Phylogeny and sequestration of iridoid glycosides in selected genera of the Mecininae (Coleoptera, Curculionidae) with particular focus on their host plant relationship

Dissertation

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vorgelegt von Christian Ulrich Baden
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To Whom It May Concern:

I have read over the Ph.D. thesis of Christian Baden entitled, '*Phylogeny and sequestration of iridoidglycosides in selected genera of the Mecininae (Coleoptera, Curculionidae) with particular focus on their host plant relationship*'. As a native speaker of English, I attest to the quality of Mr. Baden's English.

Sincerely,

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Aufführung der in Anspruch genommenen fremden Hilfen

In den Kapiteln 2 und 3 hat Herr Dr. Stephan Franke die GC, GC/MS und HPLC/MS mit mir zusammen und teilweise alleine bedient. Des Weiteren hat er bei der Zuordnung der chemischen Stoffe geholfen.

In Kapitel 4 hat Dr. Ralph Peters mir bei der Realisierung des Olfaktometers und bei der statistischen Auswertung geholfen.

In Kapitel 5 hat mich Viola Boxberger an einigen Tagen bei den Arbeiten im Feld unterstützt.

Wenn Käfer nicht von mir gesammelt wurden, so ist dies innerhalb der Arbeit vermerkt.

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Zusammenfassung

Iridoidglykoside (IGs) sind sekundäre Pflanzeninhaltsstoffe, die in einer Vielzahl von Pflanzenfamilien vorkommen und deren biologisch aktive Form das toxische Aglycon ist. Die Toxizität des Aglycons beruht auf seiner denaturierenden Wirkung auf Nukleinsäuren, Aminosäuren und Proteinen, welche es alkyliert und so irreversible Querverbindungen erzeugt. Die wohl am besten untersuchten und am weitesten verbreiteten IGs sind Aucubin und Catalpol.

Trotz ihrer Toxizität gibt es viele spezialisierte Herbivore, die sich von Pflanzen, die IGs produzieren, ernähren und sich auf diesen entwickeln. Die meisten dieser herbivoren Insekten sind Schmetterlinge (Lepidoptera) und Käfer (Coleoptera). Zusätzlich sequestrieren die meisten dieser Vertreter die IGs sogar um sich selbst gegen Prädatoren und Bakterien zu schützen. Tatsächlich zeigen alle bisher untersuchten spezialisierten Gattungen dieses Verhalten. Innerhalb der Mecininae (Coleoptera, Curculionidae) gibt es mehrere Gattungen, die ausschließlich auf IG-haltigen (Aucubin, Catalpol und Antirrhinosid) Pflanzen leben: *Mecinus* GERMAR 1821, *Rhinusa* STEPHENS 1829, *Gymnetron* SCHÖNHERR 1825 (alles Mecinini), *Cionus* CLAIRVILLE & SCHELLENBERG 1798 und *Cleopus* DEJEAN 1821 (beide Cionini). Die hier vorliegende Arbeit hat sich eingehend mit diesen Gattungen beschäftigt.

Zunächst wurde ein Stammbaum der 27 zur Verfügung stehenden Arten auf der Basis von mitochondrialer (COI/II) und nukleärer (EF-1α) DNS erstellt. Dieser Stammbaum zeigt zum Einen, dass die Gattung *Rhinusa* nicht monophyletisch ist, sondern dass die Arten, die sich auf *Verbascum* und *Scrophularia* entwickeln, näher mit der Gattung *Gymnetron* verwandt sind, als mit den anderen *Rhinusa*-Arten. Desweiteren gehören die *Gymnetron*-Arten die auf *Plantago* leben zu der Gattung *Mecinus*, genau wie Caldara (2001) es postulierte. Weiterhin sind *Cionus schultzei* und *Rhinusa tetrum* ab. *plagiellum* eigenständige Arten letztere als *Rhinusa fuscescens* bereits von ROSENSCHÖLD (1838) beschrieben. Beide wurden bisher eher als Unterarten oder Aberrationen angesehen. Ein gewichtigeres Resultat ist aber, dass es bei den Mecinini nur wenige Wirtpflanzenwechsel gab und diese jedes Mal eine Radiation nach sich zogen während es bei den Cionini regelmäßiger zu Wirtspflanzenwechseln kam, welche sich aber nur zwischen *Verbascum* und *Scrophularia* abspielten.

Im Weiteren wurden chemische Analysen durchgeführt, um festzustellen, welche Rüsselkäfer-Arten IGs sequestrieren und bis zu welcher Konzentration dies geschieht. Es zeigte sich, dass die untersuchten Mecinini Arten weder Aucubin, Catalpol noch Antirrhinosid sequestrieren, was etwas Besonderes ist, da sie damit die einzigen auf IG-haltigen Pflanzen spezialisierten Insekten-Gattungen sind, die die Stoffe nicht sequestrieren. In den Cionini konnten dagegen IGs festgestellt werden, wobei *Cionus* Aucubin und Catalpol sequestriert und *Cleopus* nur Catalpol. Diese Tatsache deckt sich mit der Ökologie der Käfer, da die Mecinini einen sehr versteckten Generationszyklus besitzen und sich meist endophag ernähren, während sich die Cionini ektophag ernähren – sogar die auffällig gelben Larven. In der Gattung *Cionus* wird Catalpol immer effizienter sequestriert als Aucubin, jedoch kann beobachtet werden, dass Arten, die auf *Scrophularia* leben, normalerweise mehr Aucubin als Catalpol im Körper einlagern und es bei Arten, die auf *Verbascum* leben, genau umgekehrt ist. Diese Verteilung wird sogar zwischen den Populationen der einzigen Art, die auf beiden Pflanzen vorkommt (*Cionus hortulanus*), beobachtet. Die Ursache hierfür liegt aber nicht in den Konzentrationen der Wirtspflanzen, beide Arten zeigen ein ähnliches Konzentrationsverhältnis zwischen Aucubin und Catalpol.

In einem weiteren Versuch wurde untersucht, ob das unterschiedliche Verhältnis der sequestrierten IGs ihre Ursache in den Pflanzen, oder in den Käfern hat. Dafür wurden befruchtete *C. hortulanus* Weibchen einer Population von *S. nodosa* gesammelt und für einen Generationszyklus auf *S. nodosa* bzw. *V. nigrum* gehalten. Während dieser Zeit wurden alle Lebensstadien der Käfer bis auf die Eier sowie die Wirtspflanze selbst beprobt. Es zeigte sich, dass die Unterschiede im Konzentrations-Verhältnis von Aucubin und Catalpol von der Pflanze abhängig sind und nicht vom Metabolismus der Käfer. Die Analysen ergaben nämlich wiederum, dass Tiere von *Scrophularia* mehr Aucubin und Tiere von *Verbascum* mehr Catalpol beinhalten. Dieses Verhältnis lässt sich auch hier nicht mit den Gehalten in den Pflanzen erklären, da sich jene kaum unterschieden. Die Konzentrationen in Männchen und Weibchen unterschieden sich nicht signifikant. Allerdings waren die Unterschiede in den IG-Konzentration selbst zwischen Käfern der gleichen Population, die sich an derselben Pflanze entwickelt hatten, sehr hoch.

Weiterhin wurde ein olfaktorischer Versuch durchgeführt, um die Rolle der Pflanzendüfte für die Monophagie der Gattung *Cionus* zu erforschen. Untersucht wurden drei an *Scrophularia* lebende Arten (*C. alauda*, *C. tuberculosus* und *C. scrophulariae*) und eine von *Verbascum* (*C. nigritarsis*) sowie je eine Population von *C. hortulanus* von *Scrophularia* bzw. *Verbascum*.

Getestet wurden die Arten auf *Scrophularia* und *Verbascum*. Bei diesem Versuch zeigten alle Arten eine olfaktorische Reaktion auf die Pflanzendüfte und alle präferierten ihre Wirtspflanze. Die Wirtspflanzensuche ist also mit dem olfaktorischen Sinn gekoppelt. Da immer die eigene Wirtspflanze präferiert wurde, ist davon auszugehen, dass sich die Präferenz mit jedem Wirtspflanzenwechsel neu ausgebildet hat, oder diese Arten zu Reversionen neigen.

Abschließend wurde noch getestet, ob *C. hortulanus* Populationen von *S. nodosa* im Freiland auf *V. nigrum* überleben können (wie es schon unter Laborbedingung in dieser Arbeit gezeigt wurde), um zu testen, ob hier bereits eine ökologische Speziation stattfinden könnte. Zusätzlich zu dieser Art wurde auch noch *C. tuberculosus* getestet, dessen Wirtpflanze *S. nodosa* ist. Jedoch gibt es einige alte Quellen, die diese Art auch an *V. nigrum* gefunden haben. Dafür habe ich Populationen aus dem Freiland entnommen. Die Tiere wurden individuell markiert und in einer Gegend mit circa 500 *V. nigrum* Pflanzen freigelassen. Das Monitoring dieser Tiere zeigt, dass *C. hortulanus* sich bald über die Pflanzen der Umgebung zu verteilen begann und sich dort augenscheinlich etablierte, während *C. tuberculosus* bald fast vollständig verschwunden war. Vermutlich toleriert letztere Art *V. nigrum* nur kurzzeitig als Wirtspflanze. Eine eventuelle ökologische Speziation der Art *C. hortulanus* konnte aber nicht festgestellt werden.

Summary

Iridoide glycosides (IGs) are secondary plant compounds which occur in a high number of plant families. Their biologically active form is the toxic aglycone. The toxicity is caused by its denaturing effect on nucleic acids, amino acids and proteins, which get alkylated and thereby irreversible cross-linked. The best studied and most widespread IGs are aucubin and catalpol.

Despite their toxicity many herbivores are specialised on IG containing plants. Most of these insects are Lepidoptera and Coleoptera. Actually all insect genera studied so far which are specialized on IG containing plants sequester them for their own protection against predators and pathogens. Within the Mecininae (Curculionidae) several genera are living exclusively on IG producing plants: *Mecinus* GERMAR 1821, *Rhinusa* STEPHENS 1829, *Gymnetron* SCHÖNHERR 1825 (belonging to the Mecinini), *Cionus* CLAIRVILLE & SCHELLENBERG 1798 and *Cleopus* DEJEAN 1821 (both Cionini). The present study investigated these genera in detail.

First, a phylogeny of 27 available species was constructed based on mitochondrial (CO I/II) and nuclear (EF1-α) genes. The phylogeny demonstrates first that the genus *Rhinusa* is not monophyletic: The species living on *Verbascum* and *Scrophularia* are more closely related to the genus *Gymnetron* than to the other *Rhinusa* species. Furthermore the *Gymnetron* species living on *Plantago* should be placed in the genus *Mecinus*, just as Caldara (2001) suggested. In addition, the species *Cionus schultzei* and *Rinusa tetrum* var. *plagiellum* are distinct species and not just subspecies or aberrations. The latter species has already been described by ROSENSCHÖLD (1835). The most interesting result, however, is that there have only been few host plant switches in the Mecinini whereas they occurred frequently in the Cionini. In the Mecinini every host plant switch lead to a radiation on the new plant, but in the Cionini it did not, as they seem to switch easily between *Scrophularia* and *Verbascum* as host plants.

In a second experiment I performed chemical analyses to investigate which species sequester IGs and if so to what extent they do. The results show that the analyzed Mecinini species do not sequester IGs (aucubin, catalpol and antirrhinoside) which is remarkable as this is the first record of insect genera specialized on IG containing plants which do not sequester these compounds. Both tested Cionini genera sequester IGs: *Cionus* both aucubin and catalpol and

Cleopus only catalpol. This difference between the tribes perfectly fits with the ecology of the weevils, as the Mecinini live very cryptically and mostly endophagously, whereas the Cionini live ectophagously with conspicuously colored larvae. Catalpol is always sequestered more efficiently than aucubin by *Cionus*, nevertheless the concentrations of aucubin are mostly higher than those of catalpol in species living on *Scrophularia* and in weevils living on *Verbascum* it is the other way around. This pattern can even be observed within a single species: *C. hortulanus*, the only species living on both plant genera. Interestingly this pattern cannot be observed in the plants themselves, as both possess nearly equivalent concentrations of aucubin and catalpol.

Furthermore I studied whether the different patterns of IG sequestration are caused by the plants or the weevil's metabolism. For this fertilized females of the same *C. hortulanus* population taken from *Scrophularia* were reared on *S. nodosa* respectively *V. nigrum* for a whole life cycle. During that time samples from every life stage (except eggs) of the beetles were collected together with plant samples. The same IG concentration patterns as before could be observed; the different patterns thus depend on the plants. Yet, as was the case with the previous experiment these different patterns could not be observed in the plants but only in the weevils. The differences in the IG concentrations in the plants are only marginal. Furthermore no differences could be detected between male and female weevils. Even in individuals of the same population reared on the same plant a very wide range of different IG concentrations was measured. But it is now clear that the different sequestration patterns are caused by the host plants and that the reason cannot to be found in differences in the weevils' metabolism.

As a fourth experiment olfactometer tests were conducted to investigate whether plant odors play a role in the host plant finding of the weevils of the genus *Cionus*. Three species living on *Scrophularia* (*C. alauda*, *C. tuberculosus* and *C. scrophulariae*), one from *Verbascum* (*C. nigritarsis*) and two populations (one from each plant species) of *C. hortulanus* were tested. We analyzed the weevils' preference for both plant genera *Verbascum* and *Scrophularia*. In this experiment every weevil species showed an olfactory reaction to plant odours and a preference for their own host plants. And the host plant use is linked with a specific olfactory reaction. Because the host plants were preferred in each case it is likely that the preference has been newly developed with every host plant switch – which have been rather frequently between *Scrophularia* and *Verbascum* in *Cionus'* phylogeny.

Finally a dispersal test was conducted with the species *C. hortulanus* and *C. tuberculosus* taken from *S. nodosa* in the field to investigate if they are able to live on *Verbascum*, too. This had already been shown in the present study for *C. hortulanus* under laboratory conditions. For *C. hortulanus* it was tested if populations from *S. nodosa* are able to establish on *V. nigrum* or if there is ecological speciation taking place while *C. tuberculosus* was tested to see whether ancient publications are right which suggest that this species is living on *V. nigrum*, too. I individually marked roughly one hundred weevils per species and released them in an area with about 500 plants of *V. nigrum* but no *S. nodosa*. The monitoring showed that *C. hortulanus* started to disperse over the area and established there whereas *C. tuberculosus* had disappeared almost completely after a few days. Thus *C. tuberculosus* is probably able to tolerate *V. nigrum* only for a very short time as host plant. Signs of a possible ecological speciation of *C. hortulanus* into two species could not be detected.

General Introduction

The iridoid glycosides aucubin and catalpol

In addition to the products of the primary metabolism, all higher plants produce secondary metabolites. These are chemical compounds of most variable structures which have diverse functions for the plants. Lignin for example has a skeletal and cutin an anti-desiccating function. There are moreover compounds that attract pollinator, some to repel herbivores and others to kill pathogens. Yet, there are not only the "inventions" of new toxins against herbivore attacks, but, on the other hand, insect species got adapted to those toxins, which may even serve sometimes as their attractants. There are specialised herbivores even for the most dangerous plants and their toxins. Those insects are not only able to eat toxic plants they are specialised on, but some can even sequester the plant's toxins for their own benefits. Most of these adapted insects are specialised on a small group of chemicals or sometimes even just on one. As already mentioned sometimes they are especially adapted to the secondary metabolites of a plant so that this metabolite acts as an attractant for the herbivore, and therefore its function is conversed. In some cases this attractiveness can increase to the point that the toxins become disadvantageous for the plant and its fitness decreases because of its secondary metabolites (Konno et al. 1999, Ode 2006).

Secondary metabolites belong to diverse chemical compounds. Most common are terpenoids, alkaloids and flavonoids (Wink 2003). One group are the monoterpenes to which the iridoid glycosides (IGs) belong. These IGs are deterrent against many herbivores and pathogens. They are produced by many different plant families, e.g. the Apocynaceae, Caprifoliaceae, Gentinaceae, Lamiaceae, Loganiaceae, Menyanthaceae, Oleaceae, Pedaliaceae, Plantaginaceae, Rubiaceae, Scrophulariaceae, Valerianaceae and Verbenaceae (Wink 2003). An overview over the IGs is given by El-Naggar & Beal (1980) and Boros & Stermitz (1991): there are hundreds of IG structures known whose common characteristic is a cyclopentane ring as skeletal structure (fig. I1). The biologically active structure of the IGs is their aglycone. Therefore they are stored inside the plants in their inactive glycoside structure to avoid an autotoxication. To convert the inactive form into the active one the plants possess the enzyme β-glycosidase which cleaves the molecule into its β-glycosidic bond - glucose and aglycone. Konno et al. (1999) reported that plants store the β-glycosidase and the IGs in different cell organelles to avoid the mentioned autotoxication by its own secondary

metabolites. If the cell is damaged by a herbivore the enzyme is released and the IGs are cleaved - the active aglycone is set free. The enzyme appears in the insect guts as digestive enzyme, too. Hence, the generation of aglycones happens in the insect as well. This strengthens the process of conversion into toxins. The toxic effect of the aglycones is caused by their denaturing actions against nucleic acids, amino acids and proteins by alkylating them. The irreversible cross links generated thereby are stronger than those caused by glutaraldehyde. The denaturing effects can not only harm the herbivore but leads to an unpalatability of the leaf (Konno et al. 1999, Kim et al. 2000). In addition, the enzyme DNA polymerase is inhibited, so the new synthesis of DNA is hindered (Pungitors et al. 2004). Furthermore, aucubin (fig. I1) is as glycoside deterrent to herbivores, just as other IGs, because of its bitter taste (Biere et al. 2004).

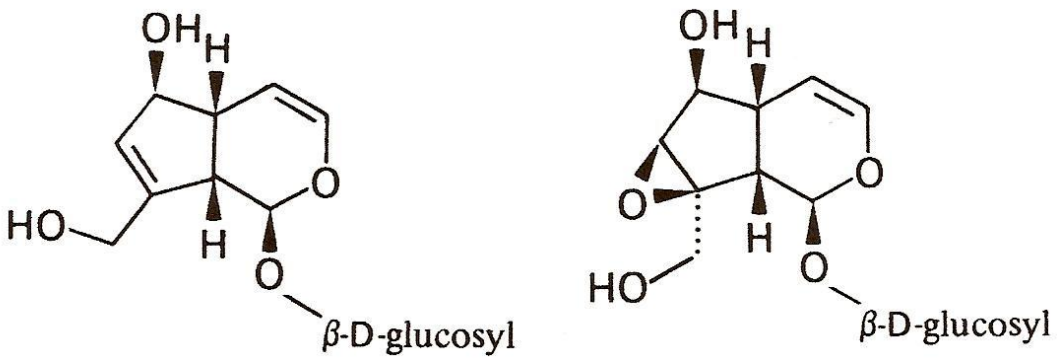

Figure I 1 Shown are the iridoid glycosides aucubin (left) and catalpol (right).

Aucubigenin is the aglycone of the perhaps best examined IG, aucubin (fig. I1). Aucubigenin inhibits amongst others the cytochrome P-450 and induces the loss of the α-helical structure of that enzyme (Bartholomäus & Ahokas 1995). The ability of the aglycone to permeate cell membranes enforces toxic effects inside the herbivores (Bartholomäus & Ahokas 1995). A further property is the antibacterial effect against approved test-organisms (Rombouts & Links 1956, Ishiguro et al. 1983, Davini et al. 1986) and against the entomopathogen *Bacterium thuringiensis* (Baden & Dobler 2009). Aucubin does not only act as a deterrent itself but is also a precursor of another IG used against herbivores: catalpol (fig. I1) (Damtoft 1994). The aglycones of IGs are not only antibacterial but also fungicidal against accepted laboratory fungi and some phytopathogenic ones (van der Sluis 1983, Davini et al. 1986, Marak et al. 2002a, Biere et al. 2004). An inhibition of yeast could not be detected (Davini et al 1986) nor of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*

(Baden & Dobler 2009). The inactive IGs even enhance the growth of some fungi, as they may use them as carbon source (Marak et al. 2002a, Marak et al. 2002b, Biere et al. 2004). Inhibitions of phytopathogenic fungi are known, too (Marak et al. 2002b).

