms of shoot

and root growth, 2) have reduced regrowth ability (tolerance) and 3) whether such costs are more pronounced under nutrient stress. Regrowth involves the storage of resources in plant parts that are relatively free from herbivore attack (van der Meijden et al. 1988, Iwasa and Kubo 1997). Plants that are frequently consumed have low shoot:root ratios and a well developed regrowth mechanism, whereas plants that are rarely consumed have high shoot:root ratios and a poor regrowth mechanism (van der Meijden et al. 1988). Defence and regrowth are alternatives in the struggle of plants against herbivore damage (van der Meijden et al. 1988, Fineblum and Rausher 1995, Strauss and Agrawal 1999). Assuming that resources are limited, defence and regrowth compete with each other for resources and can both be costly in the sense that they slow down the growth of the plant in an herbivore-free environment.

We investigated the effect of different levels of two defence compounds in *Plantago lanceolata*, the iridoid glycosides (IGs) aucubin and catalpol, on the plant's ability to regrow under different nutrient conditions. In natural populations IG levels range from undetectable to ca. 9% of the dry weight (Bowers 1991). Variation in the constitutive IG amount within and among populations in *P. lanceolata* is partially genetically determined (Bowers and Stamp 1992, 1993, Adler et al. 1995, Marak et al. 2000). Aucubin and catalpol are carbon-based secondary metabolites. The biosynthetic costs of these IGs are high (Gershenzon 1984), but previous studies on fitness costs of IGs in *P. lanceolata* in the absence of herbivores and pathogens have produced mixed results. No costs of IGs could be detected in terms of negative among-genotype correlations between the level of IGs and aboveground biomass of plant growth (Adler et al. 1995), but in another study costs were found in terms of lower reproductive dry weight and a smaller number of inflorescences produced by plants selected for high levels of leaf IGs (Marak et al. 2003). None of these studies addressed effects on root growth or the ability to regrow after defoliation.

For this study we used *P. lanceolata* plants that had been artificially selected for high and low leaf IG concentrations (Marak et al. 2000). The advantage of using these selection lines is that pleiotropic costs of defence can be measured without any confounding effects of linkage disequilibrium (Strauss et al. 1999, Siemens et al. 2002).

We performed a regrowth experiment to answer the following questions: 1) Are there fitness costs in terms of root and shoot growth and reproduction associated with the production of high IG levels? 2) Is the production of high IG levels in the absence of natural enemies associated with a lower regrowth capability? 3) Are these costs more pronounced under nutrient stress?

Material and Methods

Plants

Plantago lanceolata L., ribwort plantain, is a rosette-forming, self-incompatible, wind-pollinated, perennial herb which overwinters as a basal rosette and in the spring and summer produces numerous leaves and spiked inflorescences at the end of fibrous stalks (Cavers et al. 1980, Primack and Antonovics 1982). It has nowadays a worldwide distribution and large ecological amplitude. In the Netherlands, it is a common plant of roadsides and moist and dry grassland (Haeck 1992). Among the secondary plant compounds produced by *P. lanceolata* are the two IGs (IGs) aucubin and catalpol (Duff et al. 1965, Bowers and Stamp 1992, Adler et al. 1995). These IGs are known to have a deterrent effect on pathogens (Marak et al. 2002a) and generalist and non adapted specialist insect herbivores of *P. lanceolata* (Bowers and Puttick 1988, Bowers 1991). However, they function as oviposition cues for the specialist

butterfly *Junonia coenia* (Pereyra and Bowers 1988) and *Melitaea cinxia* (Nieminen et al. 2003) and as feeding stimulants for at least some checkerspot butterflies (Bowers 1983).

The *P. lanceolata* plants used for this experiment were the offspring of 16 full-sib crosses. Eight of these crosses were made between parents with low levels of leaf IG ("L crosses") and eight between parents with high levels of leaf IG ("H crosses"). The parents originated from two selection lines previously created after four generations of artificial selection for low and high leaf IG concentrations (Marak et al. 2000). Previous studies have shown that average leaf IG levels differ ca. four-fold between lines, but that considerable variation is present among individual plants within these lines as well (Marak et al. 2000, 2003). On average, offspring from L crosses are thus expected to have lower leaf IG levels than offspring from H crosses, but considerable variation is expected within these sets of crosses as well, providing a range of IG levels among crosses used in this study.

Set up

Sixty seeds from all the 16 crosses were germinated on water agar in a growth cabinet (L:D 14:10h; 25/15ºC). After 14 days, seedlings were transplanted into plastic pots (diameter 13.0cm, height 11.2cm, volume 970ml) with sand. All pots were placed on saucers so that nutrient solution spilled from the bottom of the pot could be reabsorbed by the soil or plant.

The experiment consisted of three treatments (Table 6.1): a clipping-and-regrowth treatment (C), a treatment allowing root weight estimation in treatment C at the time of clipping (E) and an unmanipulated growth treatment (U). In treatment C, shoots were clipped just above the caudex and harvested eight weeks after transplantation (T8). Roots were allowed to regrow new shoots for another five weeks until roots and regrown shoots were finally harvested 13 weeks after transplantation (T13). Since we lack weight measurements for the roots of these plants at the moment of clipping (T8), in treatment E an additional set of plants from each cross was grown under identical conditions, which was entirely harvested at T8. Finally, to allow comparison of growth and chemistry of clipped and regrown plants with those of unmanipulated plants, an additional set of plants from each cross was grown under identical condition as C but without clipping: this set was harvested at T13 (treatment U).

Table 6.1 Overview of the treatments to investigate regrowth capacity in different genotypes of *Plantago lanceolata*. All treatments were performed under nutrient poor and nutrient rich conditions.

Time (T)	Treatment		
(weeks)	**C**	**E**	**U**
↓	Clipping and Regrowth	Root growth Estimation	Unmanipulated
0	Germination	Germination	Germination
2	Transplantation	Transplantation	Transplantation
8	Shoot harvest	Shoot and Root harvest	-
13	Shoot and Root harvest	-	Shoot and Root harvest

Every treatment was performed under two nutrient conditions; rich and poor. The first two weeks all the plants received the same amount of nutrients (50ml of a 1/32 strength Hoagland solution) to germinate well. After two weeks we started the two nutrient levels. Half of the pots of each treatment received a low nutrient level: 50ml of a 1/32 strength Hoagland's solution from T2-T4 and a 1/16 strength Hoagland's solution from T4-T13 (poor). The other half of the pots received a high nutrient level: 50ml of a 1/8 strength Hoagland's solution from T2-T13 (rich). Full strength Hoagland's solution contained: 5mM $Ca(NO_3)_2$, 5mM KNO_3, 1mM KH_2PO_4, 2mM $MgSO_4$, 174µM $C_{10}H_{12}FeN_2O_8Na$, 93µM H_3BO_3, 18µM $MnCl_2$, 1.5µM $ZnSO_4$, 0.6µM $CuSO_4$ and 1.0µM Na_2MoO_4.

All plants were watered individually three times a week. During the last five weeks (after the first harvest of treatment C), pots of treatment U (not clipped) received 100ml solution to meet their increased water demands without lowering soil nutrient concentration. In total the experiment consisted of 16 genotypes x 3 treatments x 2 nutrient levels x 6 replicates, i.e., 576 plants.

Measurements

For all plants we scored the day they produced their first inflorescence. After eight weeks (T8) the shoots of all the plants in treatment C and the whole plants of treatment E were harvested. For all the harvested plants we measured the number of rosettes, number of leaves and the length and width of their longest leaf. The roots were rinsed clean. After harvesting, all plants were separated into roots, vegetative shoots and reproductive parts and all fractions per plant were put at -80˚C before freeze-drying. After drying, the plant parts were weighed to determine their dry mass. The leaf samples from each plant from treatment C were used for HPLC analyses. Similarly, at T13, roots, vegetative shoot parts and reproductive shoot parts of all plants in treatment C and U were harvested and the same parameters were measured as above. Leaf samples of all plants from treatment C and U (T13) were used for HPLC analyses.

Since we wanted to relate regrowth ability of plants from different crosses in treatment C to both their IG levels and their root dry weight at the time of defoliation we estimated dry weights of roots in treatment C at T8. First, we fitted general linear models to estimate how well root dry weight of plants in treatment E could be predicted from their shoot dry weight and the cross from which they originated. Both shoot dry weight and cross significantly affected root dry weight under nutrient-poor conditions ($F_{1, 62} = 358.8$, $P<0.001$ and $F_{15, 62} = 4.76$, $P<0.001$, respectively, interaction n.s.) and under nutrient-rich conditions ($F_{1,59} = 89.23$, $P<0.001$ and $F_{15, 59} = 3.91$, $P<0.001$, respectively, interaction n.s.), explaining 84% and 68% of variation in root dry weight under nutrient-poor and rich conditions, respectively. Then, the parameter estimates from these analyses were used to estimate root dry weight of plants in treatment C from their shoot dry weight in treatment C and the cross from which they originated.

Chemical analyses

Samples of all the dry leaves (from treatment C at T8 and from treatment C and U at T13) were ground to a fine powder with a ball mill (type MM 301, Retsch GmbH & Co., Haan, Germany). Ground leaf material (25mg) was extracted in 10ml of 70% MeOH and was shaken overnight. The crude extract was filtered using Whatman filter paper #4 and the filtrate was diluted ten times with Milli-Q water. The concentrations of the IGs aucubin and catalpol were analysed by HPLC using a Bio-Lc (Dionex Corp., Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4 x 50mm) and analytical column (4 x 250mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD).

NaOH (1M) and Milli-Q water were used as eluents (10:90, 1ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Peaks were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunnyvale, USA).

Statistical analyses

The effects of Line (selection line from which parents of the crosses originated), Cross (nested within Line), Nutrient level and their interaction effects on the different plant traits were analyzed using GLM (Generalized Linear Models) (SAS v. 8.2, procedure GENMOD, SAS Institute, Cary, NC) with a normal error distribution and an identity link function. Dependent variables were transformed prior to analysis if necessary to improve normality. The number of leaves and all weight measurements were ln-transformed and the IG levels were square root-transformed. In addition, we used (Pearson) correlations to look for associations between (cross means for) leaf IG levels and (cross means for) growth, reproduction, or regrowth capacity. Allocation costs of chemical defence in terms of growth, reproduction, or regrowth would show up either as Line effects for these traits in GLM (lower performance by plants of the H crosses), or as negative correlations between cross-means for these traits and cross-means for leaf IG levels.