All present research assume that only the aglycones have an antibacterial and fungicide effect and that the aglycone of aucubin is more effective than the one of catalpol (Marak et al. 2002a).

With regard to potential medical application the IGs are a group of interest, too. Aucubin for example is able to ease chronic inflammation (Jeong et al. 2002). Liu and colleagues (2006) discovered that catalpol increases the number of presynaptic proteins and therefore the amount of signal molecules rises up in the hippocampus.

Selected plant genera with iridoid glycosides

As mentioned before the IGs are widely distributed secondary metabolites in the plant kingdom. In the present study we will focus on species of the Plantaginaceae. The Plantaginaceae were newly arranged by Albach et al. (2005) and we should go a little more into detail here. In that study molecular phylogenies were constructed in which some genera of the Scrophulariaceae belong to the Plantaginaceae. In the present study the fact that the genera *Linaria* and *Veronica* now belong to the Plantaginaceae is of particular interest. *Scrophularia* and *Verbascum* remain in the Scrophulariaceae. Together with *Plantago* these genera are of special interest in the present studies.

In 1966 Wieffering had already the idea that aucubin and catalpol can be taken as phylogenetic markers of the Scrophulariaceae and closely related groups. In 1982 Kaplan and Gottlieb expanded the range and took IGs as phylogenetic markers for all dicotyledons. Rønstedt et al. published in the year 2000 a chemotaxonomy of the genus *Plantago*. In that study it was observed that the subgenera of *Plantago* can be clearly distinguished by their IGs and that the close relationship between *Plantago* and *Veronica* is obvious regarding their secondary metabolites. Nevertheless Albach et al. (2005) did not support this conclusion. They stated that these two genera are exceptional within the subfamily but without a closer relationship. It is remarkable that aucubin and catalpol are the main IGs of *Plantago* (Rønstedt et al. 2000) and exist in all *Veronica* species (Taskova et al. 2002a). Jensen et al. (2005) could not identify aucubin and catalpol in *Chamaedrys* – a subgenus of *Veronica*. The studies of the

chemotaxonomy of *Plantago* was continued by Rønstedt et al. (2003) and Taskova et al. (2002b) and those of *Veronica* (Taskova et al. 2006), as well. The aim of these studies was to complete the knowledge neglecting a new grouping of the genera. After all, their precise relationship has still to be ascertained.

Aucubin and catalpol can also be found in other genera like *Verbascum* and *Scrophularia* (Sesterhenn et al. 2007, Willinger & Dobler 2001), though the production of IGs in these genera did not generate the intention to use them for a chemotaxonomy, yet.

The genus *Linaria* produces the IGs antirrhide and antirrhinoside (Handjieva et al 1993). Most studies on these IGs were performed on *Antirrhinum majus*, which is eponymous for their IGs (Beninger et al. 2007).

Iridoid glycosides in tritrophic interactions

No other plant was examined so many times as *Plantago lanceolata*, regarding its IG content – aucubin and catalpol. In addition to these two this plant produces 8-epi-loganinacid and gardoside (Rønstedt et al. 2003). Secondary metabolites play such a major role in the genus *Plantago* that on that basis taxonomy on species level could be done (Rønstedt et al. 2000, Taskova et al. 2002b, Rønstedt et al. 2003). These plants produce so many of these IGs that they can make up to 7 % of the plants dry weight. In studies on the IG distribution in the individual plants it was observed that the amount of IGs is rising over the course of summer with a climax in autumn. The result is that the younger plants in spring are the worst protected life stage in the plant's lifecycle (Bowers et al. 1992, Bowers & Stamp 1993, Fuchs & Bowers 2004). Though the IG distribution inside the plant is not the same for aucubin and catalpol, the concentration of catalpol is higher in the younger leaves and lower in the older ones while the aucubin concentration peaks in the middle aged (Bowers & Stamp 1993, Adler et al. 1995). There is no correlation between the size of the plant and its IG amount (Bowers & Stamp 1992). It has to be assumed that the synthesis of IGs is not connected with high costs for the plant (Darrow & Bowers 1997).

That IGs play an important role as deterrents against herbivores is certain if we consider that catalpol is increasingly produced in times of herbivore attacks and poor nutrients (Darrow & Bowers 1999, Fuchs & Bowers 2004, Prudic et al. 2005). When the leaves are damaged an induction of the IG production takes place within days in the above ground parts of the plant.

Such an induction was detected for the root systems, too, when the rots were attacked by Elateridae larvae of the genus *Agriotes* (Coleoptera). Consequently, the catalpol concentration rises in the roots, whereas the level remains the same in the leaves or rises or falls depending on the plant individual (Wurst et al. 2008). In numerous studies no induction could be detected at all, if anything a drift from aucubin to more catalpol occurred or in some cases even a decrease of the IG amount. There is no valid explanation for this so far (Bowers & Stamp 1993, Stamp & Bowers 1996, Stamp & Bowers 2000). Every induced change in the IG concentration was smaller than the natural alteration caused by the ageing of the plants (Bowers & Stamp 1993, Stamp & Bowers 2000). In very young plants it could even be demonstrated that no induction takes place after a herbivore attack, but an increased growth rate was the response. At the same time the IG content even decreased in favour of growth (Barton 2008). One explanation for that could be that aucubin is phytotoxic too and can repress the growth of roots and germs (Pardo et al. 1998).

The content of catalpol increases relatively to aucubin in response of a herbivore attack as already described. A reason for that could be that aucubin is the precursor of catalpol. The change in the concentrations could be a benefit, because catalpol is more efficient against herbivores and aucubin against microbes (Marak et al. 2002, Fuchs & Bowers 2004). Catalpol is for example very effective against nectar thieves on *Catalpa speciosa* (Bignoniaceae), in this case catalpol is in the nectar. Only adapted nectar thieves are able to consume this nectar without any disadvantage. This is of special interest for the plant too, because these adapted insects are needed as pollinator. On the other hand, generalists lose their locomotion after consumption of only small amounts of catalpol (Stephenson 1982). A generalisation could lead to problems here because the mortality of the specialised larvae of *Junonia coenia* (Lepidoptera, Nymphalidae) increases in correlation with the aucubin contents whereas catalpol has no influence here (Adler et al. 1995). Both IGs show negative effects on the larval development of many other non-specialised herbivores (Bowers & Puttick 1988).

But not only benefits for the plant are caused by a high concentration of IGs: On the one hand, they deter generalist herbivores, on the other hand, they act as stimulants for the oviposition of specialised insects (Bowers 1983, Bowers 1984, Pereyra & Bowers 1988, Klockars et al. 1993). If the IG amount is high in the plants the sequestering insects have an advantage too: there are many different secondary metabolites inside the plant but they only sequester very selectively a few of them, even within a chemical class (Wahlberg 2001). In the case of IGs it is mainly aucubin and catalpol, whereas an adaptation in terms of sequestration is not or

marginal existent (Bowers & Puttick 1988). Besides the often mentioned Lepidoptera species an example for IG sequestering insects are the leaf beetle of the genus *Longitarsus* (Coleoptera, Chrysomelidae) (Willinger & Dobler 2001). These species sequester only between 0.4 – 1.5 % of their dry weights which is comparatively little. Anyhow, the sequestration of these beetles is quite effective. Their IG amount is higher than the one of the plants – which is normal for sequestrating insects (Stermitz et al. 1986, Mead et al. 1993, Willinger & Dobler 2001). Furthermore, some species are able sequester catalpol more effectively than aucubin (Bowers & Collinge 1992, Bowers & Stamp 1997a). Another way to cope with IGs is to excrete them with the faeces. An example for this is the saw fly *Tenthredo grandis* (Tenthredinidae, Hymenoptera). In that species it is possible to detect catalpol in the larvae, prepupae and exuviae but aucubin only in the faeces (Bowers et al. 1993). A third possibility to deal with IGs is to metabolise them. Bowers & Collinge (1992) showed that *J. coenia* does only sequester 0.8 - 3.8 % of the ingested IGs, but in the faeces only traces were found. Therefore, they assumed that the IGs must be metabolised. Camara (1997) detected that the same species sequester 69.5 % of the plant's amount consequently it is unclear whether the differences are side-effects of the different studies or whether this is the normal variability in this species. One explanation could be that Bowers & Collinge used larvae which were just before pupation and the pupae themselves possess only low amounts of IGs because the last instar larvae are metabolising the IGs or excreting them with the meconium (Camara 1997). The same is true for some larvae of the Geometridae (Bowers & Puttick 1986, Stermitz et al. 1988, Bowers 2003). A fourth variant of the adaptation to IGs is the utilisation of glycine which is present in the guts of many Lepidoptera. Glycine binds toxins like oleuropine or aucubigenine and inhibits their denaturing effects by occupying their reactive centres (Konno et al. 1996, Konno et al. 1998).

All these adaptations are known for specialised herbivores but there are some generalists which have these abilities to a certain degree, too. Sequestration is possible for many specialised insects whose growth is not inhibited by the existence of aucubin and catalpol, in contrast to generalists (Bowers and Puttick 1988, Puttick & Bowers 1988, Camara 1997). Some species are rather inhibited if no IGs are included in their food (Bowers 1984). As mentioned before the mortality of specialised species is raised by the presence of catalpol (Adler et al. 1995) but the advantage is still larger than the costs. The presence of IGs reduces the pupae's resting time and has no effect on the weight of the pupae for specialists but the weight decreases for generalists' pupae significantly (Harvey et al. 2005). For the generalist *Tribolium castaneum* (Coleoptera, Tenebrionidae) the presence of catalpol in its food has not

only a negative effect on metamorphosis if it is injected in the larvae's thorax but the adult beetle will have deformed wings and an abnormal position of legs and wings on the thorax (Pungitore et al. 2004).

The greatest advantage the sequestering insects have is the defence against predators. The bitter taste of aucubin and catalpol is a deterrent against birds in the larvae of *Euphydryas phaeton* (Lepidoptera, Nymphalidae) (Bowers 1980, Bowers 1981, Bowers & Puttick 1986), although the aucubin content of these larvae is only about 0.2 % of their dry weight (Belofsky et al. 1989). This low amount is of special interest because even generalist herbivores are sometimes able to sequester IGs in traces and maybe even these traces have an advantageous effect for these species (Bowers & Stamp 1997a). The relative amount of IGs is in some species about a magnitude higher: *Euphydryas cynthia* sequesters up to 2.3 % of their dry weight for example (Franke et al. 1987). And the larvae of the often mentioned *J. coenia* sequesters up to 7 or even 20 % of their dry weight, even though the adults do not contain any IGS at all. *Ceratomia catalpa* (Lepidoptera, Sphingidae) sequesters up to 15 % of its dry weight, particularly in their hemolymph: up to 50 % (Bowers & Collinge, Bowers & Stamp 1997a, Bowers 2003). With such a high concentration a possible autointoxication can take place, even if the IGs are stored in their glycoside structure (Camara 1997).

There must be reasons for the sequestration and the tolerance of the associated costs because there is the possibility to metabolise or tolerate the IGs. In the past many invertebrate predators were tested for a possible deterrent effect of IGs. To mention only some examples: the wolf spider *Lycosa carolensis* (Lycosidae) (Theodoratus & Bowers 1999) as well as the spring spider *Phidippus audax* (Salticidae) (Strohmeyer et al. 1998) avoid the IG containing larvae of *J. coenia*. Such a deterrent effect is known against ant species, as well; an effective protection against several ant species is achieved at a level of 1.5 % IG of the herbivores dry weight (Dyer & Bowers 1996). The bug *Podisus maculiventris* (Heteroptera, Pentatomidae) avoids IG containing food if possible and eats it only if there is no choice (Bowers & Stamp 1997b). In ecological studies the bug eats as much IG containing *J. coenia* larvae as other food but the growth is inhibited by it. The reason for this balanced food-choice could be that the nutrient mixture of *J. coenia* was maybe better than that of the alternative larvae (Bowers & Stamp 1997b). *J. coenia* larvae are avoided by *Polistes fuscatus* (Hymenoptera, Verspidae) in long term studies, too (Stamp 1992). The centipede *Lithobius forficatus* (Chilopod, Lithobiidae) is also deterred by IG containing prey while the earwig *Forficula auricularia*

(Dermaptera, Forficulidae) is not, at least not at the low concentrations tested (Baden & Dobler 2009).

The only tested nematode, the entomopathogene *Heterorhabditis bacteriophora* (Heterorhabiditae), is neither deterred nor killed by aucubin and catalpol (Baden & Dobler 2009).

Hence, it was discovered that the Hymenoptera *Polistes dominulus* (Vespidae) likes the IG containing larvae of *J. coenia* better if they were fed with them in their own larval stage than if they had never been in contact with these larvae before. If this is a kind of imprinting or only addiction is unclear but the deterrence of predators by IGs seems not to function here (Rayor & Munson 2002).

Altogether, all results about the deterrent effect of IGs against predators show a partly diffuse impression of the benefits of aucubin and catalpol sequestration. Yet, it becomes more and more apparent that sequestered IGs are no wonder weapons but provide a defence against at least some predators and bacteria.

Only the benefits of aucubin and catalpol were mentioned so far but there is evidence that the IG antirrhinoside plays a similar role. This IG occurs in *Antirrhinum majus* for example (Plantaginaceae) on which the generalists *Lymantria dispar* (Lepidoptera, Lymantriidae) and *Trichoplusia ni* (Lepidoptera, Noctuidae) are living. At a concentration of 3.3 % of the plant's dry weight the growth of *L. dispar* is inhibited but the growth rate of *T. ni* is increased (Beninger et al. 2008). *L. dispar* is negatively influenced by other IGs, too (Bowers & Puttick 1988, Bowers & Puttick 1989). A hint that the sequestration of antirrhinoside is a benefit for the insects is that the aposematic larvae of *Meris paradoxa* and *Lepipoly spec.* sequester this IG. The cryptic adults have no detectable IGs in their bodies. The larvae's antirrhinoside concentration ranges between 3 % and 11 % of their dry weight (Boros et al. 1991).

The second IG of *Antirrhinum majus* is antirrhide. Although the similarity of the names and their chemical structure may suggest it, antirrhide is not known to be the precursor of antirrhinoside. These IGs occur in different plant parts and have their concentration climax in different times of the year. Antirrhinoside occurs in the aboveground parts of the plants and in its roots. Antirrhide is only detectable in the leaves where its synthesis takes place (Beninger et al. 2007). The concentration of antirrhinoside decreases in the leaves during the florescence while the amount of antirrhide increases (Høgedal & Mølgaard 2000). The antirrhinoside content is highest in the flowers and buds followed by the younger leaves (Beninger et al.

2007). There are no correlations of the light conditions, the temperature or the available water with the IG content (Høgedal & Mølgaard 2000). After all, a similar shift in the concentrations of aucubin and catalpol is found in *Plantago lanceolata*.

Weevils of the subfamily Mecininae and their phylogeny

The Curculionidae (weevils) are a family with an incredible richness of species. There are 4600 genera with over 51000 species described so far which amounts to more than 80 % of the Curculionoidea. They are able to use nearly every plant and plant part as food, because of their nearly incomparable radiation. As adults they are living on plants while their larvae (which have reduced legs) often live inside the plants or on its roots (Rheinheimer 2002, Oberprieler et al. 2007). This radiation began presumably in the middle Cretaceous (about 112 – 93.5 million years ago) and was caused by the radiation of the angiosperm plants. The radiation of the families of the Curculionoidea took place before this instance - about 166 million years ago (McKenna et al. 2009).

The family concept of the Curculionidae is highly debated among experts and the phylogenies presented so far have the most diverse basis (Oberprieler et al. 2007). The biggest problem is the very low sample size (as measured by the number of genera and species) of these phylogenetic trees (Hundsdoerfer et al. 2009). In the study of McKenna et al. (2009) for example “only” 135 genera each with only one species were involved for the whole Curculionoidea (5800 described genera, 62000 known species). The result is indeed a large tree but it contains only 2.3 % of all genera.

The Mecininae are a subfamily of the Curculioninae (Coleoptera, Curculionidae) and consists of two tribes which are in a sister group relationship: the Cionini and the Mecinini. They will be used as taxonomic units in the present studies following the taxonomy established by Caldara (2001) despite the fact that they are not monophyletic in the molecular tree of McKenna et al. (2009). There are six genera described in the Mecinini: *Mecinus*, *Gymnetron*, *Rhinusa*, *Rhinusamiarus*, *Cleopomiarus* and *Miarus*. In the Cionini there are four genera: *Cionus*, *Cleopus*, *Stereonychus* and *Cionellus*. Of these the genera *Rhinusamiarus*, *Cleopomiarus* and *Cionellus* are not found in Central Europe.

As for the host specificity of the genera *Mecinus* GERMAR (1821), *Gymnetron* SCHÖNHERR (1825), *Rhinusa* STEPHENS (1829), *Cionus* CLAIVILLE & SCHELLENBERG (1798) and *Cleopus*

DEJEAN (1821) it is well established that they are all mono- or oligophagous on already mentioned IG containing plants (Plantaginaceae and Scrophulariaceae) (Freude et al. 1983, Sprick 1997, Caldara 2001). Therefore this present study is working only with these five genera.

Caldara published a new revision of the tribus Mecinini in the year 2001. I will follow in some instances this new taxonomy and in other cases the old one (Freude et al. 1983). An explanation, which taxonomy is used will be given in the text.

According to Caldara (2001) there are over 150 described species of the Mecinini living in the Palearctic. The first drastic change exerted by him was raising the subgenus *Rhinusa* to a genus instead of keeping it as a subgenus of *Gymnetron*. Hence, this was a taxonomical change and not a phylogenetic one - this change will be adopted in this study. Caldara's phylogenetic tree is based upon 34 morphological characters and the result reflects very well the ecology of these weevils. In addition to the mentioned major change, he moved the *Gymnetron* species living on *Plantago* to the genus *Mecinus*. The result is that now *Mecinus* species are living on *Linaria* and *Plantago*, *Rhinusa* occurs on *Verbascum*, *Linaria* and *Scrophularia* and *Gymnetron* only on *Veronica* (Freude et al. 1983, Sprick 1997, Caldara 2001). The most common German *Gymnetron* species which belongs according to Caldara now to *Mecinus* are *Gymnetron pascuorum* and *G. labile*. In a later study Caldara (2008) suggested that *Mecinus* is basal in the phylogeny of the Mecinini followed by *Rhinusa*, *Gymnetron* and the exotic genera: (*Mecinus* (*Rhinusa* (*Gymnetron* (…))). That is a novum because his older model (2001) postulated the relationship (*Mecinus* (*Gymnetron* (*Rhinusa* (…))).

In the present study I adopt the change concerning *Rhinusa* but the transfer of *Gymnetron* species to the genus *Mecinus* will be tested in this study, therefore I still refer to the old system here.

The genera *Cionus* and *Cleopus* live on IG containing plants, too: *Verbascum* and *Scrophularia* (Freude et al. 1983, Räther 1989). Their taxonomy on genus-level seems to be clear to a large extent. The only change during the last decades is that Urban (1930b) mentioned a *Cionus fraxini*, which is now *Stereonychus fraxini* (Freude et al. 1983). Apart from that, Caldara (2001) suggested a phylogeny in which *Cleopus* is basal in the Cionini followed by *Stereonychus* and *Cionus* and *Cionellus* – with the last two in a sister group relationship: (*Cleopus* (*Stereonychus* (*Cionus*, *Cionellus*))).

The ecology of the Mecininae

About the ecology of the Mecinini many papers were published in the last two decades. Hence, only about those species which are considered for a biological control management of neophytes. Those neophytes are mainly *Plantago* and *Linaria* which threaten the agriculture in North America. Therefore most facts presented here concern these potential candidates for biological control.

The first *Rhinusa* species which appeared in Northern America were transported there accidentally together with agricultural commodities – just as their host plants many years before. The first reports of *Rhinusa* are about *Rhinusa antirrhini* 1917 and *R. netum* 1950 (both living on *Linaria*) in Canada (Smith 1959). The next species already came as biological control agents against *Linaria* between 1990 and 1995: *R. linariae* and *Mecinus janthinus*. Almost the same dates apply for the USA, supposedly also those of the two mentioned accidental introductions (Wilson et al. 2005). The action of *R. antirrhini* seems to be very promising: this species is able to reduce the number of fertile *Linaria* seeds about 50 % (Newman & Thomson 2005). One of the main problems is the low dispersal rate of these weevils. *M. janthinus* for example has a described rate of up to six metres a year (Anthony 2006). Another problem is the inconsistency of the results because Wilson et al. (2005) wrote about a dispersal rate for the same species of up to three kilometres in four years. They mention the high mortality of this species in winter as the biggest problem.

For the species living on *Plantago* the available records for their first appearance in North America are similar: *Gymnetron pascuorum* 1952 (Dickason 1968). In New Zealand it was tested whether *Cleopus japonicus* could reduce the rampant *Buddleja davidii*, to my knowledge without any known success (Brockerhoff et al. 1999, Withers et al. 2003, McNeill et al. 2005).

The lifecycle of these weevils deserves a detailed description as it will be important for their chemical ecology. The weevils in the tribe Mecininae have in common that their larvae exhibit a very cryptic way of life. They are living in galls, buds, stems, seeds or in the roots of their host plants (Scherf 1964). Caused by their cryptic life cycle only little is known about the larvae of many species of this tribus.

In comparison, the knowledge about the *Mecinus* species is relatively good. The larvae of these species are living inside the stems of their host plants *Linaria* and *Plantago*. They do not

even leave the stems for pupation. A production of galls could happen eventually for some species like *M. collaris* living on *Plantago maritimus*. These species lay their eggs on the stem of a plant and the larvae gnaw themselves into it. Inside the plant they produce a long spindle-shaped gall of several centimetres in length, in which the larvae live, the pupation takes place and the pupae overwinter. This species has one generation per year (Scherf 1964). *Mecinus pyraster* does not produce any galls. In this species the eggs are laid high inside the stem of *Plantago lanceolata*. The up to 3.5mm long larvae feeds from there towards the ground inside the stem and down to the root collar. The upper part of the stem is often cut by an adult beetle before the development of the larvae so that the stem is of no importance for the plant anymore. The pupae rest near to the root collar for 5-8 days and the imagines overwinter at this same spot. Therefore, in this species the adult weevils overwinter, and not the pupae. There is one generation per year (Scherf1964). The lifecycle of the species living on *Linaria* (*M. janthinus* and *M. heydeni*) are very similar to this description. *M. heydeni* causes spindle-shape galls and *M. janthinus* produces none (Scherf 1964). Wilson et al. (2005) observed that *M. janthinus* females lay up to 45 eggs but only one per day and that the imagines overwinter inside the stem and not the pupae. Both species have one generation per year (Scherf 1964).

The genera *Gymnetron* and *Rhinusa* have larvae on almost every plant part; these are root, stem, bud or seed miners. The eggs are laid inside the plants organs in which the larvae live and the pupation takes place. In some species conspicuous galls occur. For example in *G. villosulum* which lives on *Veronica beccabungae* and lays its eggs on the plant's ovaries. After 5 or 6 days the larvae hatch and live inside the unripe seed capsules. These swell up to 8mm and become red-greenish coloured. The flowers and the petals remain unchanged. In *R. thapsicola* on *Verbascum* the fruiting body gall is even parted in several chambers. *G. erinaceum* on *Veronica spicata* and *R. asellus* on *Verbascum*, on the other hand, form the already known spindle-shaped stem-gall. Some species produce red sprout galls of enormous dimensions with several larvae living in it, *R. hispidum* on *Linaria* for example. Other species determine galls on the roots of *Linaria* like *R. collinum* and *R. linaria*. These species plunge their eggs in the soil and the larva feeds themselves into the roots. Up to forty galls on the roots of a single plant were counted, sometimes they even merge. The other species live separately or with few together in the capsules of unripe seeds of the genera *Verbascum, Linaria, Scrophularia* or *Plantago*. It is hardly possible to recognize from the outside which ones are infected and which not. To those weevils species belong: *R. antirrhini, R. netum, G. beccabungae, R. bipustulatum, R. tetrum, G. pascuorum* and *G. ictericum*. All described

species have one generation per year and overwinter inside the plants as pupae or adult weevils (Fabre 1922, Urban 1930a, Scherf 1964). *R. antirrhini*, *R. linariae*, *R. netum* and *G. pascuorum* were found in the soil litter in wintertime, too (Dickason 1968, Wilson et al. 2005). For *R. antirrhini* a gall production inside the fruiting body was described by Wilson et al. (2005).

It should be explained shortly how Mecinini manage to reduce competition, because many species of the Mecinini are living on the same host plants. The example we will look at is *M. pyraster* and *G. pascuorum* on *P. lanceolata*. The larvae of both species live inside the plant but in different parts of it: *M. pyraster* inside the stem and *G. pascuorum* inside the seed capsule. This could induce competition because Scherf (1964) described that *M. pyraster* gnaws off the upper part of the stem, which is fatal for *G. pascuorum*'s way of living. The distribution of *M. pyraster* is widespread whereas the one of *G. pascuorum* is rather aggregated, because it changes only rarely the plant during oviposition. The female of *M. pyraster* changes the plant frequently. Therefore competition is rather low because the species fill different ecological niches (Hamid et al. 2005).

The larvae of the Cionini live legless ectophagously on the plants after giving up their endophagous lifestyle (Prell 1925). They own segmental crawling bulges, which can be viewed as primitive abdominal legs (Prell 1925). That would be the third time in insects that this kind of abdominal legs evolved independently (Lepidoptera and Hymenoptera are the other two instances). The larvae of Cionini are covered by a viscous layer that gives them a slug like appearance – just smaller. The pupation takes place freely on the plant in an amber coloured cocoon formed of secretions. One of the characteristics of that cocoon is that it looks like the buds of *Scrophularia* (Scherf 1964, Räther 1989).

After the description of the different species I will have a close look at the larvae's secretion. One thing is common for all Cionini: their larvae are so similar that no morphological characters have been found so far to distinguish them (Scherf 1964, Räther 1989).

The genus *Cleopus* is represented with two species in Germany: *C. pulchellus* on *Scrophularia* and *C. solani* on *Verbascum*. *C. pulchellus* lay their eggs on *S. nodosa* between its leaf epidermes. There are descriptions that a female has laid 286 eggs in 23 days. The larvae feed on the underside of the leaves on the mesophyll, the upper epidermis remains intact. The cocoons are often located on the plant's stem or on the under surface of the leaves. The eclosion is through a little round hole. The larvae seem to be nocturnal – just as the

imagines (Räther 1989). The larvae of *C. solani* are living on *Verbascum* like the adults. The way of living is just as the one of *C. pulchellus* only on another host plant. Both species have one generation per year and overwinter as imagines (Scherf 1964, Räther 1989). The exotic species *Cleopus japonicus* manages to have two or even three lifecycles per annum in New Zealand (Brockerhoff et al. 1999, Withers et al. 2003). For this species McNeill and colleagues (2005) determined the amount of leaves eaten by this species. Such an investigation has never been done with European species, therefore it will be a reference point for us: The larva eats during its fourteen days long larval stage about 2-3,5cm^2 leaves and as an imago 1-2cm^2 per week, whereupon the amount is reduced when reaching maturity (the lifetime is about 300 days). Withers et al. (2003) have calculated that the descendants of 3100 eggs will eat after eight years 2.5 hectare *Buddleia* leaves per annum.

The species *Cionus alauda* (fig. I2) lives on the genus *Scrophularia* and lays up to four eggs in the leaf parenchyma. The oviposition hole in the leaf is closed by a special secretion. The enclosed larvae live on the upper surface of the leaves (Scherf 1964). In contrast to *C. scrophulariae* whose larvae are living on the underside of the leaves of the same plant (just like *Cleopus*), but on the flowers, buds and young fruits, too. They do not lay their eggs in the leaves but in the flower buds. The holes in the buds are filled with secretion after oviposition, just like *C. alauda* does. The first larval instar lives inside the bud, but the second one leaves the bud to eat ectophagously. The pupation takes often place in cocoons on the leaves' undersides or near the seed heads which are similar to each other. The enclosure procedure is like the one of *C. alauda*. This species was already found on exotic Scrophulariaceae plants in Europe, like *Buddleia*. It is one of the largest *Cionus* species in Germany with 4.5mm body length (Dimmock 1882, Scherf 1964, Räther 1989). A species looking very similar to *C. scrophulariae* (fig. I3) is *C. tuberculosus* (fig. I4) which lives on the same host plant. This species lays its eggs inside the leaf veins and stalks. The method of oviposition is just like the ones mentioned before. It was observed that a female laid 165 eggs in 39 days. The first larval instar eats on the underside of the leaves, the later instars feeds on the upper surface of the

Figure I 2 *Cionus alauda* on *Scrophularia nodosa*

leaves and on nearly all above ground plant parts. The pupation is like the one of *C. scrophulariae* and the adults feeds on all above ground plant parts like their larvae (Scherf 1964, Räther 1989). The species *C. hortulanus* (fig. I5) lives on *Scrophularia* and on *Verbascum* as well. When they live on *Scrophularia* they lay two eggs in the perianth of the buds. The larvae live on both plants ectophagously on the leaves and inflorescences. Actually, there is hardly any information about the larvae's life. It seems similar to the larvae of the already mentioned species. *C. olens* lives on *Verbascum* and has an oviposition behaviour which is different to all other mentioned ones, definitively depending on their host plant: The eggs were placed separately on the very hairy sides of the leaves into which pits were gnawed before oviposition that were totally covered by trichomes. These trichomes desiccate and the areas become brownish. If the leaves are heavily haired the eggs were laid between the trichomes without any pits. The enclosed larvae feed under the trichomes on the leaf surface. To do so the larvae cut the trichomes and their feeding corridor is covered by brownish matted trichomes. Under this hair roof the larvae lives without the covering viscous layer that the other species always have. If the larva leaves this protective hair layer a viscous layer is formed in very short time. Even the pupation takes place under the protective hair roof (Scherf 1964). Living on *Verbascum* as well are *C. olivieri* and *C. thapsus* – the larvae of the first species are living on the under surface of the leaves, whereas the larvae of the second are living on the buds and flowers of the plant, which is severely damaged by that. The pupation takes place near the inflorescences or in it (Scherf 1964). About the same species Fabre (1922) wrote that 5-6 eggs were laid in a bud, but the first larval instar disengaged itself from it. About the larvae and life of the other species (e.g. *C. nigritarsis* living on *V. nigrum* – fig. I6) hardly anything is known by now.

Figure I 3 *Cionus scrophulariae* on *Scrophularia nodosa*

Figure I 4 *Cionus tuberculosus* on *Scrophularia nodosa*

For all *Cionus* species one generation per annum has been described and it is always the adults which overwinter (Scherf 1964, Räther 1989). Only Cunningham (1980) observed a

second generation in England and he wrote exclusively of cannibalism between *Cionus* larvae on *Verbascum*.

For the completeness the lifecycle of *Stereonychus fraxini* should be mentioned here, too. Its larvae are feeding ectophagously as well and live on *Fraxinus excelsior*. The species forms a secretion cocoon for pupation just as the other Cionini (Scherf 1964). These weevils can occur on the trees even before it has developed a single leaf. The eggs were laid on the under surface of the leaves. At this place the viscous layer covered larvae feeds later on, too. Should a larvae fall off the tree it will pupate if it is already old enough. In the South of Europe it occurs even on *Olea europae* and can perform up to three lifecycles a year - in Germany only one (Urban 1930b).

Figure I 5 *Cionus hortulanus* on *Scrophularia nodosa*

After this introduction into the lifestyle and ecology of these selected weevils of the Mecininae the description of the viscous layer of the Cionini larvae should be done. Fabre (1922) observed and described the lifecycle of *C. thapsus* very detailed. He described the secretion layer as extremely gluey and observed that the larvae can tape themselves to a surface with it. Furthermore, it is very elastic and so viscous that it does not desiccate even at high temperature or dryness. This viscous glue is secreted from the gut and is emitted from the rear of the larvae. From there it is dispersed over the larva's back by the wavelike movements of locomotion. The larva can produce and disperse so much secretion that the layer can be regenerated in only a few minutes. During the nearly twenty hours long pupation the following happens: the larva produces three different materials. Firstly, it produces a sort of glue to fix itself to the plant. Secondly, a secretion is produced to harden the normally viscous layer, which gets wiped off afterwards. Lastly, the secretion forming the cocoon is appearing. Fabre

Figure I 6 *Cionus nigritarsis* on *Verbascum nigrum*

observed all that in his garden but is this verified by other scientists? Prell (1925) had observed the secretion of the Cionini larvae, too (fig. I7). He described that colourless secretion does not cover the whole larvae but only the upper side of it down to its pleura. Furthermore, the secretion is not glued on the larva but lays like a coat over it. That is caused by some bristles on the back of the larva which hold the secretion which does not bedew the larva's skin. Particularly many and robust bristles are on the side of the larva's back that prevent the fluxion of the layer off the back. Moreover the spiracles remain open and the respiration is assured. More of those bristle rows are placed between head and thorax and further at the seventh abdominal segment of the larva, both together with the mentioned ones on the sides to hold the viscous layer in its place. This viscous layer can only be produced if the larva is well nourished (fig. I8), without the layer it will dry up very fast. Thus at least the secretion serves as protection against the sun and desiccation (Prell 1925).

Figure I 7 *Cionus tuberculosus* larvae on *Scrophularia nodosa*; *Cionus* larvae feeding on aboveground parts of the plants and are covered by a viscous secret.

The mentioned fast regeneration of the viscous layer is carried out by the eighth abdominal segment caving itself deeply into the larva's body. Thereby the seventh segment arches until it is a large bulge, the backside of the ninth segment then slides under the eighth. Caused by that movement the anus is adjusted first up and then skew to the front. From this position the anus catapults a liquid onto the back of the seventh segment. Thereafter the larva goes back to normal state to repeat the procedure after short time. The liquid gets to the viscous layer which gets dispersed by the peristaltic movement of the larva. This movement occurs by its normal locomotion because it moves its abdominal legs wave like from the front to the back. In addition, the prosoma swings, supposing for orientation and to gauge the substrate (Prell 1925). Before pupation the larva tighten itself and gets into a bulged position. At this point the

bristles are inserted on the sides and the viscous layer flows down and covers the whole larva. At that time the larva is still fully flexible and can move in times of trouble yet will lose most of the secretion. During pupation the secretion flows from the body to serve as fixation glue (which contradicts Fabre's observations). For pupation the larva secrets a more viscous secretion off its anus which is laid over the seventh segment. That secretion is moved to the front by peristaltic movements while new secretion is produced at the back. The anus alternates between the sides of the seventh segment for the deposition of the secretion which becomes alternatingly dispersed on the larva. This is done for some time until the whole back is covered by a colloidal mass. With the help of the mouthparts it gets dispersed even on the underside. At the end the lava is totally covered with a milky white film whose characteristics are different to the normal larva's coating. The old layer dissolves in water within one day, the new one not within several weeks, for example. This colloidal mass solidifies relatively fast and the larva is forming its cocoon and detaches the contact with the mass fully by doing that. Because the larva gets its bristles out of the mass doing so, many microscopic little holes appear in the cocoon these are needed for the air circulation. These holes were described for the first time by Dimmock (1882). Through these breathing holes the air comes in which is needed for the enlargement of the still soft cocoon. When the slowly hardening cocoon has its form, the larva is still producing secretion to strengthen the walls. This procedure lasts for hours during that time the cocoon's colour is changing from milky white to amber brown (fig. I9). Prell (1925) guessed that the most probable production place of the larval secretion might be the peritrophic membrane of the midgut. The Vasa Malpighii could not be the place according

Figure I 8 *Cionus tuberculosus* late larvae on *Scrophularia nodosa*

Figure I 9 Pupae of *Cionus tuberculosus* between buds of *Scrophularia nodosa*

to Prell, because no indication of the secretion can be found there. In contrast to the end gut the midgut is very rich in apocrine glands. Hence, there might be a parallel to the Membrana peritrophica occurring in other beetles (genus *Orycetes* for example) implying a change of its function. Tristram (1978) discovered this membrane actually in *Cionus scrophulariae* and described it as a diffuse peritrophic membrane which secrets in the end of the middle gut. This membrane produces only the larval secretion and not the secretion for the cocoon. The cocoon secretion is produced at the end of the middle gut, too, but in peculiar bands which are produced in deep crypts. These bands for the cocoon consist of a microfibrilliated chitin-protein-mixture. These are produced by adenocytes independently from its neighbours. The fibrillate compounds are formed at the tips of microvilli. The whole activity can be described as the following: The larva stops eating until the gut is empty and the crypts of the midgut are formed. Each crypt has very long microvilli and is filled with secretion just after an hour of starving (Tristram 1978).

The larvae's slimy covering reminds very much of some leaf beetle ones, for instance of the genera *Oulema* or *Crioceris*. Whereas in these genera faeces are admixed, the weevils of the tribus Cionini separate secretion and faeces very strictly, and the secretion gets only produced if the gut is empty (Prell 1925).

Purposes of the present study

The significant differences of the tribus Mecinini and Cionini are clearly comprehensible because their whole lifecycles differ in nearly each point. The Mecinini live as larvae and as adults cryptic or even inside the plants specialised to single plant parts, whereas the Cionini live freely and exposed on the plants in every life stage. Astonishing is the very poor camouflage of the Cionini larvae but of the adults, too. This case is particular for the larvae. Surprisingly, they are yellow and conspicuous looking and the production of the secretion must be a great effort. The common characteristic of these two tribus is that they both are living on iridoid glycoside containing host plants. Even the close relationship of the genera lets a further research on this species appear very attractive, because a different handling of the toxic IGs seems to be very likely considering the different ecology of these weevils.

In the present study I focused on two basal fields of research which are both very promising regarding the present knowledge. Firstly, I clarified whether some species of the Mecininae

are able to sequester the IGs aucubin and catalpol, and if so to what extent. That is very interesting because the secondary metabolite must have had a great influence in the specialisation in these IG containing host plants. To decide about the evolution of host use and possible adaptations to IG sequestration I constructed a phylogenetic tree of the Mecininae genera living IG containing plants. This made it also possible to judge the plausibility of Caldara's taxonomic rearrangements (2001, 2008) in the light of independent molecular data. For both investigations it is aspired to use as many Northern German species as possible, if feasible two different populations of each to assess the ecologic and genetic variability of the weevils.

C. hortulanus was especially interesting as this is living on both host plants *Verbascum* and *Scrophularia*. Here I compared four populations , two from each plant. One open question is, if there is cryptic speciation taking place in the species *C. hortulanus*. Both the phylogenetic and the chemo-ecological study will clarify that. I added ecological studies to understand better the adaptations to two different host plants. One of these studies is an olfactory test. I tested here, whether populations from *Scrophularia* prefer *Scrophularia* or *Verbascum* and vice versa. I have done this olfactory test with all species occurring in Northern Germany to have a comparison with the results of *C. hortulanus* and to understand the monophagous lifestyle of the other species, too. I interpreted the results of that study in the context of the phylogeny of these weevils. In a last investigation I transferred *C. hortulanus* from Scrophularia to *Verbascum* to document their behaviour and a possible settlement on an unfamiliar host plant.

I added a last chemo-ecological purpose as *C. hortulanus* does sequester IGs from its host plants. The aim is to clarify if there is an explanation caused by the host plant or the metabolism of the weevils for differences in the sequestration rates. Therefore female weevils from *Scrophularia* are added to *Scrophularia* and *Verbascum* plants to analyse the chemo-ecology of a whole lifecycle on the differing plants. In favour to do so, the IG contents of the starting females, the larvae, the pupae and the adult offspring together with plant materials of every sampling time were analysed, compared and discussed.

The results of all five studies hopefully give a good overview about the phylogeny and chemical-ecology of the genera *Mecinus, Rhinusa, Gymnetron, Cleopus* and *Cionus*; additionally, a detailed knowledge about the ecology and chemical-ecology of the species *Cionus hortulanus*.