Table 6.2 Effects of nutrient level, selection line and cross (nested within selection line) on leaf concentrations of allelochemicals (aucubin, catalpol, total iridoid glycosides) and plant characteristics (leaf area of the longest leaf, number of leaves on the main rosette, number of side-rosettes and number of flowering stalks) in three groups of *Plantago lanceolata* plants. Values are quasi-F values from GLM analyses of deviance (* P<0.05; ** P<0.01; *** P<0.001).

	df	Aucubin	Catalpol	Total IG	Leaf area	# Leaves	#Rosettes	# Stalks
Untreated plants T8								
N: Nutrient	1	200.1***	12.4**	130.7***	356.9***	291.8***	20.7***	25.5***
L: Line	1	55.3***	35.9***	94.4***	5.0*	83.6***	20.2***	0.5
C: Cross(L)	14	1.7	5.1***	1.9*	2.3**	1.5	1.3	1.6
N*L	1	6.3*	0.7	3.9	7.3*	6.8*	17.2***	0.5
N*C(L)	14	0.6	1.2	0.9	0.8	1.4	1.4	1.6
Error	153							
Untreated plants T13								
N: Nutrient	1	90.6***	36.7***	89.5***	428.2***	401.5***	68.2***	288.5***
L: Line	1	58.2***	30.4***	62.2***	41.6***	69.2***	17.0**	7.0*
C: Cross(L)	14	0.3	1.2	0.4	1.7	3.3***	2.5**	3.5***
N*L	1	25.6***	10.9**	25.2***	24.7***	17.7***	22.2***	7.3*
N*C(L)	14	0.3	0.5	0.3	1.3	1.5	0.6	3.0***
Error	159							
Clipped and regrown plants T13								
N: Nutrient	1	612.8***	4.8*	631.0***	238.7***	436.8***	40.7***	300.5***
L: Line	1	24.6***	0.7	36.3***	22.4***	88.8***	10.6***	4.7*
C: Cross(L)	14	0.3	8.0***	0.2	2.8**	1.9*	2.0*	4.8***
N*L	1	7.6*	0.4	9.1**	15.0**	18.3***	17.2***	8.2*
N*C(L)	14	0.3	2.8**	0.2	1.7	0.8	1.3	1.9*
Error	159							

Results

Differences in leaf iridoid glycoside levels between the crosses

Leaf IG concentrations after eight weeks (at T8) were approximately twofold higher in plants from the high crosses than in plants from the low crosses (Table 6.2; Fig. 6.1C). Leaf IG concentrations at T8 also varied significantly among crosses within lines (from which the parents originated), resulting in a three- to four-fold range of variation in mean leaf IG concentrations among offspring groups from the 16 different crosses. Both variations in leaf aucubin and in leaf catalpol concentrations contributed to differences in total IG levels between the lines (Table 6.2, Fig. 6.1A and B). Leaf IG concentrations were 1.7 fold higher under nutrient-poor conditions than under nutrient-rich conditions (Table 6.2, Fig. 6.1C), but the relative difference between high and low crosses was unaffected by nutrient supply (no interaction nutrient x line, Table 6.2).

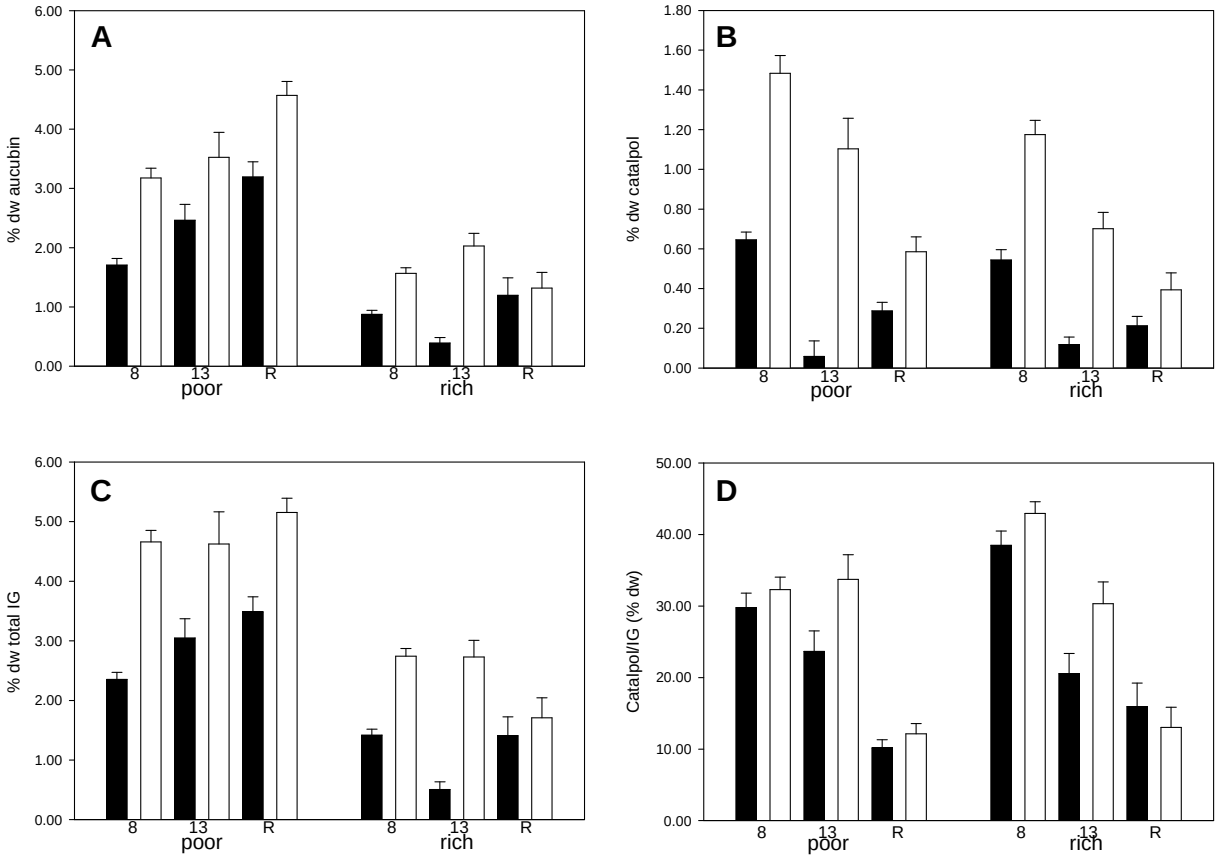

Figure 6.1 Leaf iridoid glycoside percentages of aucubin (A), catalpol (B), total IG (C) or catalpol to total IG ratio (D) of the dry weight, of *Plantago lanceolata* lines selected for low (black bar) and high (white bar) leaf iridoid glycoside concentrations. Plants were grown under nutrient poor or rich conditions and harvested at T8 (8), T13 (13) or regrown from T8 until T13 (R).

Leaf IG concentrations in plants that had regrown after clipping (C) differed from those of untreated plants (U) at the final harvest (T13) in two ways. First, only under nutrient-poor conditions, regrown leaves of plants from high crosses again had higher IG concentrations than those from low crosses. Under nutrient-rich conditions, leaf IG concentrations of plants from high crosses were as low as those from low crosses (nutrient x time interaction, Table 6.2). Second, under nutrient-poor conditions, aucubin concentrations in leaves of regrown plants (C) were higher than in untreated plants (U), but catalpol concentration were lower, resulting in a lower catalpol-to-total IG ratio (Fig. 6.1D).

Table 6.3 Effects of nutrient level, selection line and cross (nested within selection line) on the dry weight of different plant parts and the ratio of root:total biomass in three groups of *P. lanceolata* plants. Values are quasi-F values from GLM analyses of deviance (* P<0.05; ** P<0.01; *** P<0.001).

	df	Leaves	Stalks	Shoots	Roots	Total	Root fraction
Untreated plants T8							
N: Nutrient	1	1006.6***	28.4***	1026.7***	161.9***	432.7***	103.8***
L: Line	1	0.1	0.1	0.1	2.4	1.4	5.5*
C: Cross(L)	14	2.6**	1.5	2.5**	4.7***	3.6***	4.0***
N*L	1	1.0	0.1	1.0	2.5	3.0	1.5
N*C(L)	14	0.5	1.5	0.5	1.1	0.8	1.7
Error	153						
Untreated plants T13							
N: Nutrient	1	861.5***	232.1***	193.5***	2.9	1172.8***	1445.8***
L: Line	1	0.6	0.1	0.1	0.2	0.2	2.0
C: Cross(L)	14	2.9***	4.7***	19.4***	3.1***	1.9*	2.2*
N*L	1	0.8	0.1	0.0	0.7	1.8	1.5
N*C(L)	14	2.0*	3.3***	20.4***	1.9*	1.2	1.0
Error	159						
Clipped and regrown plants T13							
N: Nutrient	1	678.4***	169.2***	1248.8***	0.8	728.3***	511.9***
L: Line	1	0.0	0.1	0.0	4.0	1.6	7.7*
C: Cross(L)	14	2.4**	4.4***	1.3	1.8*	1.6	1.4
N*L	1	0.0	0.0	0.2	1.4	3.0	1.7
N*C(L)	14	1.0	2.4**	1.0	1.1	0.7	1.2
Error	159						

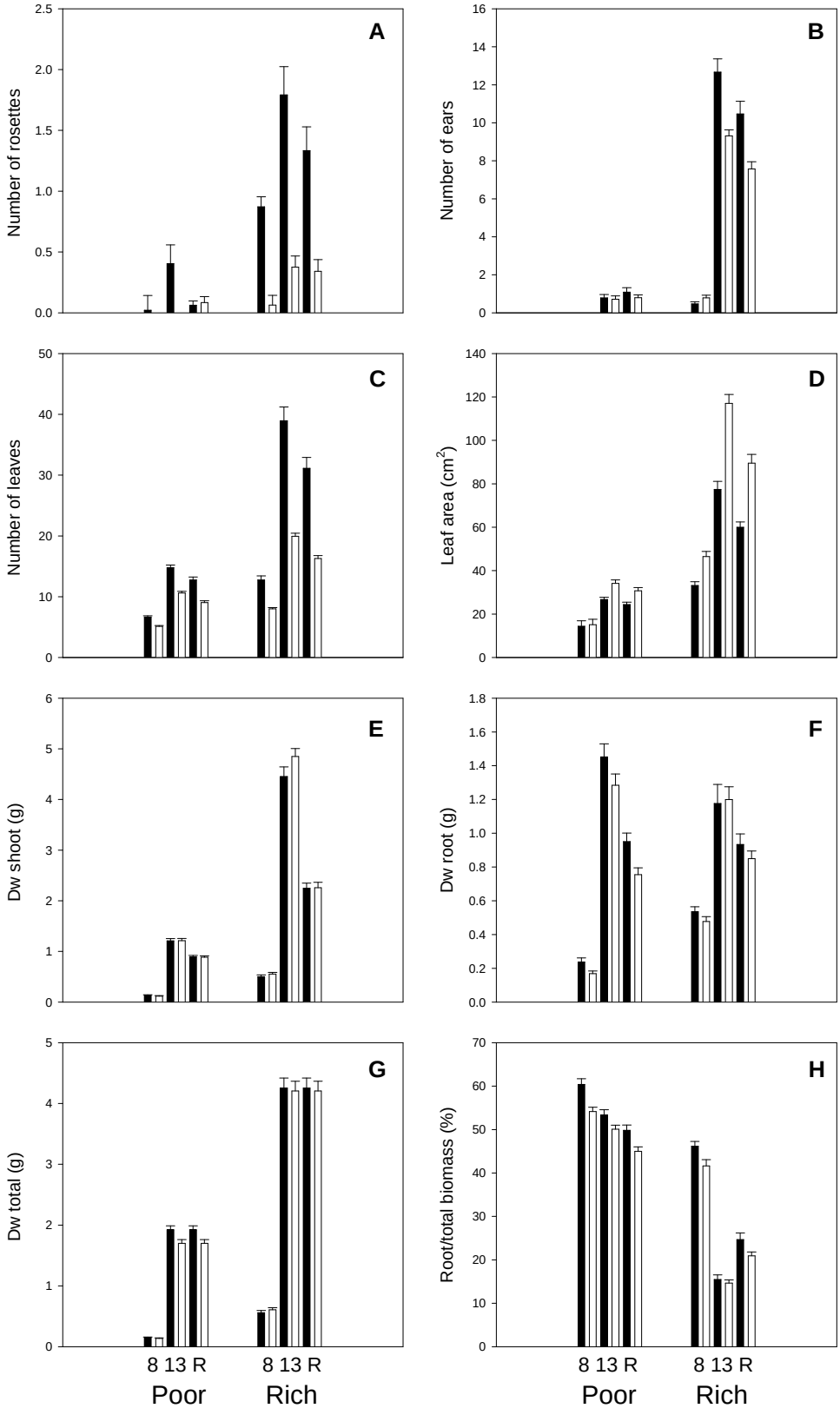

Figure 6.2
Mean number (± s.e.) of side rosettes (A); number of ears (B); number of leaves (C); area of the longest leaf (D); total dry weight shoot (E); total dry weight root (F); root to total biomass (G) of *Plantago lanceolata* lines selected for low (black bar) and high (white bar) leaf iridoid glycoside concentrations. Plants were grown under nutrient poor or rich conditions and harvested at T8 (8), T13 (13) or regrown from T8 until T13 (R).