Chapter 1

The phylogeny of some Mecininae (Coleoptera, Curculionidae) genera based on CO I/II and EF1-α gene sequences

Abstract

The taxonomy and phylogeny of the Mecininae is one of the many unresolved problems in the phylogeny of weevils. In 2001 Caldara published a revision of the Mecinini that established *Rhinusa* as a genus of its own and transferred all *Gymnetron* species living on *Plantago* to the genus *Mecinus*, yet many taxonomists remained doubtful whether they should adopt the new taxonomy. We here establish a phylogeny of the Mecininae based on 27 species representing the genera *Cionus*, *Cleopus*, *Stereonychus*, *Mecinus*, *Gymnetron* and *Rhinusa* to test the plausibility of these rearrangements and to solve remaining uncertainties. Our results clearly show that the *Gymnetron* species feeding on *Plantago* are in fact nested within the genus *Mecinus* and should be included in this genus as Caldara proposed. However, the genus *Rhinusa* cannot be delimited as proposed by Caldara (2001) according to our phylogeny since it was not resolved as a monophyletic group. Rather our data suggest that the *Rhinusa* species living on *Verbascum* and *Scrophularia* should be transferred to the genus *Gymnetron*. In addition, our data confirm that *Cionus schultzei* is a species of its own and not closely related to *Cionus hortulanus*. Furthermore, *Rhinusa tetrum* aberration *plagiellum* rather seems to be a species than an aberration as hypothesized by ROSENSCHÖLD (1838): *Rhinusa fuscescens*. Host plant switches were apparently rare in the Mecinini and resulted in groups of several related species using the same host. In contrast, host plant switches in the Cionini must have occurred more frequently.

Introduction

The Curculionidae constitute a family with an immense number of species: over 4.600 genera containing more than 51.000 species have been described so far. They are able to use nearly every organ of every plant as a nutriment resource due to their almost incomparable radiation (Rheinheimer 2002, Oberprieler et al. 2007). The systematics of the Curculionidae is highly controversial: the phylogenies and taxonomy concepts published so far have diverse fundamentals and statements (Oberprieler et al. 2007). A main problem many molecular phylogenies have is the marginal sampling considering the high number of species (Hundsdoerfer et al. 2007). For instance, the phylogeny of McKenna et al. (2009) had a sampling of 135 genera of the Curculionoidea with only one species each; compared to the 5.800 known genera in this group it is only the tip of the iceberg. The outcome of this study is a huge phylogeny, and maybe the best we have so far, but only 2.3% of the described genera are included. Consequently it is obvious that the knowledge about the phylogenies regarding the subfamilies and genera is very low either.

The subfamily of the Mecininae consists of the two tribus Cionini and Mecinini; they are in a sister group relation. Caldara published a phylogeny of the subfamily Mecininae at genus level in 2001. In this study we use Caldara's published phylogeny, even though the Mecininae are not monophyletic according to McKenna et al. (2009). We rely to the extensive morphological studies of Caldara. There are six monophyletic genera described in the Mecinini: *Mecinus, Gymnetron, Rhinusa, Miarus*, *Rhinusamiarus*, and *Cleopomiarus*; and four are described in the Cionini: *Cionus*, *Cleopus*, *Stereonychus* and *Cionellus*. But *Rhinusamiarus*, *Cleopomiarus* and *Cionellus* are not native in Central Europe (Caldara 2001). The special case of the genera *Mecinus*, *Gymnetron*, *Rhinusa*, *Cionus* and *Cleopus* is that they all live on iridoid glycoside containing host plants (Freude et al. 1983, Sprick 1997, Caldara 2001).

In the same study Caldara published the phylogeny of the Mecininae genera (2001) he revised the tribus Mecinini. The Mecinini contain over 150 known Palaearctic species and the main change he had suggested is that *Rhinusa* is ennobled to a full genus (from a subgenus status of *Gymnetron*). That is a taxonomic and not a phylogenetic revision, but he made phylogenetic changes, as well. He placed the *Gymnetron* species living on *Plantago* into the genus *Mecinus*. After these changes the new phylogeny followed the host plant choice of the weevils: *Mecinus* on *Plantago* and *Linaria*; *Gymnetron* on *Veronica*; *Rhinusa* on *Verbascum, Linaria* and *Scrophularia*. Following this study the phylogeny of the Mecinini is as follows:

(*Mecinus* (*Gymnetron* (*Rhinusa* (…)))). In a later study of Caldara (2008) the phylogeny is different: (*Mecinus* (*Rhinusa* (*Gymnetron* (…)))). 2010 Caldara made a phylogeny of the genus *Rhinusa* based on morphological and host plant data and revised Reitter (1916) who suggested *Rhinusa* to be parted into four groups. After the new revision it is parted into ten subgroups, but only one is the same in both publications.

The phylogeny of the Cionini is mentioned by Caldara (2001) in this way: (*Cleopus* (*Stereonychus* (*Cionus*, *Cionellus*))). *Cionus* and *Cleopus* are living on *Scrophularia* and *Verbascum*, and *Stereonychus* on *Fraxinus*. Over the last decades, there have been only a few taxonomic changes within these tribus. For instance, that Urban (1930) wrote about *Cionus fraxini* instead of *Stereonychus fraxini* how this species is now called.

In our study we want to verify the taxonomic changes done by Caldara (2001) and his phylogenies (2001, 2008), particularly the changes of *Gymnetron pascuorum* and *G. labile* to *Mecinus pascuorum* and *Mecinus labile*, and if the revised tribus Mecinini conducts of monophyletic genera. Furthermore, we want to discover a few things which were not subjects of Caldara's work. The first thing is to explore if *Cionus hortulanus* is really one species and not two cryptic species, because it is feeding on *Scrophularia* and *Verbascum;* that is unique in that genus. The next aim is to investigate the number of host plant switches in the both tribus and their phylogenetic impact.

The method of choice is to produce a molecular phylogeny based on the mitochondrial genes CO I/II including the $tRNA^{leu}$ and the genomic EF1-α. These genes are widespread in the molecular studies and are commonly used, even within the Mecinini (Hernandez-Vera 2010). This study mainly examines the species which occur in Northern Germany. Therefore it is not a complete phylogeny of all species, but sufficient to obtain the answers to the questions of particular interest.

Material and Methods

Weevil sampling and collection

Twenty-seven species of the Mecininae could be included into this study. The taxon sampling took place mainly in the area of the Herzogtum Lauenburg (Schleswig-Holstein, Germany) by various collectors (Table 1.1). The weevils starved between collection and freezing (-20°C) for two days that is important to ensure that they do not have any plant material in their gut

which could lead to problems in the PCR by amplifying plant's DNA. The specimen collected by Geiselhardt and Sprick were transferred to our laboratory in ethanol.

Table 1.1 Sample localities, host plants and collectors. Letter assignments for the German states: BB = Brandenburg, B-W = Baden-Württemberg, HH = Hamburg HE = Hesse, NDS = Lower Saxony, S-H = Schleswig-Holstein

Species / Individual number	locality	Host Plant	collector
Cionus alauda 01	Büchig, B-W	*Scrophularia*	Baden, Jäckel
Cionus alauda 02	Brunstorf, S-H	*Scrophularia*	Baden
Cionus hortulanus 01	Brunstorf, S-H	*Scrophularia*	Baden
Cionus hortulanus 02	Langenlehsten, S-H	*Verbascum*	Baden
Cionus hortulanus 03	Pratjau, S-H	*Verbascum*	Baden, Jäckel
Cionus hortulanus 04	Ruit, B-W	*Scrophularia*	Baden, Jäckel
Cionus hortulanus 05	Kastoria, Greece	*Verbascum*	Hengmith
Cionus hortulanus 07	Kastoria, Greece	*Verbascum*	Hengmith
Cionus nigritarsis 01	Bröthen, S-H	*Verbascum*	Baden
Cionus nigritarsis 02	Geesthacht, S-H	*Verbascum*	Baden
Cionus olens 01	Waghäusel, B-W	*Verbascum*	Baden, Jäckel
Cionus olens 02	Waghäusel, B-W	*Verbascum*	Baden, Jäckel
Cionus schultzei 01	Kastoria, Greece	*Verbascum*	Hengmith
Cionus scrophulariae 01	Sachsenwald, S-H	*Scrophularia*	Baden
Cionus scrophulariae 02	Treia, S-H	*Scrophularia*	Baden, Jäckel
Cionus tuberculosus 01	Brunstorf, S-H	*Scrophularia*	Baden
Cionus tuberculosus 02	Wohltorf, S-H	*Scrophularia*	Baden
Cleopus pulchellus 01	Geesthacht, S-H	*Scrophularia*	Baden, Jäckel
Cleopus pulchellus 02	Treia, S-H	*Scrophularia*	Baden, Jäckel
Cleopus solani 01	Freiburg, B-W	*Verbascum*	Dobler
Cleopus solani 02	Freiburg, B-W	*Verbascum*	Dobler
Gymnetron beccabungae 01	Maschen, NDS	*Veronica*	Meybohm
Gymnetron labile 01	Roseburg, S-H	*Plantago*	Baden, Lieberei
Gymnetron labile 03	Wohltorf, S-H	*Plantago*	Baden
Gymnetron pascuorum 01	Öjendorf, HH	*Plantago*	Baden
Gymnetron pascuorum 02	Öjendorf, HH	*Plantago*	Baden
Gymnetron rostellum 01	Neuzelle, BB	*Plantago*	Bayer

Gymnetron rostellum 02	Neuzelle, BB	*Veronica*	Bayer
Gymnetron veronicae 01	Birkenwerder, BB	*Veronica*	Geiselhardt
Gymnetron veronicae 02	Bietigheim, B-W	*Veronica*	Geiselhardt
Mecinus collaris 01	St. Peter-Ording, S-H	*Plantago*	Dobler, Laux
Mecinus collaris 02	St. Peter-Ording, S-H	*Plantago*	Dobler, Laux
Mecinus heydeni 01	Geesthacht, S-H	*Linaria*	Baden
Mecinus janthinus 02	Langenlehsten, S-H	*Linaria*	Baden, Boxberger
Mecinus janthinus 03	Höhbeck, NDS	*Linaria*	Baden
Mecinus pyraster 01	Roseburg, S-H	*Plantago*	Lieberei
Mecinus pyraster 02	Tümlauer Koog, S-H	*Plantago*	Dobler
Mecinus pyraster 03	Geesthacht, S-H	*Plantago*	Jäckel
Rhinusa antirrhini 01	Höhbeck, NDS	*Linaria*	Baden
Rhinusa antirrhini 02	Langenlehsten, S-H	*Linaria*	Baden
Rhinusa asellus 01	Nordstemmen, NDS	*Verbascum*	Sprick
Rhinusa asellus 02	Waghäusel, B-W	*Verbascum*	Baden, Boxberger
Rhinusa asellus 03	Kastoria, Greece	*Verbascum*	Hengmith
Rhinusa bipustulatum 01	Linkenheim, B-W	*Scrophularia*	Baden, Boxberger
Rhinusa collinum 01	Rheingau-Taunus, HE	*Linaria*	Petschenka
Rhinusa collinum 02	Langenlehsten, S-H	*Linaria*	Baden
Rhinusa linariae 01	Geesthacht, S-H	*Linaria*	Baden
Rhinusa linariae 02	Langenlehsten, S-H	*Linaria*	Baden, Boxberger
Rhinusa netum 01	Höhbeck, NDS	*Linaria*	Baden
Rhinusa tetrum 01	Geesthacht, S-H	*Verbascum*	Baden
Rhinusa tetrum 02	Waghäusel, B-W	*Verbascum*	Baden, Jäckel
Rhinusa tetrum 03	Toulon, France	*Verbascum*	Dobler
Rhinusa tetrum plagiellum 01	Kastoria, Greece	*Verbascum*	Hengmith
Stereonychus fraxini 01	Wohldorf, HH	*Fraxinus*	Hengmith
Stereonychus fraxini 02	Wohldorf, HH	*Fraxinus*	Hengmith
Trichosirocalus troglodytes	Tümlauer Koog, S-H	*Plantago*	Dobler

DNA extraction, PCR and sequencing

DNA was extracted with the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN) following protocol. The obtained DNA extract was stored in the fridge at 5°C or in the freezer at -20°C. The only variation of this protocol was to puncture the weevils' abdomen and to incubate them individually in 180µl Buffer ATL and 20µl Proteinase K over night at 55 °C at beginning. After the first centrifugation the weevils were removed. The benefit of this extraction modification is that most weevils are still intact as vouchers. Three main gene parts were amplified: the first two were fragments of the mitochondrial CO I/II (1630bp if the whole data was obtained) and the third the anterior part of the genomic EF1-α (around 650bp).

Table 1.2 Used primers for the CO I/II and EF1-α sequencing.

Used primers	**Sequences of the primers**
TS 2183	5' - TGT AAA ACG ACG GCC AGT CAA CAT TTA TTT TGA TTT TTT GG - 3'
TS 2798	5' - TGT AAA ACG ACG GCC AGT GG(AT) ATA CC(AT) CGA CGT TAT TCT GA(CT) TAT CC - 3'
TA 3020	5' - CAG GAA ACA GCT ATG ACC GGA GCT TAA ATC CAA TAC ACT ATT CTG CC - 3'
TA 3380	5' - CAG GAA ACA GCT ATG ACC GAT CA(GA) TAT CAT TGA TG(AGT) CCA AT - 3'
TA 3661	5' - CAG GAA ACA GCT ATG ACC CCA CAA ATT TCT GAA CAT TGA CCA - 3'
TA 3772	5' - CAG GAA ACA GCT ATG ACC GAG ACC ATT ACT TGC TTT CAG TCA TCT - 3'
EFS 149T	5' - TGT AAA ACG ACG GCC AGT GA(AG) AA(AG) GA(AG) GC(ATCG) CA(AG) GA(AG) ATG GG - 3'
TEF-VIA	5' - CAG GAA ACA GCT ATG ACC GGG AGA CG(ATCG) AGG GG(TC) TT(TC) TC(ATCG) GT(ATCG) GG - 3'
VIA korr-T	5' - CAG GAA ACA GCT ATG ACC G(AG)A GAC GAA GAG GTT T(GC)T C(AG)G T(AG) G - 3'
TEF-INS	5' - TGT AAA ACG ACG GCC AGT CC(ATCG) AC(ATCG) GA(GA) AA(GA) CCC CT(ATCG) CGT CTT CC - 3'
EFA 1106-T neu	5' - CAG GAA ACA GCT ATG ACC GTA TAT CCA TTG GAA ATT TGA CC(ATCG) GG(GA) TG - 3'
M13	5' - TGT AAA ACG ACG GCC AGT - 3'
M13rev	5' - CAG GAA ACA GCT ATG ACC - 3'

The CO I/II (including the $tRNA^{leu}$) segment was amplified using the primers TS 2183, TS 2798, TA 3020, TA 3380 TA 3661 and TA 3772; the primers' sequences are mentioned in table 1.2. For the EF1-α gene the four primers EFS 149T, TEF-VIA, VIA korr-T and EFA 1106-Tneu were used (table 1.2). The following pairings were employed: EFS 149T with one

of the other three EF1-α primers and for CO I/II TS 2183 with TA 3020 and TS 2798 together with TA 3380, TA 3661 or TA 3772. For the sequencing PCR these primers are M13-tailed.

The polymerase chain reaction contained PCR Rxn buffer, $MgCl_2$, dNTPs (Roth), primer (Eurofins MWG operon respectively MWG-Biotech AG) and Taq DNA Polymerase Recombinant (all others Invitrogen), because of the very diverse used mixtures which were needed to get all PCR products, they are not mentioned here. All PCRs were performed using the Mastercycler (Eppendorf). The PCR procedure for CO was as follows: initial denaturation for 2min at 95°C; 35 cycles of 45sec at 95°C, 1min at between 41°C and 51°C, 1min or 2min at 72°C; for 1 min (sometimes 2min); final elongation for 10min at 72°C. Additionally a touchdown PCR was used sometimes even with upgrading temperature. The profile was just as the one mentioned but with 38 cycles. The successful temperature ranges were: 49-41.2°C; 50-43.6°C, 51-43.4°C; 40-47.4°C, 42-45.8°C and 42-49.6°C. The PCR procedure of EF1-α was just the same as for CO I/II, with an annealing temperature range of 41°C to 63°C, and an elongation step which lasts for one or two minutes at 72°C. The touchdown technique was used here more frequently. The annealing temperature ranges were here: 55-45°C; 56.4-49°C; 58-54.8°C; 58.4-51°C; 60.4-53°C; 61,4-58,3°C; 62.4-55°C; 64.4-57°C; 45-56.4°C; 53-60.4°C; 53-64.4°C; 57-64.4°C; 58.3-61.4°C and 60-67.4°C. For the EF1-α amplification two-step PCRs were carried out, too. With annealing temperatures of 45°C for 10 cycles and 51°C for 25 cycles, respectively 60°C for 20 and 67°C for 15 cycles. Reamplifications and nested respectively semi-nested PCR were performed using the same PCR profiles.

After a positive result of a gel electrophoresis the PCR products were expurgated with the PCR-Cleaning Kit "QIAquick PCR Purification Kit" by Qiagen. Cycle sequencing in both directions was carried out either on a Li-Cor (Li-Cor 4200L, MWGBiotech, Ebersberg) or with an ABI-Sequenator 3100 (Applied Biosystems, USA). The sequencing step of the second method was done by the sequencing service of the Universitätsklinikum Hamburg-Eppendorf.

In the sequencing PCR procedure for the Li-Cor we used the Thermo Sequenase Primer Cycle Sequencing Kit by Amersham (Amersham, Braunschweig) and the infrared marked primers M13 (IR 800nm) and M13rev (IR 700nm) (table 1.2). The sequencing PCR profile was: 2 min on 95°C and then 20 cycles of 30 sec 95°C, 15 sec 56°C, 2min at 65°C, after that the samples got denatured at 95°C. Afterwards they were mixed with Li-Cor-Stop and immediately frozen. The polyacrylamide gels used by this sequencer were 66cm long and 0.2mm thick, the voltage was 2000. The runs were fulfilled overnight.

The second sequencing method was done by a sequencing service as mentioned before. But the sequencing PCR was conducted in our laboratory. It was performed with a PCR mix which contains primers, the PCR-product, 2.5 x Sequencing Buffer for Version 1.1 and BigDye Terminator Cycle Sequencing Mix Version 1.1. The PCR procedure was 1min at 96°C and then 30 repetitions: 10sec at 96°C, 10sec at 56°C and 4min at 60°C. The products were cleaned by using DyeEx 2.0 Spin Kit (Qiagen).

It was avoided to obtain pseudogenes by the separation of the mitochondrial and genomic DNA for two individuals of the genus *Mecinus*. After the sequencing we compared the gained sequences with the others to find potential pseudogenes.

The gel-pictures done by the Li-Cor sequencer were analysed using E-SEQ (a Li-Cor product). The raw sequencing data of both sequencing methods were edited using SEQUENCHER 4.5 (Gene Codes Corp., Ann Arbor, Michigan, USA). With this program it is possible to verify the sequences by the included chromatograms. Alignment of the data was accomplished visually. As an out-group the sequences of *Trichosirocalus troglodytes* were used.

Evolutionary tree construction

The data for CO I/II and EF1-α were analysed separately and together. The phylogenetic analyses were carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) with settings as follows: every run had three Markov Chains of the Markov Chain Monte Carlo (MCMC) for three million generations each. Sampling frequency was every 100 generations and a heating parameter value of 0.5 were chosen, the burnin were 7500. A general model of DNA substition (the GTR) with gamma-distributed rate variation was used. The rate partitions were set variably. The posterior probabilities of each bipartition of the consensus tree were observed.

The last tree was obtained by merging the CO I/II and EF1-α for every individual, and then MrBayes was just used as described before. This tree was achieved with heating parameter values of 0.05, 0.5 and 1.0.

Results

The analysis of the COI–COII fragment included 28 species, 56 individuals and up to 1630 characters, and the analysis of the EF1-α fragment had 26 species, 50 individuals and up to 650 characters.

Three of the obtained trees done with heating parameter values of 0.5 are shown in the figures 1.1, 1.2 and 1.3: the tree of the EF1-α gene segment (Fig. 1.1), the analysed CO I/II fragments (fig. 1.2) and the overall tree including both genes (Fig. 1.3). The posterior probabilities of the nodes are presented in these figures, too. The trees and the posterior probabilities of the nodes are comparable to those done with heating parameter values of 0.05 and 1.0; therefore the trees are highly similar.

Figure 1.3 illustrates the phylogram from Bayesian analysis of the complete data set that consists of up to 2280bp. Within the Mecininae the genera *Rhinusa* and *Gymnetron* are not monophyletic just as in the other trees. *R. collinum* and *R. netum* are mixed together as well as *Gymnetron beccabungae* and *G. veronicae*. The basal state of the Mecinini could not be dissolved, but all other species-groups are supported with posterior probabilities of over 0.79, most of them are 1.0. Within the Cionini all individuals accumulate in their species groups. *Stereonychus* and *Cleopus* forming a sister group of *Cionus*. The most basal *Cionus* species are *C. alauda*, *C. scrophulariae* and *C. schultzei* in just that order. Only the posterior probabilities are different, the sister group relationships of the genera are only at 0.75, but all others are better than 0.84 many are just 1.

The EF1-α fragment based phylogeny (Fig. 1.1) is very poorly dissolved in the genus *Cionus*. In this phylogeny *Stereonychus* is the basal genus and *Cionus* and *Cleopus* are sister groups. *Cionus alauda* and *C. scrophulariae* are basal for *Cionus*. All related posterior probabilities are over 0.98 in this area. Within the Mecinini the individuals of one species are clustering, only *Rhinusa netum* and *R. collinum* are mixed up. The genera *Rhinusa* and *Gymnetron* are not monophyletic in this phylogeny; the belonging posterior probabilities are over 0.72, mostly over 0.9.

The analysis of the COI/II fragments most closely resembled the results the full dataset except for the branches of *Mecinus* and the according *Gymnetron* species (Fig. 1.2). The species are clustering very well together, but it is impossible to distinguish between *Gymnetron veronicae* and *G. beccabungae* and *Rhinusa netum* and *R. collinum* again. In this phylogeny *Rhinusa* and *Gymnetron* are not monophyletic just as in the other phylogenies. The posterior probabilities

are in the important nodes not less than 0.79. The Cionini are grouped in this phylogeny just as in the phylogeny done with the whole dataset. The worst posterior probability is at 0.64, but all other important ones are between 0.96 and 1.0.

Discussion

Despite the incompleteness of the taxon sampling the phylogeny presented here shows many very interesting results. Beginning with the two tribus it has to be mentioned that the results are independent of the question if the Mecininae are monophyletic (Caldara 2001) or not (McKenna et al. 2009). Both tribus are monophyletic as every phylogeny presented here shows considering that we have not sampled every genus inside these tribus. The resolution of the EF1-α based phylogeny (Fig. 1.1) is rather poor for the Cionini. A possible reason could be that it is obtained by around 650 base pairs which is probably not enough. But after all, it is obvious that there are three genera and the only surprising part is that *Stereonychus* is the sister groups of *Cleopus* and *Cionus* and not in between the branches of *Cleopus* and *Cionus* as it is in the phylogeny of Caldara (2001). The resolution of the tribus Mecinini is better and the individuals of one species are clustering fairly well together. The main prediction of this phylogeny is that the genus *Rhinusa* is not monophyletic. The *Rhinusa* species on *Scrophularia* and *Verbascum* are clustering with *Gymnetron* and the ones from *Linaria* with the *Mecinus*. Another insight is that the *Gymnetron* from *Plantago* are ranged just within *Mecinus*.

The phylogeny based on CO I/II (including the $tRNA^{leu}$) shows a more differentiated picture (Fig. 1.2). *Cionus hortulanus* is a clear species, and the cluster mixes up one individual taken from *Verbascum* (see *C. hortulanus* 02) into the cluster of weevils from *Verbascum*; thus there is no cryptic speciation to be seen here. All other *Cionus* individuals are differentiated into their species. The basal species is *C. alauda* followed by *C. scrophulariae* and *C. schultzei*. And *Stereonychus* is together with *Cleopus* a sister group of *Cionus*. Furthermore, the Mecinini the picture is nearly the same as in the EF1-α based phylogeny.

The combined phylogeny shows as expected the most differentiated result of these three phylogenies (Fig. 1.3). *Mecinus* and the *Gymnetron* from *Plantago* are clustering together. So Caldara (2001) is right and these formerly *Gymnetron* species belong to the genus *Mecinus*. This is an important result because the entomologists had no common nomenclature for these weevils since 2001. Another branch includes the *Gymnetron* species from *Veronica* and

Rhinusa from *Verbascum* and *Scrophularia*. A third branch is represented by the *Rhinusa* species from *Linaria*. Here is another crucial result: the genus *Rhinusa* is not monophyletic because the *Rhinusa* from *Linaria* is a sister group of the other *Rhinusa* and *Gymnetron* species. So far this was not discovered or mentioned by any author, and depicts an utterly new concept. This concept is very verisimilar because of the high support of the branches of the tree. It is also very likely, because the species with the same host plant are clustering together, which means that there were only a few host plant switches needed for this mentioned phylogeny. We do not now the basal species of this tree because the resolution is not good enough here, but we do know the three branches of the represented Mecinini tree. If we take the *Gymnetron* on *Veronica* and *R. bipustulatum* from *Scrophularia* as own branches, every branch has its own host plant. Only in one branch mixing up can be found and that are the *Mecinus* species *M. janthinus* and *M. heydeni* from *Linaria* which occur on the branch only with *Plantago* feeding species. To sum it up, it can be said that the host plant is a good phylogenetic marker for the Mecinini. There are only two species-clusters which cannot be solved: one is *Gymnetron beccabungae* with *G. veronicae* and the other is *Rhinusa netum* and *R. collinum*. It is highly likely that this is because of a short time period since the speciation events took place, possibly so short that there was no species specific gene differentiation in CO I/II and EF1-α so far.

The most important result is that *Rhinusa* is not monophyletic. The evidence is fairly obvious and even better if we take the host plants into considartion. The congruence even expands if we claim that *Verbascum*, *Veronica* and *Scrophularia* belong to the Scrophulariaceae and *Linaria* to the Plantaginaceae (Olmstead et al. 2001, Albach et al. 2005). Consequently, we have three branches: a *Mecinus* branch, a *Rhinusa*/*Gymnetron* branch with Scrophulariaceae as host plants and one *Rhinusa* branch with Plantaginaceae as host plants. In light of this we would favour taxonomy with all those Mecinini feeding on Scrophulariaceae belonging to the genus *Gymnetron*. The ones feeding on *Linaria* would remain in *Rhinusa*. The four or ten Rhinusa groups mentioned by Reitter (1916) respectively Caldara (2008) could not be verified here because of the small species sampling size. But we can now rebut Reitter's groups which were mixed up in their host plant choices.

The tested *Gymnetron* on *Plantago* are belonging to *Mecinus* just as Caldara mentioned (2001). We do not know, if the phylogeny of the Mecinini is (*Mecinus* (*Gymnetron* (*Rhinusa* (…)))) (Caldara 2001) or (*Mecinus* (*Rhinusa* (*Gymnetron* (…)))) (Caldara 2008) is the right one, because our combined phylogeny does not show the wanted resolution in the basal area.

In the CO phylogeny *Mecinus* and *Rhinusa*/*Gymnetron* are sister groups, but in the EF1-α phylogeny *Mecinus* is not basal for the Mecinini as mentioned by Caldara (2001, 2008).

Our phylogeny of the tribus Cionini is not the same as Caldara's (2001). *Stereonychus* is in two trees together with *Cleopus* in a sister group relationship with *Cionus*. The ancestral host plant of at least *Cionus* seems to be *Scrophularia*, because the three basal species are feeding exclusively on it. The species feeding on *Verbascum* and *Scrophularia* are mixed up, therefore it has to be mentioned that many host plant switches occurred inside this genus. Consequently, the host plant is not a good phylogenetic marker within *Cionus* because it switches too often between *Verbascum* and *Scrophularia*. This result is by every means worth mentioning, as this happens not very often.

Furthermore, our knowledge about some certain species increases. For instance, there seems to be no evidence for a cryptic speciation in *Cionus hortulanus*. But it is noteworthy that an individual from northern Germany is clustering with two individuals from Greece (all three from *Verbascum*) instead of other ones from Germany. Therefore, for a better understanding it is necessary to do ecological experiments, as well.

Quite interesting cases are *Cionus schultzei* and *Rhinusa tetrum* ab. *plagiellum*. *Cionus schultzei* was indicated in the work of Wingelmüller (1937) as a possible race of *Cionus hortulanus*, as our data show it is clearly a correct species which is positioned far away from *C. hortulanus* in the phylogeny. *Rhinusa tetrum* ab. *plagiellum* is mentioned by Reitter (1916) as an aberration described by GYLLENHAL, but our data point out that is an own species already described by ROSENSCHÖLD as *Rhinusa fuscescens*.

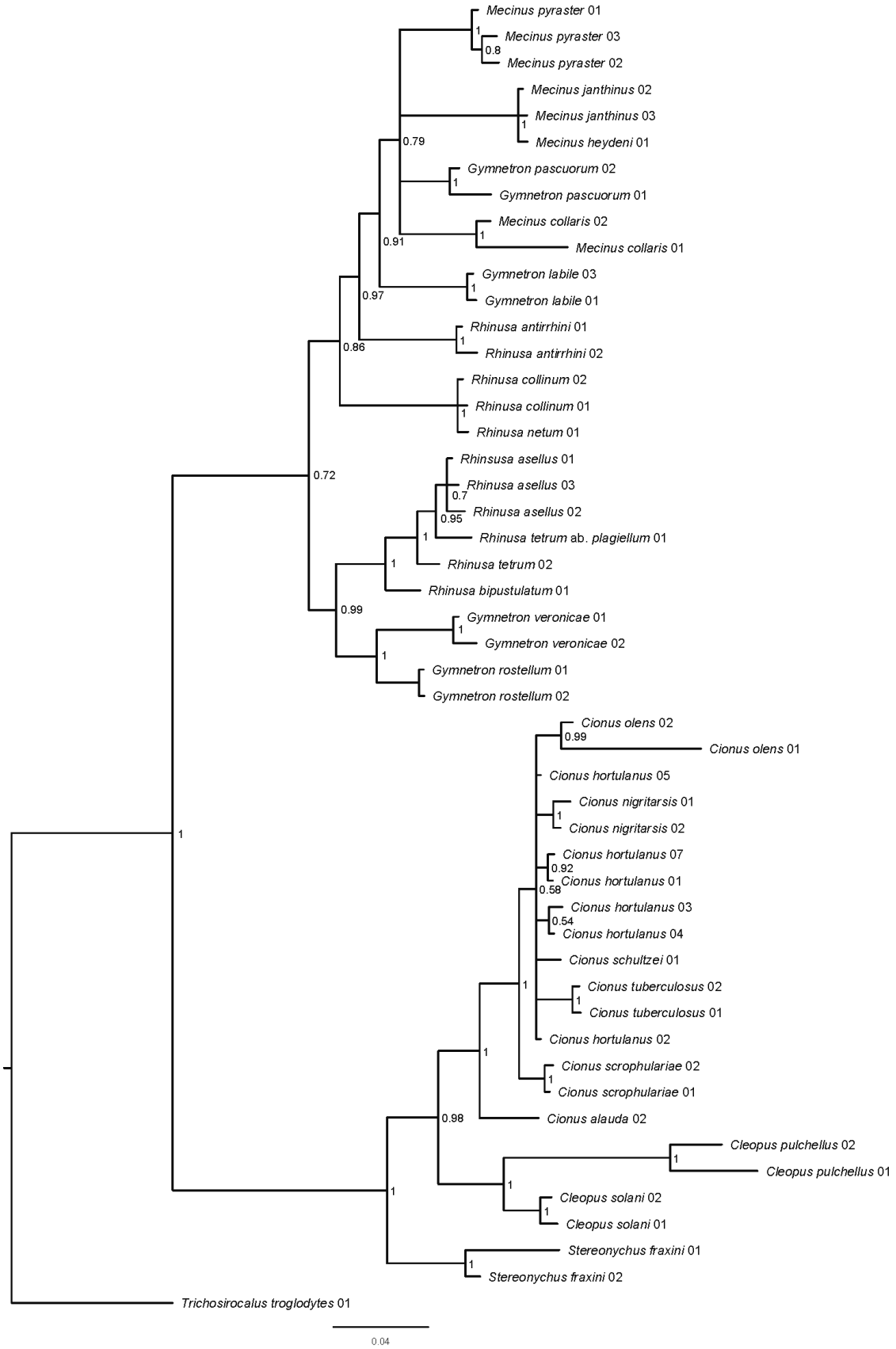

Figure 1.1 Phylogenetic reconstruction derived from Bayesian algorithms based on the nuclear EF1-α gene (~650bp). The posterior probabilities are given.

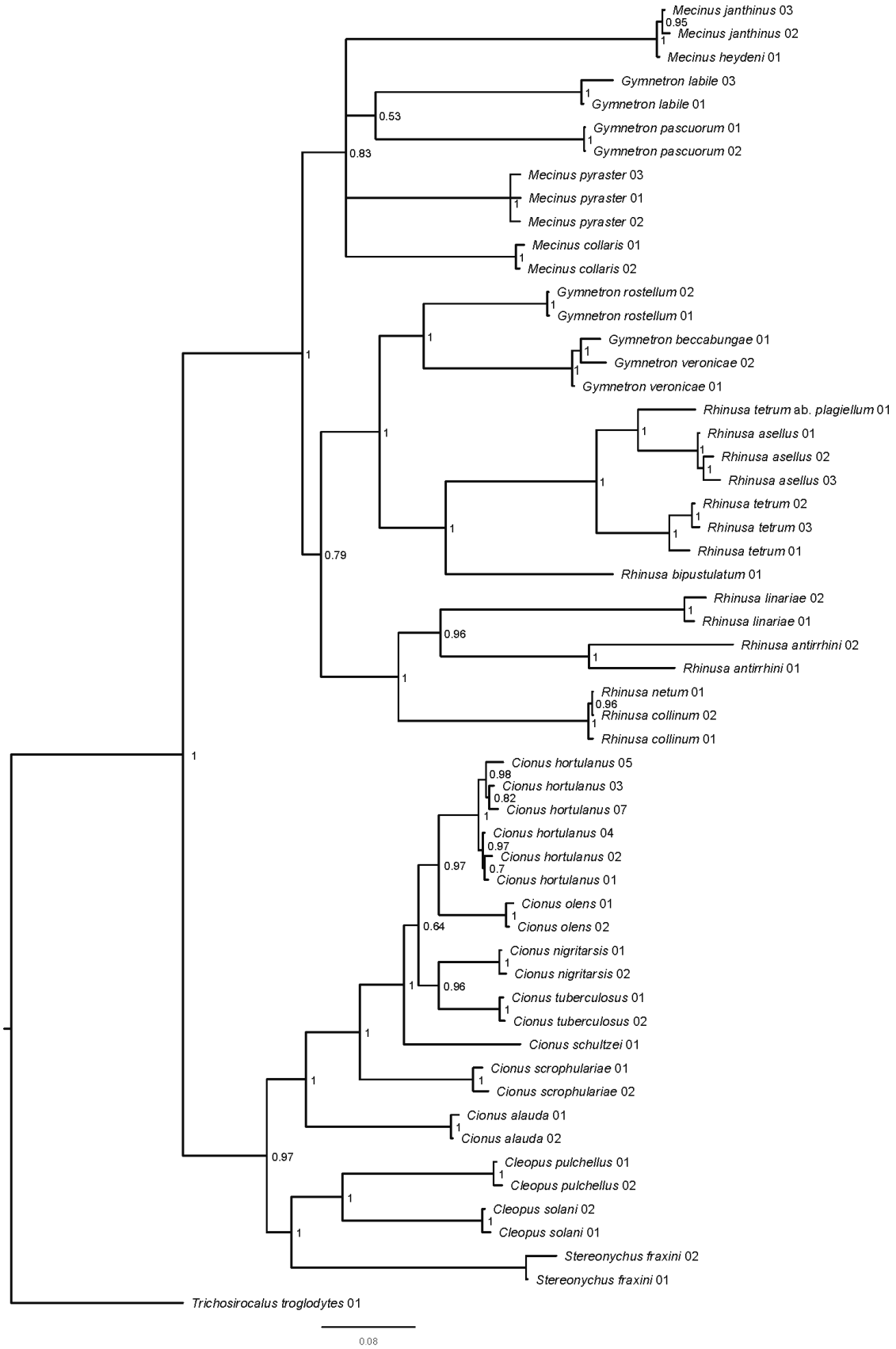

Figure 1.2 Phylogram estimated using Bayesian algorithms based on mitochondrial CO I/II (mostly ~1630bp). Posterior probability values are given.

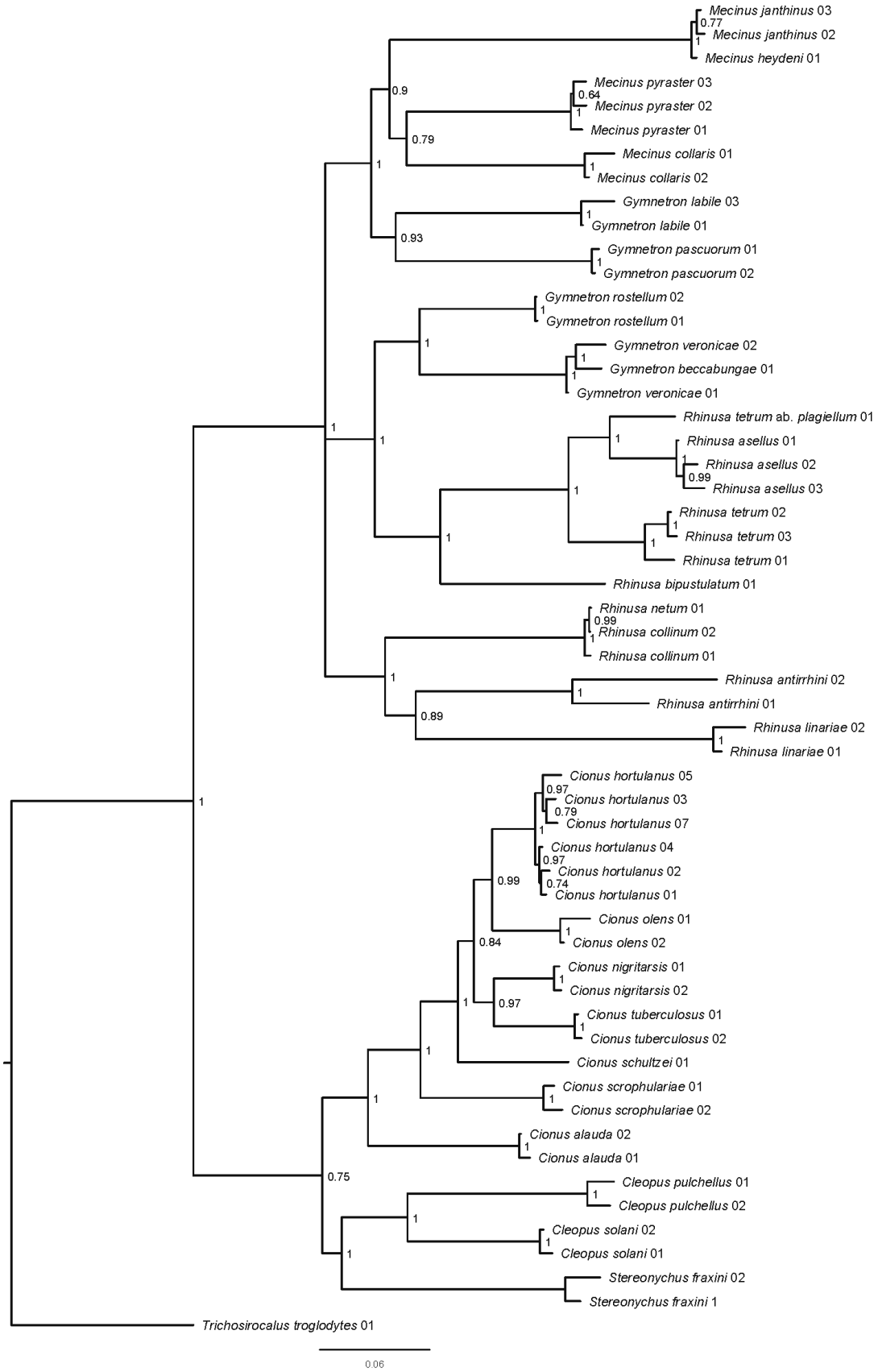

Figure 1.3 Phylogeny of the Mecininae derived from Bayesian algorithms based on a combined CO I/II (mostly ~1630bp) and EF1-α (~650bp) analyses. The posterior probabilities of the nodes are given.