Growth costs of iridoid glycosides

Total dry weight of plants varied significantly among crosses, both at T8 and T13 (Table 6.3), but plants from high crosses did not have a lower total dry weight than plants from low crosses (Table 6.3; Fig. 6.2G), indicating that there was no growth cost of producing high levels of leaf IG. This is confirmed by the absence of a negative correlation between cross means for leaf IG and total dry weight at T8 (nutrient-poor: r=-0.31, n=16, P=0.24; nutrient-rich: r=-0.13, n=16, P>0.5) or T13 (nutrient-poor: r=-0.17, n=16, P>0.5; nutrient-rich r=0.34, n=16, P=0.20). However, biomass allocation patterns and plant architecture differed between plants from high and low crosses. First, plants from high crosses invested relatively less in roots: at T8 their root mass fraction (root/total biomass) was significantly lower than that of plants from low crosses (Table 6.3; Fig. 6.2H). They also tended to produce less root mass, but this difference was not significant (Fig. 6.2F and 6.3A). Second, shoot architecture differed between plants from high and low crosses. Plants from high crosses produced significantly fewer side rosettes and leaves per plant (Table 6.2; Fig. 6.2A, C) but the leaf area (length x width) of their longest leaf was significantly larger than that of plants from low crosses (Table 6.2; Fig. 6.2D). Third, at T13, when most plants under nutrient-rich conditions had initiated reproduction, plants from high crosses produced fewer flower stalks, than plants from low crosses (Table 6.2; Fig. 6.2B). This indicates a potential reproductive cost of high leaf IG production, although the reduced number of flower stalks was not accompanied by a significantly lower reproductive biomass (Table 6.2 and 6.3).

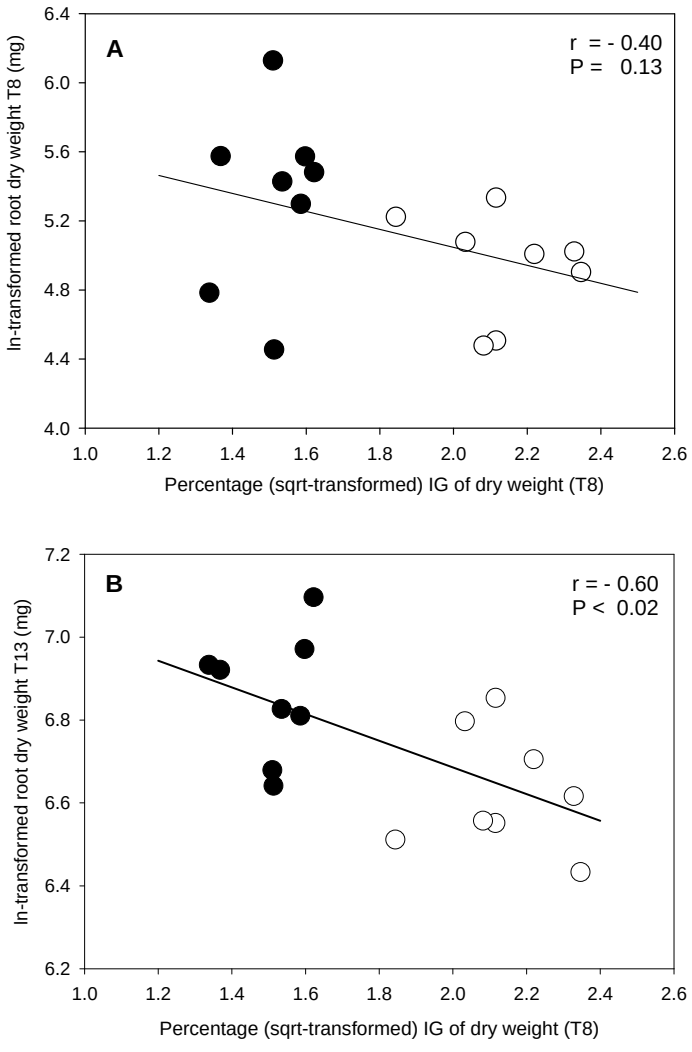

Figure 6.3 The iridoid glycoside concentrations in leaves of *P. lanceolata* at T8 (A) and T13 after clipping (treatment C) (B) plotted against the ln-transformed root dry weight in mg at these times. The root dry weight at T8 is the estimated weight for treatment C. Low (white) and high (black) cross means, all under nutrient poor conditions.

Regrowth costs of iridoid glycosides

At T13, the shoot mass produced by plants that had been clipped at T8 (C) was positively correlated with their estimated root biomass at the time of defoliation (Fig. 6.4; nutrient-poor: r=0.38, n=95, P<0.001; nutrient-rich: r=0.25, n=92, P<0.05), but did not differ between plants from high and low-IG crosses (Table 6.3; Fig. 6.2E). However, plants from high-IG crosses did produce fewer inflorescences after defoliation than plants from low-IG crosses (Table 6.2, Fig. 6.2B), as was observed for untreated plants at T13. Moreover, at low nutrient supply, the root biomass of plants from high-IG crosses, that already tended to be lower than that of low-IG crosses at the time of defoliation (Fig. 6.2F), was now significantly lower than that of plants from low-IG crosses (Table 6.3, Fig. 6.2F). There also was a significantly negative correlation between the IG concentrations of the different crosses at T8 and their root biomass after clipping at T13, under nutrient poor conditions (r =-0.60, n=16, P<0.02; Fig. 6.3B). At high nutrient supply, the difference in root weight between plants from low- and high-IG crosses was not significant.

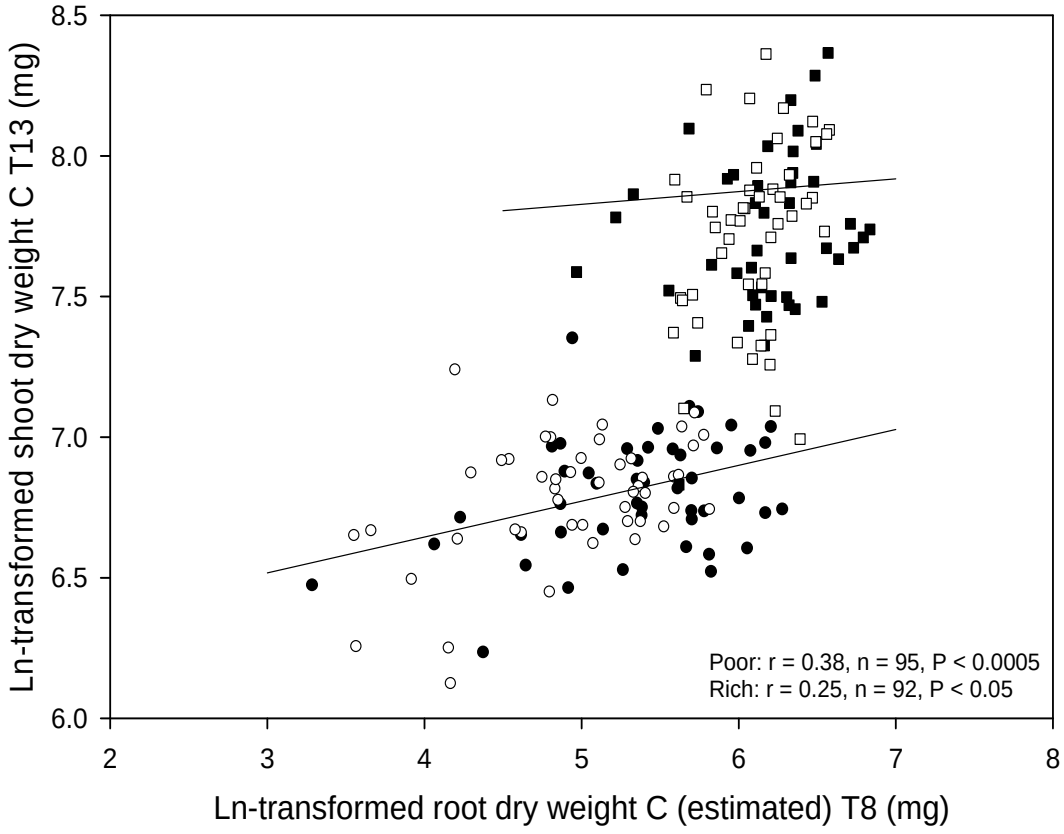

Figure 6.4 The ln dry weight of the regrown shoot at T13 against the estimated ln dry weight of the roots at T8 of *Plantago lanceolata* lines selected for low (black) and high (white) leaf iridoid glycosides, plotted under nutrient poor (circles) and rich (squares) conditions.

Discussion

Growth costs

A fundamental assumption of most plant defence theories is that limited resources lead to allocation constraints, often depicted as trade-offs between the production of secondary plant compounds and other fitness-enhancing traits. In the absence of natural enemies the production of these defence chemicals will have fitness costs. In our study we did not find trade-offs between chemical defence and growth of above ground vegetative plant parts at two nutrient levels. Plants from high-IG crosses did have significantly lower root mass fractions and tended to produce less root mass than plants from low-IG crosses, suggesting a potential cost of chemical defence in terms of fewer resources left for allocation to the roots. However, due to the large variation among crosses within selection lines, the negative association between leaf IG level and root mass was not statistically significant. The only significant cost that was observed was that plants from high-IG crosses produced fewer inflorescences under nutrient-rich conditions. This finding is consistent with other studies reporting reproductive costs of chemical defence (Berenbaum et al. 1986, Zangerl and Berenbaum 1997) and confirms results from a previous study in *P. lanceolata* based on the same selection lines (Marak et al. 2003).

Increased costs of chemical defence under conditions with environmental stress are expected, based on the arguments that a given investment in defence more strongly constrains other fitness-enhancing traits at low than at high resource levels. However, the precise effects will depend on the type of stress and the type of chemicals involved. IGs are carbon-based secondary metabolites. According to the carbon-nutrient balance hypothesis (Bryant et al. 1983, Bryant et al. 1988, Tuomi et al. 1988), plants in resource-limited environments divert their carbon reserves accumulated beyond growth requirements to secondary metabolism without a trade-off in growth (Bryant et al. 1985). Indeed, we observed higher levels of IGs under nutrient poor conditions, as previously shown in *P. lanceolata* (Darrow and Bowers 1999, Marak et al. 2003) without observing stronger reductions in growth or reproduction than under nutrient-rich conditions.