Chapter 2

Differing patterns of sequestration of iridoid glycosides in the Mecininae (Coleoptera, Curculionidae)

Abstract

We analysed several species of the weevil family Mecininae (Coleoptera, Curculionidae), that all feed on iridoid glycoside containing plants of the Plantaginaceae to investigate whether these beetles sequester these deterrent substances from their host plants. Within the Mecininae two genera were found to sequester aucubin and catalpol. They are both members of the tribe Cionini: *Cionus* CLAIRVILLE AND SCHELLENBERG and *Cleopus* DEJEAN. Three of the analysed genera, *Mecinus* GERMAR, *Gymnetron* SCHÖNHERR and *Rhinusa* STEPHENS of the tribe Mecinini, do not sequester irioid glycosides although they are present in similar concentrations in their food plants. However, in contrast to the Cionini the latter three genera have a hidden lifestyle, so that their need for antipredator defense may be lower. Both *Cionus* and *Cleopus*, sequester catalpol with a higher efficiency than aucubin. In species feeding on *Scrophularia*, however, the aucubin concentration is higher while in beetles on *Verbascum* catalpol is dominating, there are only few exceptions. This pattern can even be detected in the only species living on both plants, *Cionus hortulanus*. Remarkably, the sequestration pattern of the beetles on *Verbascum* does not follow the concentrations in the plant, since *Verbascum* just like *Scrophularia* has a higher concentration of aucubin than catalpol. A comparison with a molecular phylogeny of the group (Chapter 1) shows that the ability to sequester IGs must have a single origin at the base of the sister genera *Cionus* and *Cleopus*. However, in contrast to *Cionus* species, *Cleopus* species only sequester catalpol.

Introduction

A multitude of plant species in over 50 families of the Lamiales have the ability to produce iridoid glycosides (IGs), a group of monoterpenoid secondary compounds (El-Naggar & Beal

1980, Boros & Stermitz 1991, Wink 2003). These glycosides act as deterrents against herbivores, because of their unpalatability (Bowers & Puttick, 1986) and their ability to denature proteins and DNA (Konno et al. 1999, Kim et al. 2000). However, in parallel with their widespread occurrence in plants, the ability to sequester and accumulate them as defence compounds has evolved repeatedly in specialized herbivorous insects, too (Bowers 1991, Dobler 2001, Opitz & Müller 2009). Numerous studies could show that sequestered IGs effectively protect insects against invertebrate (e.g. Nishida & Fukami 1989, Stamp 1992, de la Fuente et al. 1995, Nishida 1995, Dyer & Bowers 1996, Bowers & Stamp 1997, Strohmeyer et al. 1998, Theoderatus & Bowers 1999, Nieminen et al. 2003, Baden & Dobler 2009, Opitz et al. 2010, Baden et al. 2011) as well as vertebrate predators (e.g. Bowers 1980, Bowers 1981, Bowers & Farley 1990) and pathogens (Rombouts & Links 1956, Ishiguro et al. 1982, van der Sluis et al. 1983, Davini et al. 1986, Stermitz 1988, Baden & Dobler, 2009).

Sequestration of the IGs aucubin and catalpol has not only been described for aposematic Lepidoptera and Hymenoptera but also in the Coleoptera for several species of the flea beetle genus *Longitarsus* (Chrysomelidae, Alticinae) which although small and rather cryptic in appearance sequester IGs up to 2 % of their dry weight (Willinger & Dobler 2001). Even this low amount shows benefits against invertebrate predators and microorganisms (Baden & Dobler 2009, Baden et al. 2011). These IG sequestering beetles live oligophagously on IGs containing host plants, for instance on species of the genera *Plantago*, *Verbascum* and *Scrophularia*. These same plants are also used as hosts by other chrysomelid and curculionid beetles. Five genera of the weevil family Meciniнae use almost exclusively IG containing plants with most of them being specialized on a single plant genus oligophagously – often the same ones as *Longitarsus* species. Meciniнae are 1.3 to 5.6 mm in length (Freude et al. 1983) and cryptic in appearance. Several species mimic bird droppings or seeds that have been left by seed eaters (fig. 2.1). Nevertheless a cryptic appearance does not preclude sequestration since the benefit of sequestered IGs may lie in the deterrence of non-visually oriented invertebrate predators or protection against pathogens (de la Fuente et al. 1995, Dyer & Bowers 1996, Baden & Dobler 2009, Baden et al. 2011).

Meciniнae on IG plants comprise the genera *Mecinus* GERMAR (with species on *Linaria* and *Plantago*), *Gymnetron* SCHÖNHERR (on *Veronica*) and *Rhinusa* STEPHENS (on *Verbascum*, *Scrophularia* and *Linaria*) all belonging to the tribe Mecinini, and *Cleopus* DEJEAN (on *Verbascum* and *Scrophularia*) and *Cionus* CLAIRVILLE AND SCHELLENBERG (also on *Verbascum* and *Scrophularia*) of the tribe Cionini. The systematic position of the tribes and

the delimitation of the genera is still unclear. The morphological analyses of Caldara (2001) supports the assumption that both tribes belong to the same subfamily, yet the molecular analyses of McKenna et al. (2009) did not confirm this. However, this new molecular phylogeny of the weevils has been criticised by Franz & Engel (2010), among other things because of the low taxon sampling. A more detailed molecular phylogeny of the genera mentioned above has been presented (Chapter 1). To avoid confusion we use the genus *Rhinusa* as it was defined by Caldara (2001) even though *Rhinusa* was resolved as a paraphyletic group in our phylogeny and was divided in two branches that coincide with differing host use of the beetles (Chapter 1). However, a taxonomic revision that incorporates these results still needs to be undertaken. In the case of *Gymnetron pascuorum* Caldara's revision as well as our molecular phylogeny supports its placement in the genus *Mecinus*, a taxonomic rearrangement that we adopt in this paper.

The aim of the present work was to investigate whether these five weevil genera have the ability to sequester iridoid glycosides present in their host plants. While in most cases aucubin and catalpol are the dominant IGs in the plants and are also the ones that are usually sequestered by specialist insects (Bowers 1991, Opitz et al. 2009, Dobler et al. 2011), we analysed for the iridoid glycoside antirrhinoside in the species living on *Linaria*, because this plant genus does not produce aucubin or catalpol. Jamieson & Bowers (2010) have already shown that *Calophasia lunula* (Lepidoptera, Noctuidae) sequesters antirrhinoside but that *Mecinus janthinus* does not. We tested the following twelve species that feed on the host plants indicated in brackets: *Mecinus collaris* (*Plantago maritima*), *M. pyraster* (*P. lanceolata*), *M. pascuorum* (*P. lanceolata*), *Rhinusa tetrum* (*Verbascum nigrum), R. antirrhini* (*Linaria vulgaris*), *Cleopus pulchellus* (*Scrophularia nodosa*), *Cl. solani* (*V. nigrum*), *Cionus alauda* (*S. nodosa*), *C. scrophulariae*

Figure 2.1 *Cionus alauda* on *Scrophularia nodosa*

(*S. nodosa*), *C. tuberculosus* (*S. nodosa*), *C. nigritarsis* (*V. nigrum*), *C. olens* (*Verbascum* spec.) and *C. hortulanus* (*V. nigrum* and *S. nodosa*), as well as the host plants of one location. To test whether the species differ in their sequestration rates depending on their host plants respectively populations, we tested two populations of each weevil species where possible and for *C. hortulanus*, the only species living on two host plants of different genera (*Scrophularia* and *Verbascum*), two populations from each plant species. The samples were analysed by gas chromatography and mass spectrometry for the presence and quantity of the IGs aucubin, catalpol and antirrhinoside. Based on our molecular phylogeny (Chapter 1) we discuss how many times and when sequestration has evolved in the evolution of these weevils and hypothesize which factors in the life history of the beetles may be supportive of this adaptation. The data are also compared to the situation observed in *Longitarsus* (Willinger & Dobler 2001, Dobler 2001).

Materials & Methods

Weevil Collection and Extraction

Weevils were collected from their host plants with the help of an aspirator at the collecting points given in table 2.1. All weevils were starved for two days before freezing to make sure that the plant material was emptied from their guts. Plant samples were taken from the field and the above ground parts were frozen for the chemical analysis.

Extraction of the insects basically followed the protocol of Gardner & Stermitz (1988) as adapted for flea beetles by Willinger & Dobler (2001). Samples were freeze dried and weighed. Plant samples were ground to a fine powder with sea sand, the beetles without it. Samples were then extracted overnight in a small amount of methanol (2-5 ml). The extracts were filtered and washed three times with 1 ml (beetle samples) or 2 ml (plant samples), respectively, of methanol the next day. The extracts were transferred into test tubes and evaporated at room temperature under a constant pressure air flow. Thereafter the residues were redissolved in 5 ml H_20 and washed three times with 2 ml $CHCl_3$ in a separatory funnel. After freeze drying of the watery phase, the residues were redissolved with 2 ml methanol mixed with 0.25 mg/ml Phenyl-β-D-glucopyranosid (PBG) and stored frozen. The PBG served as internal standard to quantify the IG content of the extracts.

Table 2.1 Collecting points and dates of the weevil populations and plant sample. BW = Baden-Württemberg, HH = Hamburg, NDS = Lower Saxony, SH= Schleswig-Holstein, UK = United Kingdom

Species sample	Host plant	Location	Date	Collector
Cionus alauda 01	*S. nodosa*	Sachsenwald, SH	10th May 2008	Baden
C. alauda 02	*S. nodosa*	Brunstorf, SH	17th May 2007	Baden
C. hortulanus 01	*S. nodosa*	Sachsenwald, SH	10th May 2008	Baden
C. hortulanus 02	*S. nodosa*	Dassendorf, SH	13th May 2007	Jäckel
C. hortulanus 03	*V. nigrum*	Pratjau, SH	15th July 2007	Baden, Jäckel
C. hortulanus 04	*V. nigrum*	Bröthen, SH	5th Aug. 2007	Baden
C. nigritarsis 01	*V. nigrum*	Geesthacht, SH	4th Aug. 2007	Baden
C. nigritarsis 02	*V. nigrum*	Bröthen, SH	16th July 2007	Baden
C. olens 01	*V. spec.*	Waghäusel, BW	14th July 2008	Baden, Jäckel
C. olens 02	*V. spec.*	Waghäusel, BW	14th July 2008	Baden, Jäckel
C. scrophulariae 01	*S. nodosa*	Treia, SH	11th May 2008	Baden, Jäckel
C. tuberculosus 01	*S. nodosa*	Büchig, BW	18th June 2007	Baden, Jäckel
C. tuberculosus 02	*S. nodosa*	Sachsenwald, SH	24th June 2007	Baden
Cleopus pulchellus 01	*S. nodosa*	Sachsenwald, SH	10th May 2008	Baden
C. solani 01	*V. nigrum*	Freiburg, BW	9th April 2007	Dobler
C. solani 02	*V. nigrum*	Waghäusel, BW	14th June 2007	Baden, Jäckel
Mecinus collaris 01	*P. maritimus*	St.Peter-Ording SH	2nd May 2007	Dobler
M. collaris 02	*P. maritimus*	Sussex, UK	August 2007	Laux
M. pascuorum 01	*P. lanceolata*	Wulksfelde, SH	24th May 2008	Baden
M. pascuorum 02	*P. lanceolata*	Öjendorf, HH	5th May 2007	Baden
M. pyraster 01	*P. lanceolata*	Tümlauer Koog SH	6th May 2008	Baden, Laux
Rhinusa antirrhini 01	*L. vulgaris*	Pevestorf, NDS	7th, Sept. 2007	Baden
R. antirrhini 02	*L. vulgaris*	Bröthen, SH	16th July 2007	Baden
R. tetrum 01	*V. nigrum*	Geesthacht, SH	6th Aug. 2008	Baden
V. nigrum 01	head, buds	Langenlehsten, SH	31st Aug. 2008	Baden
V. nigrum 02	Leaves	Bröthen, SH	3rd Aug. 2008	Baden
L. vulgaris 01	whole plant	Pevestorf, NDS	6th Sept. 2008	Baden
P. lanceolata 01	whole plant	Tümlauer Koog SH	6th May 2008	Baden
P. maritima 01	whole plant	St.Peter-Ording SH	6th May 2008	Baden
S. nodosa 01	Leaves	Sachsenwald, SH	10th May 2008	Baden

Chemical Analyses

For IG quantification a gas chromatograph (GC) equipped with PTV-Injector and FID was used (Hewlett Packard 6890 Plus). Before injection, the extracts were silylised with a mixture of BSA, TMCS and TMSI (3:2:3) (SUPELCO, Sigma-Aldrich CO). Fifty µl of the weevils' extracts were evaporated at room temperature under a constant pressure air flow, redissolved with 25 µl of the silylation reagent and incubated immediately in an oven at 70 °C for 30 min. One µl of the silylised sample was diluted in 50 µl iso-octan, and 1.5 µl of the mixture were injected into the GC. The oven program was: start temperature 40 °C for 1 min, heating by 30 °C/min up to 160 °C and further in 5°C steps up to 300 °C. The injector program started at 50 °C and heated up to 300 °C after 0.2 sec. The FID-detector had a flow rate of 40 ml/min H_2, 450 ml/min pressurised air and 50 ml/min N_2.

For the detection of the IGs a mass spectrometer (VG 70 SE, VG Analytical, UK) connected to a GC (Hewlett Packard 6890 Plus; with a PTV-Injector) was used with the same programs as detailed above. The IGs were identified by comparisons of the retention time and mass spectra with pure IGs. Quantities were calculated by comparing the IG peaks with the PBG peak of known, concentration.

Results

A total of 24 beetle samples of 13 species and 6 plant samples of 5 species were analysed for their aucubin, catalpol and antirrhinoside content (table 2.2). In all plant samples the IG aucubin could be detected, whereas catalpol was missing in *P. maritima* and *L. vulgaris* and antirrhinoside was only detectable in *L. vulgaris*. The highest concentration of IGs could be found in *L. vulgaris*, due to a high proportion of antirrhinoside. The highest concentration of aucubin and catalpol was found in *P. lanceolata*. In *V. nigrum* a comparison of the IG concentration in head and buds (the feeding site of all life stages of *Cionus* and *Rhinusa tetrum* larvae) with the one of the leaves (the feeding site of *Cionus* and *Cleopus solani*) did not reveal any differences. The percentage of IGs per dry weight of *V. nigrum* and *S. nodosa* was comparable.

Table 2.2 IG concentrations in plant (underlined) and corresponding beetle samples. The number of beetles extracted and the dry weight (DW) are given as well as the percentage of aucubin, catalpol and antirrhinoside of the sample's dry weight. n.d. = not detectable

Plant and beetle Species	**Sample size / DW (mg)**	**Aucubin (%) DW**	**Catalpol (%) DW**	**Antirrhinoside (%) DW**
L. vulgaris 01	- / 681	0.01	n.d.	0.44
R. antirrhini 01	15 / 25.7	n.d.	n.d.	n.d.
R. antirrhini 02	15 / 14.3	n.d.	n.d.	n.d.
P. lanceolata 01	- / 1306	0.11	0.10	n.d.
M. pascuorum 01	40 / 13.8	n.d.	n.d.	n.d.
M. pascuorum 02	40 / 17.1	n.d.	n.d.	n.d.
M. pyraster 01	10 / 17.7	n.d.	n.d.	n.d.
P. maritima 01	- / 651	0.04	n.d.	n.d.
M, collaris 01	10 / 12.6	n.d.	n.d.	n.d.
M. collaris 02	8 / 12.6	n.d.	n.d.	n.d.
S. nodosa 01	- / 416	0.15	0.01	n.d.
C. alauda 01	10 / 17.9	0.55	0.14	n.d.
C. alauda 02	10 / 17.5	1.26	0.38	n.d.
C. hortulanus 01	10 / 45.8	0.36	0.29	n.d.
C. hortulanus 02	10 / 61.8	0.48	0.17	n.d.
C. scrophulariae 01	7 / 53.0	0.22	3.13	n.d.
C. tuberculosus 01	10 / 51.3	0.42	0.19	n.d.
C. tuberculosus 02	10 / 20.9	1.92	0.11	n.d.
Cl. pulchellus 01	10 / 7.2	n.d.	2.68	n.d.
V. nigrum 01 (buds)	- / 775	0.11	0.01	n.d.
V. nigrum 02 (leaves)	- / 778	0.11	0.01	n.d.
C. hortulanus 03	10 / 53.6	0.88	1.62	n.d.
C. hortulanus 04	8 / 27.9	0.8	6.14	n.d.
C. nigritarsis 01	10 / 32.5	0.40	1.53	n.d.
C. nigritarsis 02	10 / 25.6	0.75	0.63	n.d.
C. olens 01	10 / 41.3	0.08	2.23	n.d.
C. olens 02	10 / 50.1	0.03	0.65	n.d.
Cl. solani 01	15 / 17.2	n.d.	0.34	n.d.
Cl. solani 02	12 / 13.8	n.d.	0.57	n.d.
R. tetrum 01	10 / 16.0	n.d.	n.d.	n.d.

All tested weevils of the tribus Cionini did sequester IGs, but none of the tested Mecinini did. In the Cionini the summed concentrations of aucubin and catalpol detected in the beetles was between 3 and 57 times higher than in the plant. However, in the genus *Cleopus* only catalpol was sequestered but no aucubin, while *Cionus* species sequestered both IGs, but at different ratios. Even though the IG content of their host plants did not differ significantly the concentrations of aucubin and catalpol recovered from the beetles varied dramatically – e.g. in one sample of *C. tuberculosus* collected from *S. nodosa* the ratio of 17-times more aucubin than catalpol sequestered from *S. nodosa* was close to the ratio of 15:1 detected in the plant while *C. scrophulariae* from the same plant had 14-times more catalpol than aucubin. In general, all species (with the exception of the one sample of *C. tuberculosus* mentioned above) preferentially sequestered catalpol and shifted the IG ratio towards this compound. This was even more pronounced in the species on *V. nigrum* where the ratio of 11:1 of aucubin:catalpol was reverted to a 27-times excess of catalpol over aucubin in one sample of *C. olens*. It is striking that the amount of aucubin retrieved from beetle samples collected from *S. nodosa* is higher with a maximum of 1.92 % of dry weight (DW) in *C. hortulanus* while in beetles from *V. nigrum* catalpol is dominating much more strongly with a maximum of 6.14% DW, i.e. 57-times the concentration encountered in the plants, detected in *C. hortulanus*. The latter species, which is the only one feeding on both plants, documents convincingly that the difference in sequestration efficiency must be caused by the plant and not by the beetle.

Discussion

Perhaps the most fascinating aspects of the present study is the contrast of well-developed sequestration in the genera of the Cionini, *Cionus* and *Cleopus*, compared to the total lack of IG sequestration in the closely related genera of the Mecinini, *Gymnetron*, *Mecinus* and *Rhinusa*. All species in these four genera are specialized herbivores that exclusively feed on IG containing plants in the genera *Linaria*, *Plantago*, *Scrophularia* and *Verbascum.* These plants contain the IGs aucubin, catalpol, and antirrhinoside, which are sequestered as antipredator defense by many other herbivores (e.g. Boros et al. 1991; Bowers & Puttick 1986, Bowers 1983, Bowers 1984, Bowers 1988, Willinger & Dobler 2001). So far, the ability to sequester IGs seemed to correlate with the degree of specialization of the herbivores and was usually only detected in specialists on IG plants but rarely in species with a wide host spectrum (Bowers & Stamp 1997, Dobler 2001). In contrast, in the genera *Gymnetron*,

Mecinus and *Rhinusa* we face herbivorous beetles that spend their whole life cycle on IG plants yet do not make use of the potentially defensive compounds that their hosts offer.