Regrowth

Regrowth capacity implies saving and storing of energy and nutrients in organs that are relatively free from attack. These reserves can be reallocated after herbivory. Plants from which their leaves are frequently consumed, are expected to have low shoot:root ratios and a well developed regrowth mechanism, whereas plants that are rarely consumed would have high shoot:root ratios and a poor regrowth mechanism (van der Meijden et al. 1988). Since plants from high-IG crosses had lower root mass fractions than plants from the low-IG crosses, we hypothesized that plants from high-IG crosses might also have a lower regrowth capacity after defoliation. Contrary to this expectation, the shoot mass at T13 produced by plants that had been clipped at T8 (regrowth) did not differ between plants from high and low crosses, indicating that there was no cost of high IG production in terms of shoot regrowth after defoliation. However, under nutrient-poor conditions, plants that had high levels of leaf IGs at the time of clipping had significantly lower root biomass at the final harvest (T13). This could indicate that under nutrient-poor conditions plants from high-IG crosses are well able to regrow new shoots, but do so at a slightly larger expense of new root growth than plants from low-IG crosses. After a single defoliation event we would therefore not see a negative effect of IGs on shoot regrowth. But as shoot regrowth is strongly and positively correlated with root mass at the time of defoliation, we expect that after repeated defoliation

the progressively stronger reduction of root mass of high-IG plants will eventually result in a reduced capacity of shoot regrowth compared to low-IG plants.

Effect of clipping

The two IGs aucubin and catalpol differ in their biological activity (Bowers 1991, Marak et al. 2002a). Catalpol is considered the more toxic of the two for generalist herbivores (Bowers and Puttick 1988, Bowers 1991, 1992). Consequently, the relative contribution of aucubin and catalpol to the total IG pool is biologically relevant. Clipping affected the levels of the two IGs in the leaves. Except for low-IG plants under nutrient-rich conditions, all plants that were clipped (C) had a significant lower amount of catalpol than unclipped plants (U) and the ratio of catalpol to total IG was lower in regrown plantain leaves than in leaves of untreated plants. Stamp and Bowers (1994) also found that in plants with above-ground parts clipped and regrown for five weeks, the regrowth plants had a lower catalpol tot total IG ratio. After defoliation, regrown shoots may therefore be more susceptible to generalist herbivores than shoots of plants that were not defoliated.

In summary, we detected small allocation costs of chemical defence in terms of reduced numbers of inflorescences and a tendency to produce less root mass by high-IG plants. After a single defoliation event, we did not observe a trade-off between resistance and tolerance, i.e., plants with high levels of a trait (IG level) that confers resistance to generalist insects did not have lower tolerance (shoot regrowth capacity) than plants with lower IG levels. However, since high IG plants did suffer reduced root biomass after a single defoliation and since root mass strongly determines shoot regrowth capacity, we expect that trade-offs between resistance and tolerance will become apparent after repeated defoliation. Alternatively, it is possible that local adaptation results in the absence of correlations or in a positive correlation between defence and regrowth even if a physiological trade-off between growth, defence and storage exists (de Jong and van der Meijden 2000).

Acknowledgements

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Chapter 7

Summarising discussion

In this thesis I studied interactions involving a natural plant-herbivore-parasitoid complex to analyse the occurrence, mechanisms and consequences of a potential conflict between direct and indirect plant defences. The main focus in this study was to explore the effects of genetic variation in plant allelochemistry on plant preference in herbivores and on the performance of herbivores and their parasitoids in a natural system. Many studies examining the effects of plant defence on the behaviour and development of insect herbivores and their natural enemies have been performed with crop species. Domesticated plants which have been the subject of artificial selection may have altered allelochemistry and nutrient content (Benrey et al. 1998). For instance, it has been frequently observed that domesticated plants have lower levels of defensive chemicals than their wild relatives (Rhoades 1979, Evans 1993).

When does a potential conflict become a real conflict?

There are two possible conflicts between direct and indirect defences. First, when constitutive secondary metabolites and volatiles are produced through the same biosynthetic pathway, there can be an allocation trade-off between the two defence systems and this is a potential conflict. This trade-off is not necessarily a "resource allocation" trade-off, but rather in the sense that up regulation of particular metabolic branches may result in fewer precursors for other metabolic branches. If, due to this trade-off. plants that possess high levels of direct defence chemicals emit quantitatively less volatiles after herbivore damage (herbivore induced plant volatiles: HIPV), or volatiles that are less attractive to natural enemies such as parasitoids, then this suggests that there may be a conflict. However, this conflict will only exist if the natural enemies that are recruited through the release of HIPV are beneficial for the plant's fitness. I conducted preliminary experiments in which I addressed this potential trade-off between iridoid glycosides (IGs) and terpenoid volatile production, but they are not part of this thesis. The first preliminary analyses suggest that plants selected for high IGs may have lower mono/sesquiterpenoids. However, these results have to be analysed in more detail and additional experiments verifying the effects of the attraction of parasitoids are needed.

A second potential conflict arises when plant defence chemicals are either passively stored or actively sequestered by the herbivores and have a harmful effect on the performance of their natural enemies. In the case of the generalist herbivore-parasitoid interaction in this study (Chapter 3), we see that the direct defence chemicals are potentially harmful for the generalist herbivores (e.g., longer development time, lower pupal and adult weight) whereas the performance of the generalist parasitoids was not correlated with levels of allelochemicals in the herbivore's diet. In fact, some of the generalist parasitoids actually developed more rapidly and attained greater adult weights when their hosts fed on plants containing higher levels of defence compounds. Consequently, in the case of the generalists there is probably little (potential) conflict between the direct and indirect defences.

For the specialists, however, effects are more association-specific. In the case of *Melitaea cinxia*, levels of IGs in *Plantago lanceolata* actually appeared to be positively correlated with its performance. This supports the contention of several researchers (Ehrlich and Raven 1964, Bernays 1988, Malcolm 1995, Hunter et al. 1996, Schoonhoven et al. 2005) who argue that oligophagous herbivores are often better adapted than polyphages to exploit plants containing specific types of secondary compounds. This is because of a trade-off between the number of different food sources that a herbivore can use and the efficiency with which it can use each of these sources (Singer 1983, Bernays and Minkenberg 1997). Furthermore, the performance of parasitoids associated with *M. cinxia* was not negatively correlated with levels of IGs in host diet, even though high levels of these compounds were sequestrated in larval tissues of the host caterpillars (Chapter 3). Even so, fewer caterpillars in the field were parasitized by the parasitoid *Cotesia melitaearum* when they fed on plants

containing high levels of catalpol. Consequently, there may be a conflict between direct and indirect defence systems, at least where *M. cinxia* and *C. melitaearum* are concerned. However, we do not know if the variation in the attractiveness in *P. lanceolata* plants to *C. melitaearum* females reveals genetic differences in the expression of volatiles that contrast with IG levels.

In contrast with *M. cinxia*, development of the other specialist herbivore, *Junonia coenia*, was negatively correlated with levels of IGs in herbivore diet. Other studies of *J. coenia* also have shown that, although this herbivore species prefers to feed on and shows enhanced growth on IG diet in-vitro (Bowers and Puttick 1988, 1989), assimilation efficiency of the herbivore is lower on diets with high levels of IGs (Camara 1997a). Furthermore, the performance of *J. coenia* is lower when reared on plant genotypes with higher levels of IGs in the field (Adler et al. 1995). Even when the metabolic costs are high, sequestration may still be a low-cost method of dealing with ingested IGs in *J. coenia* due to the fact that larval sequestration of IGs reduces rates of predation in the field (de la Fuente et al. 1994, Dyer and Bowers 1996). In the case of *J. coenia* there may be a (potential) conflict between the direct and indirect defences of *P. lanceolata*. Although larval development time and the pupal weight of *J. coenia* are negatively correlated with IG-levels in the plant, the herbivore sequesters IGs, therefore the caterpillars may be better defended against their natural enemies (Bowers and Stamp 1997, Theodoratus and Bowers 1999, Stamp 2001, Armstrong and Stamp 2003). Further research exploring the effects of sequestration on more specialized (and possibly better adapted) natural enemies, such as parasitoids, will hopefully shed more light on the extent of conflicts between direct and indirect defences in this system.

Efficiency of the third trophic level

Many species of koinobiont endoparasitoids attack a number of stages and/or sizes of their hosts, although parasitoid development is only optimized when specific stages are attacked (Askew and Shaw 1986, Pettit and Wietlisbach 1993, Godfray 1994, Croft and Copland 1995, Elzinga et al. 2003, Harvey 2005). In chapter two I examined performance (survival, body mass and development time) of the parasitoid *Hyposoter didymator* in different instars of two host species that vary significantly in growth potential between and within the various stadia. I found parasitoid development varied significantly with the host species on which the parasitoid was reared. The optimal instar for parasitoid development also differed between the two host species we tested: for *Spodoptera exigua* the optimal instar was L2, whereas for *Chrysodeixis chalcites* L3 hosts were of the highest quality for the development of *H. didymator*. Under natural conditions, the role of plant defence in mediating spatio-temporal interactions between herbivores and their parasitoids is little studied. However, if there is a positive correlation between the levels of phytotoxins in plant tissues and development rate in their herbivores, this suggests that the temporal duration for each instar would be extended. This might benefit parasitoids by increasing the host's 'window of susceptibility' to parasitism (the 'slow-growth-high-mortality-hypothesis'; Clancy and Price 1987, Benrey and Denno 1997, Williams 1999).

When is plant defence beneficial in terms of fitness?

From the point of view of the plant, the optimal defence strategy is to be able to avoid or reduce herbivore damage to a point where fitness (e.g., seed production) is not impaired. This may depend on the relative benefits of investing in the production (constitutive or induced) of allelochemicals against the metabolic costs of their expression (van Dam et al. 2000). The balance may also differ for defence against generalists and specialists. Clearly, the most effective defences against generalist herbivores are direct defences, which are often highly

toxic to them. In addition to their toxic effects, several studies have reported that IGs function as feeding and oviposition deterrent for generalist herbivores (Kubo et al. 1985, Bowers and Puttick 1988, Puttick and Bowers 1988). However, the situation with specialist herbivores is much less clear. Not only was the development of *M. cinxia* apparently enhanced when reared on high IG lines of *P. lanceolata* (Harvey et al. 2005), but Bowers (1991) reported that IGs also function as feeding and oviposition stimulants for several specialist herbivores in the western United States.