A closer look at the life history and ecology of the two weevil tribes may provide an explanation for the lack of sequestration in the Mecinini. In contrast to the Cionini, which have an exposed life style as adults and as larvae, the Mecinini have endophagous larvae and a cryptic life style as adults, too.

In *Rhinusa* and *Mecinus* the larvae mine or gall inside the plant's stem, roots, buds, or seeds, and the adults also spend a long time inside the plant tissue, since most overwinter in the plant, and only shortly emerge in the next summer to reproduce (Scherf 1964). Because of their cryptic lifestyle we know little about these genera. Much more is known about the genera *Cleopus* and *Cionus* which have an exophagous lifecycle. Their legless larvae have a slug like morphology, with a body covered by a viscous slime layer and prolegs similar to those of the Lepidoptera and Hymenoptera (Prell 1925). They pupate freely on the plant, in a cocoon made of a hardened amber colored secretion. In species on *Scrophularia* this cocoon has similarities with the buds (Scherf 1964, Räther 1989). Of the two species of the genus *Cleopus* in Germany *C. pulchellus* lives on *Scrophularia* and *C. solani* on *Verbascum*. The slug-like larvae of the first species feed on the underside of the leaves and build their cocoons on the stem or on the leaves (Räther 1989). The species of the genus *Cionus* live on *Scrophularia* and *Verbascum*, too. The life histories of the species investigated here differ little, they all have an ectophagous lifecycle with slug-like larvae and only the eggs are placed inside the plant (Scherf 1964, Räther 1989). In *C. olens* a new adaptation appears: eggs, larvae and pupae are masked with chopped trichomes of *Verbascum* leaves. The slime layer seems to be optional, since it is only formed when the larva is not masked by trichomes (Scherf 1964).

This comparison of the life histories of the two tribes can explain why the Cionini do sequester IGs and the Mecinini do not. For the Cionini sequestration seems to be a key adaptation to an ectophagous life cycle. The Mecinini have no other known chemical defence, yet they do not need one, because their hidden life style inside the plants is another way of effectively protecting them against predators. Chemical ecology in these weevils thus correlates well with the ecology and life history of the species. On the other hand, the data presented here clearly demonstrate that sequestration of IGs is not a passive process. Rather, uptake and storage of IGs in the body need specific adaptations whose evolution are prerequisites for sequestration (Bowers 1992, Dobler et al. 2011).

Comparing the concentrations of IGs detected in the Cionini with those of other IG sequestering insects reveals that the amount of sequestered IGs in the Cionini is quite high. For example in comparison to the chrysomelid genus *Longitarsus* that is feeding on the same plant genera. The highest IG concentration in these beetles was found in *L. nigrofasciatus* from *V. thapsus* with 2.23 % DW, 0.82 % of which was catalpol (Willinger & Dobler 2001). This sample had a 16 times higher catalpol level than the plant – in the present study both *Cionus* and *Cleopus* species accumulate catalpol to a concentrations hundreds of times over the one in the host plant with a maximum of 6.94 % DW in *C. hortulanus* compared to 0.01 % in its host *V. nigrum*. It is remarkable that in *Cionus* the affinity for catalpol is very high and most striking in species on *Verbascum*. Several lepidopteran species in a similar way preferentially sequester catalpol compared to aucubin (Bowers & Puttick 1986, Belofsky et al. 1989, Bowers & Collinge 1992). But the percentage of the sequestered IGs of lepidopteran's DW is normally higher than of the *Longitarsus* beetles and the tested weevils have an average position. Some examples of the IG content will be given: Euphydryas anicia sequesters up to 9 % DW, but the mean is around 4 % (Gardner & Stermitz 1988), the larvae of Ceratomia catalpa even up to 15 % DW (Bowers & Collinge 1992) and the highest measured values is up to 20 % DW in the larvae of Junonia coenia – but the normal content is far less (Bowers & Collinge 1992, Bowers & Stamp 1997, Lampert & Bowers 2010). The two *Cleopus* species investigated here represent an even more extreme case, since aucubin was not taken up at all, but only catalpol was detected in the beetles. This example is the first one for beetles. One remarkable aspect of the *Cleopus* data is that here *Cleopus pulchellus* sequesters up to 5- 8 times more catalpol than *Cleopus solani* on *Verbascum*.

The benefits of the IG sequestration are described in many works. For other beetles (*Longitarsus*) it has been discovered that the IGs are antimicrobial against bacteria which synthesise their own β-glucosidase as *Bacillus thuringiensis* and against chilopod predators (Baden & Dobler 2009) as well as ant species (Baden et al. 2011). Several Lepidoptera species get protected against wolf spiders (Theodoratus & Bowers 1999), spring spiders (Strohmeyer et al. 1998), several ant species (de la Fuente et al. 1995, Dyer & Bowers 1996), bugs as *Podisus maculiventris* (Bowers & Stamp 1997) and several other invertebrates as described in other works as well. Though it is highly likely that the Cionini gets an anti-predatorian and antimicrobial effect as well, this might be the most prominent adaptation for their ectophagous lifecycle.

Most of the time the following rule for *Cionus* can be adapted: species on *Scrophularia* sequester more aucubin and species on *Verbascum* more catalpol – with the exceptions *C. scrophulariae* and one population of *C. nigritarsis*. This occurs even though the IG concentrations in the plants are almost the same. This correlation can even be found within the species *C. hortulanus*. The cause for the rather high differences in the ratios between and within the species can be of multitude nature. The first can be the fact that the plants have a change in their IG content over the year (Bowers et al. 1992, Bowers & Stamp 1993, Fuchs & Bowers 2004). If a different IG content of individual plants or a difference in the genetics of the weevil populations play a role is still unclear.

Most likely the differences in sequestered catalpol versus aucubin are caused by differing uptake characteristics of carriers across the gut membrane, however, the samples from *Verbascum* also document another phenomenon: here the amount of catalpol retrieved is in several species so high that it seems more likely that other compounds were converted into catalpol as was e.g. the case in caterpillars of *E. anicia* or in sawfly larvae on *P. lanceolata* (Gardner & Stermitz 1988, Opitz et al. 2010). That other undetected iridoid glycosides in *V. nigrum* may be the solution to this riddle and not just differences in the physiology of the beetle species is best documented by the comparison of *C. hortulanus* from its two host plants: when feeding on *S. nodosa* the beetles had a higher concentration of aucubin while on *V. nigrum* catalpol was higher despite similar IG concentrations in the plants. Looking on the phylogeny of *C. hortulanus* it can be seen that the separation of populations from *Verbascum* and *Scrophularia* is incomplete – therefore an evolving ability in a different sequestration can be declined here. Otherwise, the differentiation in *C. hortulanus* could be so young that we have not detected them in the done molecular phylogeny yet.

Mapping host use and sequestration ability on our molecular phylogeny of the Mecininae (Chapter 1.) reveals a simple pattern: all species in the Cionini shown here to sequester IGs form a monophyletic group and are well separated from the species in the monophyletic Mecinini that do not sequester IGs. The ability for sequestration has thus been acquired once at the base of the genera *Cionus* and *Cleopus* - a third more basal genus in the Cionini living on *Fraxinus* was not available in sufficient numbers to be included in the chemical analyses and lives on a plant not known to contain the analysed IGs. As for the differential ability to sequester aucubin versus catalpol no traces of a stepwise evolution can be deduced from the phylogeny, rather frequent host switches between *Verbascum* and *Scrophularia* are the rule and resulted in similar patterns of sequestration. To further investigate the differences in

sequestration between beetles on both plants, *C. hortulanus*, the only species feeding on both taxa represents an ideal, experimentally accessible system.

Chapter 3

Differing patterns of sequestration of iridoid glycosides in the species *Cionus hortulanus* (FOURCROY, 1785) (Coleoptera, Curculionidae)

Abstract

Weevils of the genus *Cionus* (Curculionidae, Mecininae, tribus Cionini) have recently been shown to sequester the iridoid glycosides (IG) aucubin and catalpol from a variety of host plants. In Middle Europe one species of the genus is especially interesting, because it feeds on two hosts from different genera: *Scrophularia nodosa* and *Verbascum nigrum*. Depending on the local host plant they sequester either more aucubin or more catalpol – in the same ranges as other monophagous *Cionus* species do (Chapter 2). To investigate whether this phenomenon is caused by genetic differences between *C. hortulanus* populations or rather by the availability of IGs in the host plant we collected *C. hortulanus* from *S. nodosa* in the field and reared them either on *S. nodosa* or on *V. nigrum*. We sampled specimens of all life stages of the weevils together with the corresponding plant samples and analysed them for their IG content. The analyses show that differences in the IG concentrations are specific for the host plant upon which the weevils developed. The distribution of aucubin and catalpol resembles the one found in other species of the Cionini (Chapter 2). Thus individuals from *Scrophularia* have more aucubin than catalpol while the situation is reversed in specimens from *Verbascum*. This pattern can neither be explained by the plants' IG content nor by differences between the weevils' sexes. It seems, as if the concentrations of aucubin of the weevils correlates with the amount in the plants. Furthermore, the concentrations of IGs sequestered vary widely even in one population reared on a single plant – thus in the field individuals must differ largely in their level of defensive compounds, too. Our data clearly show that *C. hortulanus* is able to develop on both plants and that we do not face two cryptic species adapted to different plant species, not does it seem to be a case of ongoing speciation.

Introduction

Within the Mecininae (Coleoptera, Curculionidae) the tribus Cionini comprises two genera – *Cionus* and *Cleopus* which both sequester iridoid glycosides (IG). While *Cionus* sequesters aucubin and catalpol, in *Cleopus* only catalpol could be detected (Chapter 2). Both these iridoid glycosides are monoterpenoid secondary metabolites which occur in over 50 plant families of the Lamiales (El-Naggar & Beal 1980, Boros & Stermitz 1991, Wink 2003). In the plants they act as deterrents against herbivores that exert toxicological effects upon consumption by denaturing DNA and proteins (Konno et al. 1999, Kim et al. 2000). They thus protect the plants against many generalist herbivores and microbes. However, several specialised herbivores are adapted to those chemical defences and can tolerate them or even sequester them for their own benefit (Dobler et al. 2011, Opitz & Müller 2009). The sequestration of the IGs aucubin and catalpol has many benefits for herbivores as they are potentially protected against some bacteria like *Bacillus thuringiensis* and some fungi (Davini et al. 1986, Marak et al. 2002, Biere et al. 2004, Baden & Dobler 2009), against some predators like birds (Bowers 1980, Bowers 1981), invertebrate predators like the chilopod *Lithobius* (Baden & Dobler 2009), spring spieders (Strohmeyer et al. 1998) and wolf spiders (Theodoratus & Bowers 1999), predatory insects like the bug *Podisus maculiventris* (Bowers & Stamp 1997), ant species (de la Fuente et al. 1995, Dyer & Bowers 1996, Opitz et al. 2010, Baden et al. 2011, submitted) and several other predators (e.g. Nishida & Fukami 1989).

Most of these studies were carried out with Lepidoptera as many sequestering species are butterflies some beetles have this ability, too. Sequestration of IGs is known for leaf beetles of the genera *Longitarsus* and *Diabolia* (Chrysomelidae, Alticinae) (Bowers 1988, Willinger & Dobler 2001) and weevils of the genera *Cleopus* and *Cionus* (Chapter 2). Some of these beetle species live on the same iridoid glycoside containing plants, for instance *Verbascum* and *Scrophularia*. The sequestered amounts of IG vary widely in these genera. While *Longitarsus* species sequester up to 2 % IG of their dry weight (DW) weevils of the genus *Cionus* have up to 7 % IG of their DW. Even the relatively low concentrations of IGs in *Longitarsus* show deterrent and antimicrobial effects (Baden & Dobler 2009, Baden et al. 2011, submitted). Thus, it can safely be assumed that the ectophagously living genera *Cleopus* and *Cionus* (which is exceptional for weevils) get an advantage caused by the sequestered IG, too.

A closer look at these weevils shows that *Cleopus* sequesters only catalpol and no aucubin. Furthermore, *Cionus* species living on *Scrophularia* tend to sequester more aucubin than catalpol while the species on *Verbascum* sequester more catalpol than aucubin – with only

few exceptions (Chapter 2). This pattern of IG sequestration could even be detected the only species living on both plants: *Cionus hortulanus*. It is still unclear, why such differences in sequestration exist. Particularly remarkable is that the host plants do not differ that much in their IG concentrations, rather they are very similar and contain both much more aucubin than catalpol (Willinger & Dobler 2001, Chapter 2).

Although *C. hortulanus* is the only species living on both plants, no clear signs of a genetic differentiation hinting to host race formation and possibly beginning speciation could be detected in a molecular phylogeny of this genus (Chapter 1). However, this possibility could not be excluded either, since only one population from *Verbascum* clustered with two populations from *Scrophularia*, whereas the remaining populations from *Verbascum* formed a cluster of their own. Thus, it still remains possible that ecological differentiation is happening but cannot yet be detected by CO I/II or EF1α as genetic markers.

In addition other questions about *C. hortulanus* biology, host choice and physiology remain open. One of the main question is whether the differences in sequestration patterns in dependence of the host plant is genetically fixed in weevil populations living on different host plants. In that case we would for instance expect that populations on *Verbascum* could better sequester catalpol than those on *Scrophularia*. On the other hand, this pattern might be explained by a different content of catalpol - like chemicals in the plants which might get metabolized to catalpol in the weevils. Lastly, the key to the observed differences could lie in not yet understood physiological processes on *Verbascum*. To get further insights in the cause for these intriguing patterns we reared *C. hortulanus* on both host plants to verify whether the differences in sequestration are caused by the plants or by the weevils themselves. Furthermore, we wanted to know more about the ecology of *C. hortulanus* to get more information about adaptions to either plant species or the possibility of ecological speciation.

To this aim, we collected *C. hortulanus* from one location in the field from *Scrophularia* and reared them for a complete lifecycle on *Scrophularia* or on *Verbascum*, respectively. At four dates we removed the different stages of their lifecycle together with some plant material. These specimens from different plants were analysed and compared for their IG content the causality of possible differences.

Material & Methods

Weevil collection and rearing

C. hortulanus were collected in spring 2008 in the Sachsenwald (Schleswig-Holstein) and its close vicinity. Weevils were exclusively collected from *Scrophularia* in areas with no Verbascum plants in the nearness. The weevils were kept together for at least a week, to ensure that every female was fertilised. The captured beetles had hatched the year before and overwintered as adults, therefore we were sure that they were sexually mature. Before we started the experiment we determined the beetles' sex, since we only needed the females.

Three potted plants of *Verbascum nigrum* and three *Scrophularia nodosa* plants were kept in a common garden at the University of Hamburg. The *V. nigrum* plants were taken from Bröthen and *S. nodosa* from Sachsenwald (both Schleswig-Holstein). After three female weevils were placed each plant, the plants were enclosed with gauze. As soon as the weevils laid the eggs on the plants their lifecycle on the plants began.

At the time of the female's insertion plant material was removed and frozen for a later chemical analysis. Then samples of the late larvae, pupae and adult offspring were taken and frozen during the course of the summer, on the day the first larvae were removed from a plant the parental weevils were removed too to avoid later confusions with adult offspring. Before freezing the larvae and the imagines were starved for two days to empty the plant material from their guts. With every weevil sample corresponding plant material was collected, too.

Chemical extractions of the iridoid glycosides

The weevil specimens (larvae, pupae, imagines) were extracted individually, e.g. if we had 10 imagines from one plant, we did 10 separate extractions. The extraction followed the protocol of Willinger and Dobler (2001) in all basic details. First of all the fresh weight of the samples was determined, and then they were freeze dried and the dry weights taken. Thereafter the plant samples were ground with sea sand, the weevils without sand. The powders were extracted in methanol overnight, using 2ml for the beetle and 5 ml for the plant samples. The samples were then filtered and washed three times with 2 ml methanol. The extracts were evaporated under constant airflow at 37 °C, the residues redissolved in 5 ml H_2O and washed three times with 2 ml $CHCl_3$ each in a separating funnel. The extracts were freeze dried

overnight and redissolved in 1ml methanol with 0.25 mg/ml Phenyl-β-D-glucopyranosid (PBG) which served as an internal standard.

Chemical analysis

To quantify the concentrations of aucubin and catalpol in the extracts a HPLC-MS (HPLC-ECI-TOF-MS, Agilent Technologies, model 6224) was used. The samples were further diluted 1:50 with H_2O and 2 µl of this dilution were injected with a flow rate of 0.4ml/min at a temperature of 25 °C. Two eluents were used: A1 (water with 0.1 % formid acid) and B1 (acetonitrile with 0.1 % formid acid). The run was structured in the following segments: time 0min ratio B1 5 %, time 1 min ratio B1 5 %, time 5 min ratio B1 80 %, time 6min ratio B1 80%, time 7 min ratio B1 5 %, time 8min ratio B1 5 %. The post time between two runs was 2 min. The IG concentration in the extracts could be calculated by comparing their peak areas to the area of the PBG peak of known amount. The iridoid glycosides could be identified by their molecular weight and by comparison to reference compounds of aucubin, catalpol and PBG.

Results

C. hortulanus could successfully complete its lifecycle on two *S. nodosa* plants but only on one *V. nigrum* plant. Development on the remaining plants failed since the plants were killed by aphids. Nevertheless, a total of 48 individuals could be retrieved from *S. nodosa* and of 29 individuals on *V. nigrum*, demonstrating that the weevils can successfully develop on both hosts (table 3.1).

Chemical analyses were done on single individuals the mean values for each life stage are indicated in table 3.1. The variability of IG concentrations is documented in figures 3.1 and 3.2, while table 3.2 gives the IG concentrations of the plants. The sampling dates document which concentration were present in the plants at the day of the removal of the weevil specimens (table 3.1 and 3.2).

Table 3.1 Developmental stages of the sampled weevils, their corresponding host plant, the number of specimen and the mean percentage of aucubin and catalpol of their dry weight. The sampling dates allow for a comparison with the weevil data in table 3.2.

Life Stage	Plant No.	quantitiy	% aucubin DW	% catalpol DW	Sampling date
Females	*S. nodosa 1*	3	0.63	0.98	8th June 2009
Females	*S.nodosa 2*	3	1.48	0.41	8th June 2009
Females	*V. nigrum 1*	2	0.30	1.82	22th July 2009
Late Larvae	*S. nodosa 1*	-	-	-	-
Late Larvae	*S.nodosa 2*	4	0.10	0.003	8th June 2009
Late Larvae	*V. nigrum 1*	3	0.15	0.14	22th July 2009
Pupae	*S. nodosa 1*	3	0.05	0.02	8th June 2009
Pupae	*S.nodosa 2*	5	0.50	0.06	8th June 2009
Pupae	*V. nigrum 1*	5	0.15	0.86	22th July 2009
Adult offspring	*S. nodosa 1*	21	2.05	0.20	17th July 2009
Adult offspring	*S.nodosa 2*	10	1.06	0.03	17th July 2009
Adult offspring	*V. nigrum 1*	19	0.46	0.96	12th August 2009

In the weevils reared on *S. nodosa* the concentration of aucubin was higher than the one of catalpol in all life stages (fig. 3.1). In the two *S. nodosa* plants the IG concentration increased over the summer from around 0.25-0.30 % DW in May to over 1 %, respectively over 2 % DW in July-August (table 3.2). The first weevil specimens, i.e. the parental females, late larvae and pupae, were collected in June from the *S. nodosa* plants. The parental females had a high amount of IGs both with over 1.5 % IG of their DW and notably the females of the first plant had more catalpol than aucubin while the relation was reversed in the females from the second plant. IG concentrations increased gradually over the course of development from 0.1 % IG in the larvae to the mean of around 0.4 % and 1.9 % in the adult offspring. Obviously even on the same plant the amounts are very variable. A closer look at the aucubin concentrations of the adults shows that in the beginning the concentrations of plant 2 was higher than in plant 1 as were the IG concentrations in the parental females, too. At the time when we took the adult offspring from the plants the situation was reversed.