Based on these observations it appears that the optimal defence strategy for plants may differ between generalist and specialist attackers. For specialist herbivores, control by natural enemies is probably more effective than direct defences, but this critically depends on the nature of the interaction between the herbivore and its natural enemy. Predators are the most efficient, because they usually kill the herbivore immediately, which has direct benefits for plant fitness. However, in nature most arthropod predators are generalists, which means that they are often not adapted to cope with exposure to high levels of allelochemicals contained in their prey. For instance, several generalist predators, including wasps, mantids and spiders are deterred by the IGs sequestered by *J. coenia* caterpillars (Bowers 1980, 1981, Theodoratus and Bowers 1999, Stamp 2001, Armstrong and Stamp 2003). By contrast, parasitoids are often more specialized than predators, and are thus assumed to be better able to cope with sequestered allelochemicals. However, in parasitoids how efficient they are also depends on several important factors including the feeding strategy of the immature parasitoid, the relative size of the adult parasitoid to its host, brood size, and ultimately how these factors affect plant fitness. Parasitoids that paralyze or kill the host immediately (idiobionts) are of course the best indirect defence for the plant, but very many parasitoids do attack hosts that continue feeding and growing throughout much of the course of parasitism (so-called koinobiont parasitoids). Most solitary koinobionts greatly reduce the amount for feeding damage by the host, and arrest host development in the penultimate instar (Jones and Lewis 1971, Vinson 1972, Harvey et al. 1999). However, in some cases usually involving gregarious koinobionts, parasitized caterpillars consume even more plant material than healthy caterpillars, presumably a form of host regulation that ensures sufficient resources are available for development of the immature parasitoids (Slansky 1986, Coleman et al. 1999). In these circumstances, the benefits of the plant 'recruiting' parasitoids as measures of indirect defence must be seriously questioned (van der Meijden and Klinkhamer 2000).

Direct defence chemicals and oviposition choice

IGs are known to function as oviposition cues for the specialist butterfly *J. coenia* (Pereyra and Bowers 1988). I examined oviposition choice in two closely related specialist butterflies, *M. cinxia* and *M. athalia* and of the specialist parasitoid *C. melitaearum*. Butterflies that lay their eggs in clusters, such as checkerspots, are expected to spend more time in discriminating amongst potentially different food plants for their offspring than solitary egg laying species (Singer 2004). A highly discriminating female that delays her oviposition by rejecting most hosts can simply lay a larger cluster when she eventually does oviposit. *M. cinxia* females clearly decide where to oviposit based on cues they obtain from the plant (chapter 4), where in *M. athalia* the surrounding of the host plants plays a bigger role (chapter 5).

In dual choice experiments *M. cinxia* clearly prefers to oviposit on plants with a higher level of aucubin (chapter 4) and these results are in agreement with a field study (Nieminen et al. 2003). However, I can exclude the possibility that the higher levels of aucubin were a consequence of induction following ovipositing, because the leaves of the plants were sampled before the oviposition. I also found that *M. cinxia* females need a certain threshold to distinguish between different IG levels (chapter 4), suggesting that the absolute level of IGs

should be above 1% of the dry weight, or, conversely, that the difference between the plants should be large enough for females to distinguish. In the field, the size of the plant is a positive visual stimulus and thus plants with more leaves received more eggs. Host plant size is an important aspect, because host plants should be large enough to support the early development of an entire brood (often more than hundred larvae) that hatch from the egg batch. Most importantly, the adult female should choose to oviposit on plants growing in aggregated stands where the larvae can easily move to adjacent plants when the natal plant is exhausted (Andrewartha and Birch 1954, le Masurier 1994). As soon as the eggs hatch, the young *M. cinxia* larvae spin a communal web on the host plant. The larvae are restricted to the natal plant until later in development (e.g., after winter diapause) when it is exhausted of tissues and the caterpillars are forced to disperse to adjacent plants (Kuussaari et al. 2004). In contrast to *M. cinxia* no correlation was found between oviposition preference in *M. athalia* for a host plant species and the average IG content of that species. This indicates that the butterflies either cannot discriminate among plants based on their IGs or that they can, but make choices based on other factors.

Despite the fact that specialist herbivores usually incur a physiological cost of dealing with secondary metabolites in their preferred host plant (Camara 1997a), several studies have shown that *M. cinxia* larvae perform better on plants with a higher level of IGs than on plants with a lower level of IGs (Harvey et al. 2005, Saastamoinen et al. 2007). Another advantage of feeding on plants with IGs, is through protection against natural enemies by sequestration of these defence chemicals (Camara 1997b, Suomi et al. 2001b). IGs can only exert direct effects on higher trophic levels if these higher trophic levels are actually exposed to the allelochemicals in their diet, e.g., the trophic level immediately below. Thus, if IGs are sequestered by the herbivores, they can directly influence the primary parasitoids and if the primary parasitoids sequester these compounds, the same is true for primary and secondary hyperparasitoids. In my study I even found traces of IGs in the fourth trophic level (Chapter 3), the first time, as far as I know, that this has been described.

Costs of direct defence chemicals

A fundamental assumption of most plant defence theories is that limited resources lead to allocation constraints, often depicted as trade-offs between the production of secondary plant compounds and other fitness-enhancing traits. In the absence of natural enemies the production, transport, storage, self-detoxification, activation and/or turnover of these defence chemicals will have fitness costs. I detected small allocation costs of chemical defence in terms of a reduced number of inflorescences and a tendency to produce less root mass by high-IG plants. After a single defoliation event, I did not observe a trade-off between resistance and tolerance, i.e., plants with high levels of a trait (IG level) that confers resistance to generalist insects did not have lower tolerance (shoot regrowth capacity) than plant with lower IG levels. However, since high IG plants did suffer reduced root biomass after a single defoliation and root mass strongly determines shoot regrowth capacity, I expect that trade-offs between resistance and tolerance will become apparent after repeated defoliation events.

Epilogue

For the plant, the most efficient means of defence against herbivores depends on the identity (e.g., feeding biology) of the attacker. Direct defence compounds are probably most effective against generalist herbivores, but often not against more specialised and thus better adapted herbivores. In this case, indirect defences, through the release of HIPV as ‘SOS signals’ are

likely to be more effective in reducing herbivore damage. The importance of indirect defences may, in turn, hinge critically on their reliability in recruiting natural enemies, and this may vary depending on the degree of structural heterogeneity in the landscape and the plant community (Cronin and Reeve 2005). Indirect plant defences are undoubtedly most reliable when the plant is intimately involved with the natural enemy, as occurs with the production of domatia, extra-floral nectarines, etc., where the natural enemy either lives on the plant itself or spends a considerable amount of time on it (Agrawal et al. 2000). When the interactions are not so intimate, for instance with natural enemies that are often some distance from the plants, HIPV may be much less reliable signals for plant damage. In this situation it may be better for the plant to invest in direct defences rather than in less reliable indirect defences. Thus far, the basis of our understanding of the role of indirect defences has been primarily based on simple lab studies or in crop systems where plants exist mostly in monocultures and often have adapted allelochemicals. Future studies should aim at exploring the relative importance of direct and indirect defences in focal plants growing in habitats that vary significantly in terms of species richness and spatial heterogeneity. This will enable us to determine how reliable HIPVs are in recruiting natural enemies in natural systems and over what kind of spatial scales they operate.

How direct and indirect defence mechanisms interact depends on the combination of species involved, as well as on the expression of genes that control the defences. A possible conflict between these two defence strategies may arise if the plants that are most attractive to natural enemies also posses stronger direct chemical defences that exhibit clear negative effects on the performance of predators and parasitoids, or when there are trade-offs between these two kinds of defences. Future studies with natural plant species should examine whether these conflicts really exist in nature.

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Nederlandse samenvatting

Plantenafweer

Omdat planten niet kunnen vluchten voor hun vijanden of zich kunnen verstoppen, moeten ze in staat zijn zich te verdedigen of vraat kunnen tolereren. Planten hebben verschillende soorten mechanische en chemische verdedigingsmechanismen ontwikkeld tegen planteneters (herbivoren). Er kunnen drie typen van verdediginsmechanismen onderscheiden worden: *directe afweer* heeft direct effect op de herbivoor, *indirecte afweer* trekt de vijanden van de herbivoren aan, en als derde kan een plant *tolerant* zijn, waardoor de plant minder last heeft van de herbivorenvraat. Daarnaast kan de plant zijn afweer voortdurend gereed hebben nog voordat hij is aangevallen door een herbivoor; dit heet *constitutieve afweer*. Andere afweermechanismen werken pas nadat de plant wordt aangevreten, de zogenaamde *induceerbare afweer*. De meesten plantensoorten gebruiken verschillende typen van afweer, mechanisch en chemisch, constitutief en geïnduceerd, die er allemaal samen voor zorgen dat de plant zich kan verdedigen tegen zijn vijanden.

Directe afweer

Onder *directe afweer* vallen alle planteneigenschappen die ervoor zorgen dat de plant minder aantrekkelijk is voor herbivoren of die de prestaties van de herbivoren negatief beïnvloeden. Directe afweer wordt verdeeld in *antixenosis* en *antibiosis*. *Antixenosis* zorgt ervoor dat insecten worden verjaagd van de plant, waardoor er minder vraat en oviposition (het leggen van eieren) optreden. Hierbij kun je denken aan de doorns van een roos, maar ook chemische stoffen op de oppervlakte van de plant die afschrikwekkend werken. Van sommige planten is het bekend dat ze eieren nabootsen op hun bladeren, om andere vrouwtjes te ontmoedigen ook hun eieren daar te leggen. *Antibiosis* zorgt ervoor dat de overleving, groei en reproductie van de herbivoren die van de plant eten, gereduceerd worden. Het wordt vaak veroorzaakt door de productie van giftige chemicaliën in plantenweefsel.

Indirecte afweer

Indirecte afweer zorgt ervoor dat de kans groter is dat natuurlijke vijanden (predatoren) van de herbivoren bij of op de plant aanwezig zijn op het moment dat de plant aangevallen wordt. Er zijn verschillende manieren waarop de plant natuurlijke vijanden van zijn herbivoren kan aantrekken. Planten kunnen bijvoorbeeld zorgen voor bescherming door speciale structuren te produceren, zoals domatia. Domatia beschermen de natuurlijke vijanden tegen slechte weersomstandigheden en tegen grotere hyperpredatoren (de vijanden van de natuurlijke vijanden). Een andere manier is om voedsel aan te bieden. Dit kunnen pollen van de plant zijn, maar sommige planten hebben ook speciale orgaantjes waar ze nectar kunnen produceren buiten de bloemen. Het aanbieden van voedsel zorgt voor een verhoogde activiteit van de predatoren op bepaalde gebieden van de plant. Een derde manier om predatoren aan te trekken, is door vluchtige geurstoffen te produceren. Een plant kan deze stoffen aanmaken als reactie op vraatschade. Predatoren kunnen dit dan associëren met de aanwezigheid van herbivoren op de plant.

Tolerantie

Wanneer een plant niet in staat is zichzelf te verdedigen tegen zijn herbivoren, kan het voordelig zijn wanneer een plant *tolerant* is. Een plant is tolerant wanneer het weefselverlies kan doorstaan met weinig verlies van fitness (de hoeveelheid toekomstige nakomelingen) vergeleken met een plant zonder schade. Een plant kan hiervoor zorgen door reservestoffen op te slaan in organen die niet aangevreten worden, bijvoorbeeld in hun wortels. Verder kunnen ze ervoor zorgen dat ze, als reactie op vraat, een verhoogde fotosynthese en opname van voedingsstoffen hebben, waardoor ze in staat zijn sneller te hergroeien.

Interacties tussen directe en indirecte afweer

Het is onwaarschijnlijk dat directe en indirecte afweer onafhankelijk van elkaar opereren in een plant. Natuurlijke vijanden van herbivoren (de *indirecte afweer*) kunnen bijvoorbeeld negatieve gevolgen ondervinden (slechtere groei, hogere sterfte) wanneer zij rupsen aanvallen die eten van planten met veel afweerstoffen (*directe afweer*). De meeste studies die kijken naar de interacties tussen directe en indirecte afweer zijn gedaan met landbouwgewassen. Landbouwgewassen hebben vaak lagere gehaltes van directe afweerstoffen dan hun wilde verwanten. Ze zijn hier vaak op geselecteerd, omdat ze dan beter smaken, want mensen houden vaak ook niet van de afweerstoffen in planten. Om een goed begrip te krijgen van de interacties tussen directe en indirecte afweer is het dus belangrijk om dit te bestuderen in een natuurlijk systeem. In dit proefschrift beschrijf ik de verschillende proeven die ik heb gedaan met een natuurlijk plant-herbivoor-sluipwespsysteem. De nadruk in dit proefschrift ligt op de directe chemische verdediging van planten en het effect hiervan op de hogere trofische niveaus, in dit geval rupsen, hun sluipwespen (parasitoïden) en de sluipwespen van deze sluipwespen (hyperparsitoïden). Parasitoïden zijn insecten, voornamelijk sluipwespen maar ook sluipvliegen, die hun eitjes op of in een ander insect leggen (de gastheer) en waarvan de larven zich voeden met het insect dat uiteindelijk sterft. Hyperparasïtoiden leggen hun eitjes in of op de larven van parasitoïden. De larven voeden zich met de larve van de parasitoïd, die uiteindelijk sterft. Je kunt onderscheid maken tussen solitaire en gregaire parasitoïden, die respectievelijk één eitje of meerdere eitjes in of op een gastheer leggen. Vervolgens kun je nog ondescheid maken tussen parasitoïden die de groei van hun gastheer direct stoppen en diegenen die hun gastheer laten leven en groeien, tot de larven van de parasitoïd volgroeid zijn. Veel parasitoïden kunnen verschillende soorten insecten aanvallen (*generalisten*), in tegenstelling tot diegenen die zich maar in één of een beperkt aantal gastheren kunnen ontwikkelen (*specialisten*).

Parasitoïden kunnen op twee manieren zorgen dat planten minder schade ondervinden van herbivore insecten. Ten eerste doden ze hun gastheren, waardoor het aantal herbivoren in de volgende generatie vermindert. Ten tweede zorgen veel parasitoïden ervoor dat individuele gastheren minder eten als ze geparasiteerd zijn.

De vraagstelling

De hoofdvraag in dit proefschrift is: wat zijn de effecten van directe chemische afweer op de voorkeuren en prestaties van generalistische en specialistische herbivoren en hun hogere trofische niveaus, in een plant-herbivoor-(hyper)parasitoïdsysteem? In de verschilende hoofdstukken van dit proefschrift komen deelvragen met betrekking tot de hoofdvraag aan bod. In **hoofdstuk 2** onderzoek ik wat het optimale gastheerstadium voor de parasitoïd

Hyposoter didymator is en of dit verschillend is voor verschillende gastheersoorten. In **hoofstuk 3** onderzoek ik wat het effect is van de directe afweerstoffen van smalle weegbree (*Plantago lanceolata*) op de groei, ontwikkeling en overleving van twee verschillende generalistische herbivoren, twee specialistische herbivoren en hun sluipwespen. Worden deze afweerstoffen opgeslagen in de herbivoren en hun sluipwespen. Zijn er verschillen tussen specialisten en generalisten? In **hoofdstuk 4** onderzoek ik of de directe afweerstoffen van smalle weegbree en de grootte van de plant invloed hebben op de eierlegkeuze van de specialistische veldparelmoervlinder. In **hoofdstuk 5** onderzoek ik welke gastheerplanten worden geprefereerd door de specialistische bosparelmoervlinder voor ovipositie. Zijn dit ook de meest geschikte voedselplanten voor haar nakomelingen en komen ze voor in de omgeving waar de vlinder te vinden is? In **hoofdstuk 6** kijk ik tenslotte of er kosten zijn verbonden aan het hebben van hoge niveaus van directe afweerstoffen voor smalle weegbree.

Het studiesysteem: Smalle weegbree, de specialistische en generalistische herbivoren en hun (hyper)parasitoïden

Smalle weegbree, *Plantago lanceolata*, is een kruidachtige plant die overal ter wereld voorkomt. Als directe chemische afweerstoffen heeft deze plant iridoïde glycosiden (IG), een groep van bittere, terpeen-achtige stoffen, waarvan de belangrijkste in smalle weegbreeaucubine en catalpol zijn. Deze stoffen zijn afschrikwekkend voor generalistische herbivoren, maar kunnen juist aantrekkelijk zijn voor specialistische herbivoren waardoor deze eerder hun eieren afzetten op planten met hoge gehaltes van deze stoffen en hun rupsen gestimuleerd worden deze planten te eten. De generalisten die ik gebruikt heb in mijn studie zijn de Floridamot, *Spodoptera exigua* en de Turkse mot, *Chrysodeixis chalcites* en hun sluipwespen *Hyposoter didymator* en *Cotesia marginiventris*. Ik heb drie verschillende specialistische herbivoren gebruikt in mijn onderzoek. Ten eerste *Junonia coenia*, een Noord-Amerikaanse schoenlappersvlinder, waarvan de rupsen alleen eten van voedselplanten die IG bevatten. Als tweede specialist heb ik de bosparelmoervlinder, *Melitaea athalia,* gebruikt. Deze vlinder heeft veel verschillende voedselplanten, maar ook die bevatten allemaal IG. De derde specialist is de veldparelmoervlinder, *Melitaea cinxia.* Deze vlinder is een rode lijst-soort in Nederland en komt nog sporadisch voor in Zuid-Limburg. De vlinders en rupsen die ik gebruikt hebben komen van Åland, een eilandengroep in het zuidwesten van Finland. Hier komt deze vlinder nog veelvuldig voor. De rupsen gebruiken hier smalle weegbree en aarereprijs (*Veronica spicata*) als voedselplant, welke beide iridoïde glycosiden bevatten. Van deze specialist heb ik ook de specialistische parasitoïden *Cotesia marginiventris* en *Hyposoter didymator* gebruikt en hun hyperparasitoïden, *Gelis agilis* en *Mesochorus* cf *stigmaticus*.

Het optimale gastheerstadium

Natuurlijke vijanden van herbivoren zijn een potentiële vorm van directe afweer voor een plant. Zoals eerder uitgelegd kunnen planten deze natuurlijke vijanden op verschillende manieren aantrekken. De efficiëntie van deze indirecte afweer is afhankelijk van het effect dat de natuurlijke vijand op de herbivoor heeft. Parasitoïden hebben vaak een optimaal gastheerstadium waarin zij hun gastheer parasiteren. In dit optimale stadium kunnen zij zich sneller ontwikkelen en/of groter groeien en hebben zij een grotere overlevingskans. In **hoofdstuk 2** heb ik gekeken naar het optimale gastheerstadium voor de sluipwesp *Hyposoter*

didymator op twee van zijn generalistische gastheren, de Floridamot en de Turkse mot. Hiervoor heb ik de sluipwesp alle stadia van de gastheren laten parasiteren. Voor de Floridamot zijn dit er vijf en voor de Turkse mot zes. *Hyposoter didymator* laat zijn gastheer doorgroeien nadat ze geparasiteerd zijn. Wanneer een kleine rups geparasiteerd wordt, eet en groeit deze dus nog verder. De larven van deze sluipwesp voeden zich met het weefsel van de rupsen en niet alleen met het hemolymfe (insectenbloed). Hierdoor hebben zij niet alleen een minimale grootte van een gastheer die geschikt is voor hun ontwikkeling, maar ook een maximale grootte, omdat consumptie van de hele gastheer nodig is om succesvol uit de gastheer te kunnen komen. Aangezien de rupsen van de Turksemot een stuk groter worden dan de rupsen van de Floridamot, was de verwachting dat de Floridamot een betere gastheer zou zijn voor *H. didymator*. Maar in tegenstelling tot de verwachting bleek de Turkse mot kwalitatief superieur aan de Floridamot wat betreft de overleving, het aantal sluipwespen dat zich tot volwassen wesp ontwikkelde en de wespgrootte. In de Turksemot kon de sluipwesp zich dus in grotere rupsen ontwikkelen dan in de Floridamot. In beide gastheersoorten was het percentage sluipwespen die zich tot volswassen sluipwesp ontwikkelde het laagst in de grootste gastheerstadia. Het succes van een parasitoïd die zich met het weefsel van zijn gastheer voedt, is dus afhankelijk van de kwaliteit en de grootte van zijn gastheer en het optimale gastheerstadium verschilt dus per gastheersoort.

De effecten van iridoïde glycosiden op de ontwikkeling van specialistische en generalistische herbivoren en sluipwespen

Eén van de directe afweermechanismen van planten tegen herbivoren is de productie van chemische stoffen. Het effect van deze stoffen hoeft niet beperkt te blijven tot alleen de herbivoor maar kan ook invloed hebben op de hogere trofische niveaus in de voedselketen, zoals de predatoren en sluipwespen van de herbivoor. In **hoofdstuk 3** heb ik gekeken naar de effecten van twee directe afweerstoffen van smalle weegbree, de IG aucubine en catalpol, op de ontwikkeling van twee generalistische en twee specialistische herbivoren en hun sluipwespen. Daarnaast heb ik gekeken of deze stoffen ook worden opgeslagen (gesequestreerd) in de weefsels van de specialist *M. cinxia* en haar parasitoïden en hyperparasitoïden. In het algemeen was de ontwikkeling van de generalistische herbivoren negatief gecorreleerd met de IG niveaus in de plant, maar dit effect was niet zo duidelijk terug te vinden bij hun sluipwespen. Bovendien, doordat herbivoren zich langzamer ontwikkelen wanneer ze eten van planten met een hoog IG gehalte, is het optimale stadium voor de parasitoïd langer beschikbaar, waardoor de kans op succesvolle parasitering groter is. Bij de specialistische herbivoren verschillen de effecten tussen *J. coenia* en *M. cinxia*. De ontwikkeling van *J. coenia* was langzamer wanneer de rups at van planten met een hoog gehalte aan IG, terwijl dit juist omgekeerd was voor *M. cinxia.* Hetzelfde patroon was te vinden in *Cotesia melitaearum*, een gregaire sluipwesp van *M. cinxia* rupsen. Ook zij ontwikkelden sneller in rupsen die aten van smalle weegbree planten met een hoog gehalte aan IG. Wanneer we kijken naar de sequestratie van de iridoïde glycosiden in *M. cinxia* en haar (hyper)parasitoïden, dan vinden we deze stoffen terug in de rupsen, poppen en adulten van *M. cinxia* en ook in de pophuiden waar de vlinder al is uitgekomen en de rupshuiden waar de sluipwespen uitgekomen zijn. Ook werden aucubine en catalpol teruggevonden in de sluipwespen *C. melitaearum* en *Hyposoter didymator* en in de hyperparasitoïden *Gelis agilis* en *Mesochorus* cf *stigmaticus*. De effecten van directe afweerstoffen werken dus het effectiefst tegen genralistische herbivoren. Maar er kan een conlict ontstaan wanneer de

chemische stoffen (directe afweer) gesequestreerd worden en een negatief effect hebben op de natuurlijke vijanden van de rupsen (de indirecte afweer).

Iridoïde glycosiden en andere planteigenschappen als ovipositieprikkels

Een andere eigenschap van IG is dat ze kunnen werken als ovipositieprikkel voor specialistische herbivoren en als voedselstimulans voor hun rupsen. In **hoofdstuk 4** beschrijf ik het effect van IG en plantgrootte (voornamelijk het aantal blaadjes), van de waardplant *Plantago lanceolata* (smalle weegbree), op het ovipositiegedrag van de specialistische vlinder, *M. cinxia* (de veldparelmoervlinder). Een eerdere studie met dezelfde soorten liet al zien dat er een verband is tussen een hoog aucubine niveau in de plant en de keuze van vlinders om hun eitjes op deze plant te leggen, maar in deze studie werd geen onderscheid gemaakt of het niveau in de plant zorgde voor de ovipositiekeuze (actieve keuze) of dat de ovipositie het hoge niveau in de plant veroorzaakte (inductie). Ik heb een aantal twee- en meerkeuze experimenten uitgevoerd met planten die verschillen in IG niveau, in kooien en in het veld. In de kooien vond ik een positieve correlatie tussen het aucubine niveau in de plant voor ovipositie en het aantal oviposities, wat aangeeft dat er een actieve ovipositiekeuze is voor planten met een hoger aucubineniveau. De resultaten laten ook een drempelwaarde zien voor de concentratie, namelijk 1% van het drooggewicht. Onder dit concentratieniveau maken de vrouwtjes geen onderscheid tussen de IG niveaus in de planten. In tegenstelling tot de kooiexperimenten, bleek in het veld de grootte van de plant belangrijker te zijn dan de hoeveelheid IG, waarbij op grotere planten (met meer blaadjes) meer ovipositiegebeurtenissen waren dan op kleinere planten, onafhankelijk van hun chemische afweerstoffen. Waarschijnlijk hangt het dus af van de omgeving welke signalen het belangrijkst zijn voor de ovipositiekeuze van de vlinder.

Dat niet alleen de chemische afweerstoffen belangrijk zijn in de ovipositiekeuze, is ook duidelijk in **hoofdstuk 5**, waarin ik gekeken heb naar de ovipositievoorkeur, habitat gebruik en geschiktheid van de voedselplant voor een andere specialist op smalle weegbree, de bosparelmoervlinder, *Melitaea athalia*. In een grote kooi (26 x 30 x 3 m) heb ik gekeken naar de ovipositiekeuze van deze vlinder. Er zijn acht verschillende waardplanten gebruikt die allemaal IG bevatten. De planten die het meest gebruikt werden zijn: aarereprijs (*Veronica chamaedrys*), gewone ereprijs (*V. spicata*) en smalle weegbree (*P. lanceolata*). Al deze planten groeien in open weidegebieden, wat overeenkomt met de plaats waar de volwassen vlinders het vaakst vliegen in het veld. Het verschil in waardplant en habitatgebruik tussen Åland, Finland (waar deze proeven zijn gedaan) en andere regio's waar deze vlinder voorkomt, kan een weerspiegeling zijn van lokale aanpassingen aan het landgebruik en de geologie van het gebied, waardoor er clusters van kleine open weidegebieden zijn ontstaan. Ondanks het feit dat alle waardplanten van *M. athalia* IG bevatten, was de ovipositiekeuze binnen de groep van waardplanten en tussen de individuele planten binnen een soort grotendeels onafhankelijk van het IG niveau in de plant. Alhoewel de vlinders specifieke plantensoorten kiezen voor hun ovipositie, is de directe omgeving van deze waardplanten belangrijker dan hun IG niveau. Planten in groepen omgeven door kale grond, ontvingen significant meer ei-groepjes dan planten in groepen omgeven door vegetatie. De rupsen van *M. athalia* hadden bijna geen voordeel van de waardplantkeuze van hun moeder. Ze ontwikkelden zich even goed op alle plantensoorten gebruikt in het ontwikkelingsexperiment, behalve op *V. officinalis* waar ze het slecht op deden. Deze plant werd dan ook voor ei-afzet het minst gekozen door de vlinders.

Kosten van iridoïde glycosiden

Veel natuurlijke plantenpopulaties hebben genetische variatie in het niveau van hun chemische afweer tegen herbivoren. In smalle weegbree is er ook variatie in IG niveau. Eén van de factoren die eraan bijdraagt dat deze variatie bestaat, zijn de zogenaamde fittness-kosten van chemische afweer, waardoor ze minder nakomelingen kunnen produceren. Wanneer deze kosten er zijn, zou dit betekenen dat in de afwezigheid van herbivoren, de productie, het transport, de opslag, de ontgiftiging en de activering van chemische afweerstoffen zou leiden tot een lagere fitness, aangezien de stoffen die gebruikt worden voor afweer niet gebruikt kunnen worden voor groei, overleving en reproductie. In **hoofdstuk 6** beschrijf ik een hergroeiexperiment waarin ik heb gekeken of er trade-offs zijn tussen resistentie en een specifiek element van tolerantie, het vermogen tot hergroei na ontbladering. Planten met verschillend IG niveau heb ik onder twee voedingscondities laten groeien, rijk en arm. Na acht weken heb ik de bovengrondse biomassa afgeknipt en de planten laten hergroeien gedurende vijf weken. De vragen die ik wilde beantwoorden zijn: 1) hebben planten met een hoog IG gehalte kosten in termen van wortel- en bladgroei, 2) hebben deze planten een verminderd hergroeivermogen na bladverlies en 3) zijn deze kosten duidelijker bij een gebrek aan voedingsstoffen (nutriënten)?

Ik vond geen verschil in totale massa geproduceerd door hoge en lage IG planten. Hoge IG planten produceerden wel minder bloeiaren (reproductie kosten) en investeerden minder in hun wortels dan lage IG planten. Na hergroei, was de wortelmassa van hoge IG planten die groeiden onder lage nutriëntencondities, significant lager dan die van lage IG planten onder dezelfde nutriëntencondities. Aangezien de wortelmassa voor hergroei positief gerelateerd is met de bovengrondse massa na hergroei, verwacht ik dat na herhaalde ontbladering, hoge IG planten er uiteindelijk niet in slagen om hun bladhergroei vol te houden. Trade-offs tussen resistentie (in dit geval het hebben van een hoog IG niveau) en tolerantie in dit systeem, zullen waarschijnlijk pas zichtbaar zijn na herhaaldelijke ontbladering.

Conclusies

Wat kan er geconcludeerd worden over de effecten van iridoïde glycosiden op de verschillende herbivoren en sluipwespen, en verschillen de observaties tussen de generalistische en specialistische herbivoor-parasitoïd combinaties? Uit de ontwikkelingsexperimenten is duidelijk naar voren gekomen dat er een groot verschil is tussen specialistische en generalistishce herbivoren. Directe afweer werkt goed tegen de generalistische herbivoren; ze ontwikkelen langzamer, worden minder groot en de kans op parisitering neemt toe (langere periode dat ze de optimale grootte voor parasitering hebben). Voor hun sluipwespen zijn geen negatieve effecten van IG gevonden. Voor het generalistische systeem bestaat er dus geen probleem tussen de directe en indircte afweer. Dit verhaal wordt anders voor de specialisten. Voor de specialist *Junonia coenia*, zijn directe afweerstoffen effectief; de rupsen groeien langzamer en hebben een lager popgewicht. Hiertegenover staat, dat deze rupsen in staat zijn de IG te sequestreren. Hierdoor worden ze oneetbaar voor veel generalistische vijanden (de indirecte afweer van de plant). In dit geval kan er dus wel een conflict tussen de directe en indirecte afweer van de plant ontstaan. Voor *M. cinxia*, is het nog weer anders. Hier hebben de directe afweerstoffen een positief effect op de ontwikkeling van de herbivoren, waardoor de tijd dat ze beschikbaar zijn voor hun natuurljke vijanden, speciaal voor de sluipwespen juist wordt verkort. Ook van *M. cinxia* is bekend dat zij IG sequestreren.

Deze sequestratie heeft geen negatieve invloed op de ontwikkeling van de parasitoïden, maar uit proeven in het veld is wel gebleken dat rupsen op planten met een hoog gehalte aan catalpol minder worden geparasiteerd dan rupsen op ander planten. In dit gevahebben hogere gehalten aan IG dus niet alleen een negatief effect op directe afweer (snellere ontwikkeling van deruspen) maar ook op de indirecte afweer (lagere kans op parasitering van de rups). Hoe een plant zich dus het best kan verdedigen, hangt voor een groot deel af van wie de aanvaller is. Voor generalisten werkt directe afweer prima, maar bij specialisten is waarschijnlijk indirecte afweer effectiever, mits de directe afweer de indirecte afweer niet tegenwerkt.

Meer onderzoek is nodig naar de effectiviteit van de indirecte afweer. Worden natuurlijke vijanden van herbivoren eerder aangetrokken door planten met hoge of lage directe afweer? De eerste resultaten van mijn onderzoek (niet opgenomen in dit proefschrift) lijken erop te wijzen dat planten met veel directe afweer, minder vluchtige stoffen uitscheiden na vraat. Maar dit is slechts gebaseerd op twee plantengenotypen, één met veel en één met weinig directe afweerstoffen. Het is ook goed mogelijk dat niet de hoeveelheid vluchtige stoffen, maar de samenstelling van deze stoffen belangrijk is voor de aantrekking van bijvoorbeeld sluipwespen. Voorts is het, wanneer de natuurlijke vijanden eenmaal door de plant zijn aangetrokken, natuurlijk nog van belang hoe effectief ze zijn in de bestrijding van de herbivoren. Een predator is het meest effectief, aangezien deze de herbivoor opeet en meteen verdere schade aan de plant voorkomt, maar predatoren zijn vaak generalisten en dus gevoelig voor de directe afweer van de plant. De effectiviteit van een sluipwesp hangt van verschillende dingen af, zoals in welk stadium van de rups valt de sluipwesp aan, is hij solitair of gregair en laat hij de rups nog groeien of verlamt hij de rups? Voor de plant is het dus van belang de juiste natuurlijke vijand aan te trekken. Hoe betrouwbaarder het signaal is dat de plant afgeeft na vraat, hoe groter de kans is dat de juiste “hulptroepen” worden gerekruteerd.

Een ander aspect van IG is dat het ovipositie stimuleert voor specialistische vlinders. Voor *M. cinxia* en *J. coenia* is het bekend dat ze vooral eieren leggen op planten met een hoog IG gehalte. Voor een plant in een omgeving met veel specialistische herbivoren, is het dus beter om niet te veel directe afweerstoffen te hebben, aangezien het de ovipositie stimuleert en in het geval van *M. cinxia* ontwikkelen de rupsen zich ook beter op planten met een hoog IG niveau. Maar op plekken waar juist geen specialistische herbivoren zijn, maar meer generalistische herbivoren zou een plant juist in zijn directe afweer moeten investeren. Dit is een effectieve afweer tegen generalistische herbivoren en doordat het hun ontwikkelingstijd vertraagt, verlengt het de tijd dat ze aangevallen kunnen worden door hun vijanden. In een situatie waar zowel specialistische als generalistische herbivoren veel voorkomen, is een intermediair niveau van directe afweerstoffen en een goede tolerantie (bijvoorbeeld hergroeivermogen) waarschijnlijk de beste verdediging.

Verdere studies aan natuurlijke plantenpopulaties, herbivoren en hun natuurlijk vijanden zijn nodig om te onderzoeken of conflicten tussen directe en indirecte afweer echt voorkomen in de natuur en wat de invloed is van vluchtige stoffen in de rekrutering van natuurlijke vijanden.

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Zonder Gregor kan niemand praktisch werk doen op het CTE. Of het nu kasruimte is die je nodig hebt, een klimaatkamer of ruimte voor kooien in de tuin, Gregor is diegene die het allemaal regelt en er ook nog voor zorgt dat er ruimte is voor iedereen. Je stond ook altijd klaar in wit marsmannenpak als mijn weegbree weer eens geplaagd werd door meeldauw of luis en zelfs nu ik hier in Finland zit, kan ik je nog steeds mailen voor bestrijdingsadvies. Bedankt! Ik wil Nicole en Kees bedanken voor hun hulp bij de GCMS, helaas staan de resultaten van dit werk nu niet in dit proefschrift (vier jaar is toch echt te kort), maar ze zullen niet verloren gaan. In dit rijtje wil ik dan ook Wiecher bedanken, die menig gasfles voor mijn metingen heeft verwisseld. Daarnaast stond je altijd klaar als ik weer een iets wilde lenen, en

heb jij heel wat bestellingen voor me gedaan. Als ik Wiecher niet kon vinden, dan was Henk altijd wel bereid om mij bij te staan bij de gasfleswisselingen. Ook voor balansen die niet meer wilden wegen, of het maken van digitale foto's onder de microscoop stond Henk klaar. Dat je *Veronica* niet opdezelfde kolom kunt meten als *Plantago*, daar kwam ik samen met Roel achter. Roel ik wil je bedanken voor al de technische ondersteuning bij de HPLC, de bereidheid altijd terug te willen komen als de HPLC weer eens zijn eigen wil had, ondanks dat het je vrije dag was en voor het uitzoeken van de juiste kolom voor het meten van *Veronica*. Ab en Gilles bedankt voor het maken van mijn deelbare kooien en het nasturen van de noodset om een opening te maken, het repareren van de zoveelste lamp in de zuurkast en het openschroeven van te strak dichtgedraaide maalbekers. Verder wil ik Suus en Chrisje bedanken voor de administratie (zelfs nu betaal je nog mijn chemicaliën), Gerda, Gerrie en Elly voor alle formuliertjes, emailtjes, pakketjes etc. die overal heen moesten, en natuurlijk voor de gezellige kletsmomentjes als ik echt even genoeg had van het schrijven en ik dan maar 0,50 € kwam halen voor de snoepautomaat.

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Haluaisin kiittää sinua, Kaisa, yhteistyöstäsi Suomessa. Ilman tutkimustasi ratamoverkkoperhosesta väitöskirjani lukua 5 ei olisi olemassa. Työssä tehdyn yhteistyön lisäksi kävimme mukavilla kävelyretkillä saaren ympäri, vietimme elokuvailtoja ja etsimme tikankonttia. Haluaisin kiittää myös Annea, Riikkaa, Kirkoa ja Suvia seurasta Nåton Biologisella asemalla.

De laatste twee jaar heb ik ook veel tijd doorgebracht in Lausanne, om daar aan mijn analyses en manuscripten te werken. Ik wil alle mensen van de Nederlandse borrels bedanken voor de gezellige avondjes. En particulier, j'aimerais remercier Anne, pour sa compagnie, son amitié, tous les pauses de café et de midi toujours aussi sympats, rendant mon séjour à Lausanne encore plus agréable. J'espère que nous resterons en contact et que nous aurons beaucoup de visites et de bavardage à skype.

Naast mijn collega's heb ik tijdens mijn OIO-periode ook veel steun gehad aan mijn "Marnix" vriendinnen, door onze gezellige jaarlijkse weekendjes weg, de landendiners, kleurendates en noem maar op zorgden jullie voor de nodige ontspanning tijdens deze tijd. Bedankt Annelies, Anneloes, Jacqueline Jessica en Linda! In het bijzonder wil ik Andrea bedanken, voor al haar vriendschap door de jaren heen! Ik ben dan ook erg blij dat jij mijn paranimf wilt zijn. Samen met Carmen en Ron en later Corine en Rien, zorgde jij ook voor de gezellige tijd op scouting. Ik wil jullie en alle kabouters dan ook heel hartelijk bedanken voor alle leuke, hectische zaterdagen, maar ook zeker voor alle weekend- en zomerkampen, hierdoor had ik toch nog altijd een beetje vakantie door het jaar heen.

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renhalfuurtjes op zondagochtend. Ook Lilian, heit en mem wil ik bedanken voor hun belangstelling en steun en Marin voor haar gezelligheid. Lieve familie, bedankt voor alle hulp!

Als laatste dan de meest belangrijke persoon. Jelmer, eerst was je mijn drukke, altijd aanwezige, troepmakende kamergenoot. Maar na een half jaar veranderde je in de nog meer aanwezige, ook in mijn huis troepmakende vriend. Je staat altijd voor me klaar, met welke vragen ik ook kom. Dan is het weer statistiek die ik niet begrijp, een vastgelopen computer of hoe je iets schrjft in het Engels. Geduldig heb je al mijn versies van mijn hoofdstukken gelezen en herlezen, mijn literatuurlijst tot op de kommma nagekeken en mijn wisselende humeur tijdens het schrijven en herschrijven om je heen geduld. Op afstand en dichtbij heb je me altijd gesteund tijdens mijn onderzoek. Jelmer, laten we samen, hopelijk voor altijd dichtbij elkaar, gelukkig blijven!

Curriculum vitae

Joanneke Reudler Talsma was born on the 6th of December 1978, in Bennekom, the Netherlands. She attended the Marnix college in Ede for her secondary education (Atheneum). In 1997 she started her study biology at Leiden University and in the second year she chose population biology as differentiation. For her specialisation in animal ecology she carried out three undergraduate research projects. The first project was at Alterra, Wageningen, where she looked with a computer model at the effect of landscape ressitance on ground-dwelling species, especially the tree frog (*Hyla arborea*), supervised by Jan Bokdam and Claire Vos. The second project was the characterisation of Antarctica bacteria; this project was done, as an exchange student, at Kent State University, in Kent, U.S.A., and was supervised by Laura Leff. The third project was carried out at Leiden University. Here she looked at occasional sex of thelytokous females with arrhenotokous males of the endoparasitoid *Venturia canescens*. The main question in this research project was whether the offspring of occasional sex was viable. This study was supervised by Vicky Schneider. She did her second specialisation, nature and environmental education, she did at Utrecht University, where she developed a method to implement the topic "sustainable development" in an education task for high school students.

In Januari 2003 Joanneke started her PhD project in the Department of Plant Population Biology (later at the Department of Multitrophic Interactions) at the Centre for Terrestrial Ecology of the Netherlands Insitute of Ecology (NIOO-KNAW) in Heteren. Under supervision of dr. Arjen Biere, dr. Jeff Harvey and prof. dr. Louise Vet, she studied the effects of iridoid glycosides in multitrophic systems. She looked at the performance of different generalist and specialist herbivore and parasitoid species and at the oviposition preferences of specialist herbivores. Part of the experiments were carried out in the field on Åland, Finland, while other experiments were conducted in the greenhouses in Heteren. The results of this project are presented in this thesis.

In June 2007 she started working as a posdoctoral researcher in the group of Johanna Mappes at the Department of Environmental and Biological Sciences of the University of Jyväskylä, Finland, to continue her work on the effects of plant chemicals, but now focussing on the warning colours of herbivore insects.

Publication list

Thesis work

J.H. Reudler Talsma, J.A. Elzinga, J.A. Harvey and A. Biere
Optimum and maximum host size at parasitism for the endoparasitoid *Hyposoter didymator* (Hymenoptera: Ichneumonidae) differ greatly between two host species. Environmental Entomology 36 (5): 1048-1053

J.H. Reudler Talsma, S. van Nouhuys and A. Biere
Oviposition cues for a specialist butterfly: plant chemistry and size. In review

J.H. Reudler Talsma, K. Torri, and S. van Nouhuys
Host plant use by the heath fritillary butterfly, *Melitaea athalia*: plant habitat, species and chemistry. Manuscript

J.H. Reudler Talsma, A. Biere, J.H B. Turin and S.C Honders
Trade-offs between chemical defence and regrowth capacity in *Plantago lanceolata*. Manuscript

J. H. Reudler Talsma, A. Biere, J. A. Harvey and S. van Nouhuys
Performance of specialist and generalist herbivores using chemically defended *Plantago lanceolata*: the sequestration of ididoid glycosides up to the hyperparasitoids. Manuscript

Pre-thesis work

M.V. Schneider, G. Driessen, L.W. Beukeboom, R. Boll, K. van Eunen, A. Selzner, J.H. Talsma and L. Lapchin 2003
Gene flow between arrhenotokous and thelytokous populations of *Venturia canescens* (Hymenoptera). Heredity 90 (3): 260-267

GRADUATE SCHOOL
Functional Ecology

On behalf of the Educational Committee of the Research School Functional Ecology, I declare that J. Reudler-Talsma, has completed the following elements of the educational programma.

	EC
1. National courses	
2003 Population Genetics, Graduate School Functional Ecology (NL)	
2002 Population Dymacis, Graduate School Functional Ecology (NL)	2.5
	2.5
2. Theoretical pre-study	
Plant defences, what to do if you cannot run?	
3. Verwey-PhD meeting	8
2006 Annual Meeting of the Graduate School Functional Ecology ('Verweij Dagen'), Lunteren; oral presentation	
2004 Annual Meeting of the Graduate School Functional Ecology ('Verweij Dagen'), Texel; poster presentation	2
	2
4. Presentations at International Conferences	
2006 September 25 – 28, Behavioural Ecology of Insect Parasitoids, Antibes, France (oral presentation)	2
2004 August 7 – 12, Symposium on Insect-Plant Relationships, Berlin, Germany (oral presentation)	2
2003 August 18 – 24, ESEB, Leeds, UK (poster presentation)	2
5. Facultative elements	
2006 April 25, Workshop: Plant Insect Interactions: from moleculair biology to ecology (oral presentation)	2
2005 March 19–23, Chemical communication: from gene to ecosystem (EPS and PE&RC)	2
2003 Chromatografie, University of Wageningen	2
2002 2 oral presentations at NIOO-days	2
Total	**31**

The required EC of the educational programme is 30.

Haren, July, 12th, 2007

Prof.Dr. F.J. Weissing
Scientific Director

NETHERLANDS INSTITUTE OF ECOLOGY

Royal Netherlands Academy of Arts and Sciences

The research presented in this thesis was conducted at the Department of Plant Population Biology and the Department of Multitrophic Interactions at the Centre for Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Heteren. This project was supported by the research council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO). This is NIOO thesis 58.

Front cover: a caterpillar of *Chrysodeixis chalcites*, an adult butterfly of *Melitaea athalia,* and two *Plantago lanceolata* plants in the oviposition choice experiment with, in the middle, an adult *Melitaea cinxia* on the feeding sponge.
Back cover: from above the parasitoids *Cotesia melitaearum, C. marginiventris* and *Hyposoter horticola* and the hyperparasitoids *Gelis agilis* and *Mesochorus* sp. cf. *stigmaticus.*