In the beetles from *Verbascum* catalpol is the predominant IG only in the larvae both IGs had similar concentrations. This is remarkable because catalpol was detectable only in traces in the host plant (figure 3.2). The parental females had a concentration of 0.3 % aucubin and 1.82 % catalpol of their DW, though they lived in the field on *Scrophularia* and had only spent the last three month on *Verbascum*. The highest detected individual concentrations of

IGs in the parental females on *Scrophularia* were for aucubin 1.1 % of a weevil from plant 2 and 1.16 % for catalpol on plant 1. Overall, the highest measured IG amount in the adult offspring was on *Scrophularia* 5.39 % aucubin and 0.48 % catalpol and on *Verbascum* 0.99 % aucubin and 1.81 % catalpol.

A comparison of male (n = 11) and female (n = 20) offspring developed on *S. nodosa* showed no significant differences between the sexes (t-test, p = 0.45 for aucubin and p = 0.088 for catalpol).

Table 3.2 Concentrations of aucubin and catalpol in the experimental plants as percentage of their dry weight. The sampling dates are allow for a comparison with the weevil data in table 3.1. A * occurs were the IG was detected only in traces.

Plant	**% aucubin DW**	**% catalpol DW**	**Sampling date**
S. nodosa 1	0.17	0.09	7th May 2009
S. nodosa 1	0.17	0.08	8th June 2009
S. nodosa 1	2.08	0.07	17th July 2009
S. nodosa 1	1.82	*	28th August 2009
S. nodosa 2	0.26	0.03	7th May 2009
S. nodosa 2	0.13	0.09	8th June 2009
S. nodosa 2	1.10	0.04	17th July 2009
V. nigrum 1	0.44	*	14th May 2009
V. nigrum 1	*	*	22th July 2009
V. nigrum 1	0.02	*	12th August 2009
V. nigrum 1	0.08	*	28th August 2009

Discussion

Several interesting patterns can be seen in our data. The first and most obvious is that weevils living on *Scrophularia* sequester more aucubin than catalpol and while in those on *Verbascum* it is the other way round. This corresponds to the pattern observed in other *Cionus* species, too (Chapter 2).

The present data give us much more insights into the course of sequestration and allow making some new interpretations. One of the insights is that the females (supposedly without eggs) do not have more IG than the males; a differing pattern of IG sequestration in different sexes is reported in other studies with insects and also for other sequestrated metabolites (Opitz & Müller 2009).

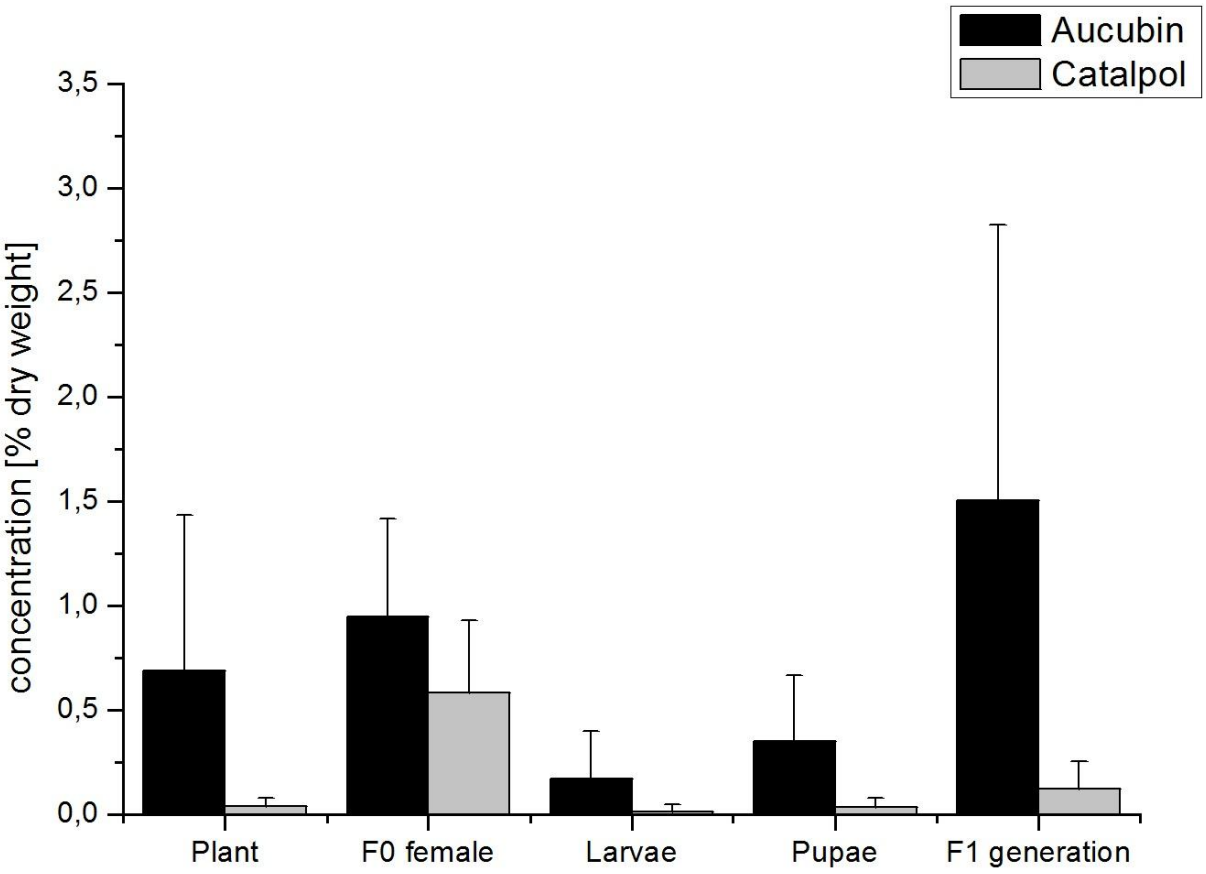

Figure 3.1 Mean concentrations of aucubin and catalpol as percentage of dry weight in the weevils reared on *S. nodosa*. The plants column shows the pooled data for all dates and both plants.

Another interesting issue is the concentration of IGs sequestered. We detected a large variability in the IG content of individuals taken from the same population and reared on the very same plants. This might be due to differences in their ability to sequester on the level of the individual and, not just on a species level. Having measured several individuals of each life stage and not just pooled individuals of a population is one of the big assets of our investigation. The range of IG concentration between individuals of one population of *C. hortulanus* perfectly fits with the range observed for the populations of different *Cionus* species (Chapter 2). This demonstrates that the differences between the populations of a species are not exceptional but have their equivalence even within populations on the level of individuals. That is quite remarkable as many investigations so far measured only the amounts of a few individuals or a few populations. We see that the individual differences may be too high to interpret a few data points as giving the complete picture of a species' sequestration rate.

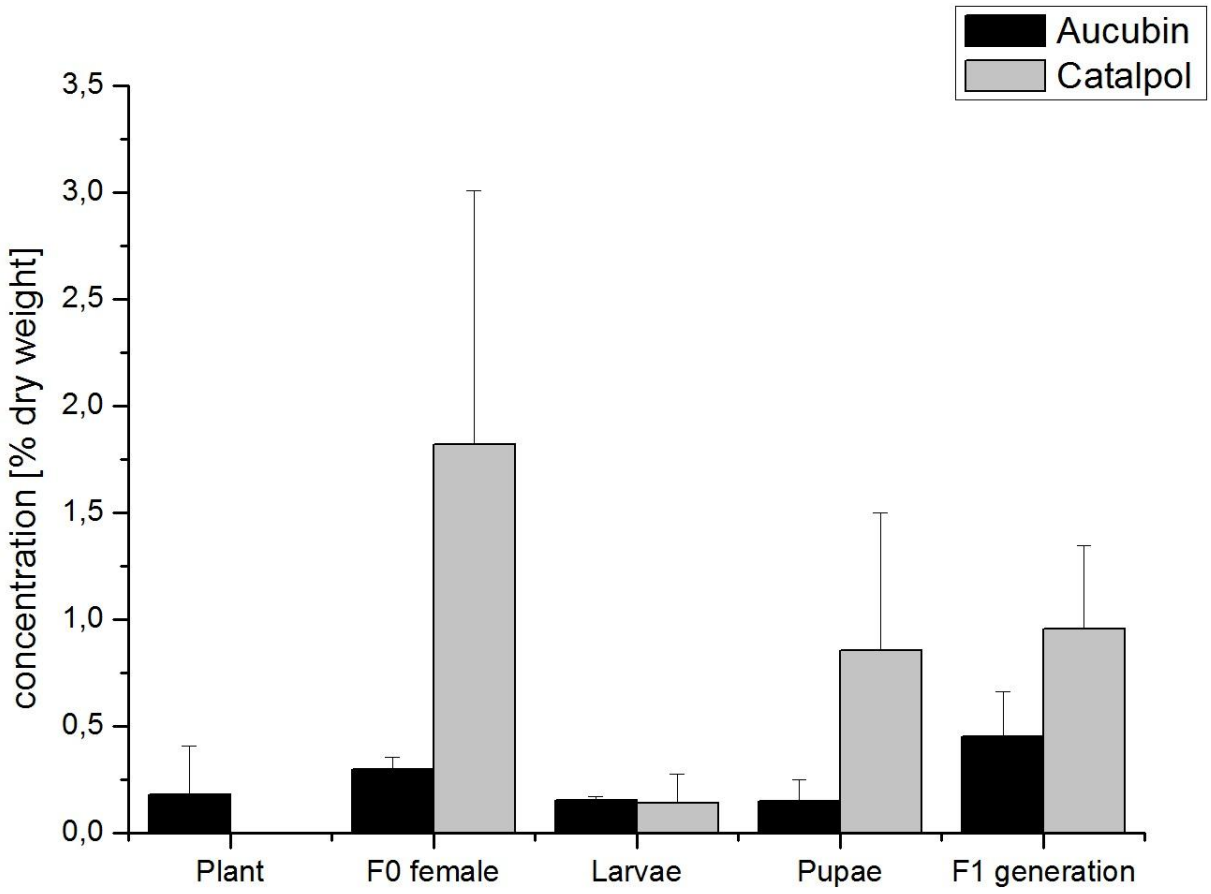

Figure 3.2 Mean concentrations of aucubin and catalpol as percentage of dry weight in the weevils which reared on V. nigrum. Catalpol was found only in traces in the plant. The plants column shows the pooled data for all dates and both plants.

The results of the present experiment support other results of the investigation of IGs in several *Cionus* species (Chapter 2), too. It is obvious that *Cionus* weevils on *Scrophularia* usually sequester more aucubin than catalpol and on *Verbascum* vice versa. The present results underline this phenomenon very clearly. Yet, a problem encountered earlier remains: the IG content of *Scrophularia* and *Verbascum* does not explain the IG distribution in the weevils. Catalpol is more often effectively sequestered as has been described not only for beetles (Willinger & Dobler 2001, Chapter 2) but also for Lepidoptera (Bowers & Puttick 1986, Belofsky et al .1989, Bowers & Collinge 1992). There are several possibilities to interpret this phenomenon; for instance, the insects may convert other so far undetected IGs into catalpol (Bowers & Puttick 1986); possibly, derivatives of catalpol (Stermitz et al. 1986, Opitz et al. 2010). A conversion of aucubin to catalpol seems possible since the two are structurally closely related (Boros & Stermitz 1991), however, in the only study that rigorously tested for such a conversion this could be excluded, because in experiments Lepidopteran larvae were fed with aucubin, they did not yield any catalpol (Bowers &

Collinge 1992). On the other hand, the weevils might be able to take up catalpol with much higher efficiency than aucubin. Different patterns of sequestration of these two IGs in related species were observed in several studies (Bowers & Stamp 1997, Willinger & Dobler 2001, Lampert & Bowers 2010). Another possibility is that *Verbascum* transforms aucubin into catalpol in a higher amount while it is consumed than *Scrophularia* does. Thus, aucubin is a precursor of catalpol in the plant's metabolism (Damtoft 1994).

A closer look at *C. hortulanus* shows that it is unique in the genus *Cionus* because it uses both *Scrophularia* and *Verbascum* as hosts. The data of the transplanted beetles here shows the same sequestration patterns as observed for populations naturally living on the two plants (Chapter 2). What does this mean if we focus on the species? First, individuals living on *Scrophularia* in the field can without problems ovipost on *Verbascum* and their offspring can successfully develop on this plant, just as on *Scrophularia*. A molecular systematic analysis did not reveal an unequivocal pattern of host race formation as possible sign of ongoing speciation (Chapter 1). Moreover, our experiment demonstrates that the differences in IG concentrations found in *C. hortulanus* collected from different hosts (Chapter 2) are not caused by differences in the physiology of the weevils but simply depend on the local host plant.

Chapter 4

The characteristics of host plant specificity and host plant switch within the genus *Cionus* CLAIRVILLE (Coleoptera, Curculionidae) inferred from olfactory tests

Abstract

The species of the genus *Cionus* are living either on *Scrophularia* or on *Verbascum* plants, and in Middle Europe only *C. hortulanus* is living on both plant genera. We performed an olfactory survey to assess the role of host plant odours for five species of the weevil genus *Cionus* CLAIRVILLE (Curculionidae, Mecininae) and to map olfactory reaction as a host plant specificity marker on the intrageneric phylogeny of *Cionus*. We investigated the respective reactions to host plants and non-host plants for five *Cionus* species, three living on *Scrophularia* (*C. alauda*, *C. tuberculosus* and *C. scrophulariae*), one living on *Verbascum* (*C. nigritarsis*) and *Cionus hortulanus* living on both plants (in separate populations). The weevils (1) show a significant olfactory reaction to plant odours and (2) prefer their host plant, i.e. host plant use is linked with a specific olfactory reaction. If mapped on the phylogeny of *Cionus* host plant switches including changes in olfactory behaviour occurred at least twice interspecifically and twice intraspecifically. We conclude that host plant specificity of phytophagous beetles is not necessarily fixed in some major lineages but has to be expected to repeatedly change in the course of intrageneric evolution. Intraspecific switches in *C. hortulanus* are less strict and we cannot prove if this is speciation in action or if it is only larvae's imprinting with the possibility of future speciation.

Introduction

Cionus CLAIRVILLE (1798) is a genus within Mecininae (Coleoptera, Curculionidae) that includes 157 phytophagous species, all feeding ectophagously on their host plants as larvae and adults. In phytophagous weevils host plant use is often specific and the connection

between beetle and plant is strong. This host plant specificity can also be found within *Cionus*. The species are oligo- or monophagous living either on *Scrophularia* or *Verbascum* species; in Europe they are monophagous for one of these plant genera, *Cionus hortulanus* is the only exception living on both. The similarity of both plants is that they produce iridoid glycosides which are sequestered by the *Cionus* species (Chapter 2, Chapter 3). One trait associated with host plant use, i.e. a marker of plant-insect interaction specificity, is olfactory reaction to this host plant and a preference of the host plant over non-host plants (Tunset et al. 1993, Cerda et al. 1999).

In known systems of phytophagous insect taxa, switches of host plant mostly occur at the base of complex lineages and are rather rare within the phylogeny of a phytophagous insect taxon (at lower levels such as species-groups, genera, tribes). These switches are often followed by a radiation, with several sibling-species living on the same plant, e.g. in *Longitarsus* (Coleoptera, Chrysomelidae) (Dobler 2001), the Donaciinae (Coleoptera, Chrysomelidae) (Kölsch & Pedersen 2008), the Mecinini (Coleoptera, Curculionidae) (Chapter 1) and *Phyllonorycter* (Lepidoptera, Gracillariidae) (Lopez-Vaamonde et al. 2003). Host plant specificity is supposed to be a major evolutionary step as it involves many adaptations (Franz & Valente 2005) such as specific olfactory reactions.

Within *Cionus* these host plant switches between *Scrophularia* and *Verbascum* happened several times (Chapter 1). However, characteristics of these switches in the context of intrageneric and intraspecific evolution have yet been left undescribed and uninterpreted.

We want to characterize the host plant use within *Cionus* and test if it is linked with quantitative or qualitative differences in olfactory reaction to control, host plant and non-host plant. These characteristics will allow some conclusions on the evolution of host plant use and switches based on the phylogenetic tree of the genus *Cionus* (Chapter 1).

Material and Methods

The following *Cionus* species from northern Germany were tested: Species from *Scrophularia nodosa*: *C. alauda* (HERBST, 1784) and *C. tuberculosus* (SCOPULI, 1763), both collected at Sachsenwald (Schleswig-Holstein, Germany), and *C. scrophulariae* (LINNÉ, 1758) from Treia (Schleswig-Holstein). Species from *Verbascum nigrum*: *C. nigritaris* REITTER (1904) from Bröthen (Schleswig-Holstein).

Cionus hortulanus (FOURCROY, 1785) from both plants: (1) from *Scrophularia nodosa* collected in woods around Sachsenwald, (2) from *Verbascum nigrum* collected in Sachsenwald, Nehmten, Langenlehsten and Besental (all Schleswig-Holstein). They were held at long day cycle (16 h light) at 16 °C and were fed with their normal host plant until the start of the experiments.

The olfactory survey was done with a wind tunnel olfactometer as described by Peters & Abraham (2009) with slight modifications. This olfactometer (Fig. 4.1) has some advantages compared to the usual Y-tube model (see Peters & Abraham 2009), most notably the fact that the tested specimen can move freely inside the tube and accordingly show more of their normal behavior. Furthermore the measured length of stay provides more information than the yes-no-choices of the Y-tube (Peters & Abraham 2009).

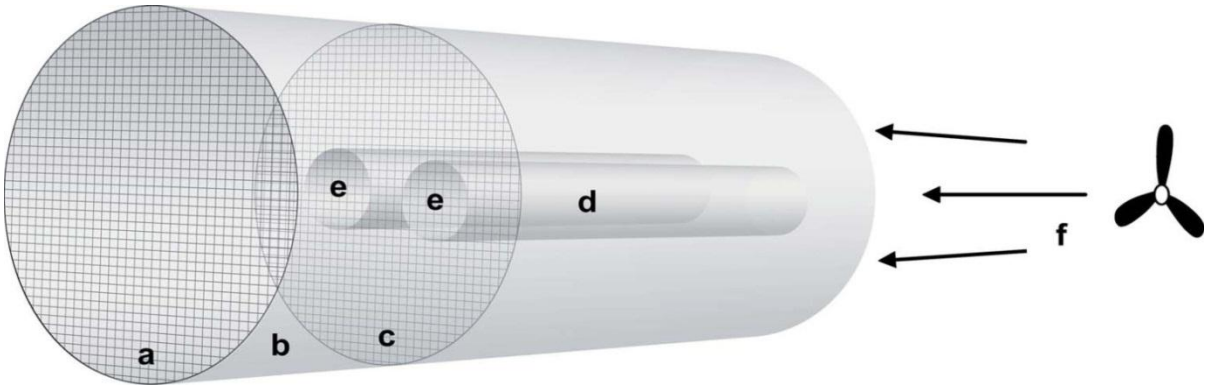

Figure 4.1: Wind tunnel olfactometer. **a.** gauze cover; **b.** experiment chamber; **c.** vertical gauze testing screen; **d.** glass substrate tubes; **e.** test sectors; **f.** airflow (Peters & Abraham 2009).

The main body of the olfactometer was a transparent acrylic glass tube with a total length of 60 cm and a diameter of almost 20 cm. The tube was parted into two chambers, an experimental chamber (Fig 4.1, b) and a substrate chamber, both with a length of 30 cm. The two parts are separated by a vertical gauze screen (Fig 4.1, c). The substrate chamber contained two glass tubes with a length of 30 cm and a diameter of 4.6 cm (Fig 4.1, d). These glass tubes were fixed with pipe clamps 3 cm from of the outer wall of the chamber. The openings of these glass tubes nearly touched the vertical gauze screen and created two test sectors on this screen (Fig 4.1, e).

In a distance of 50 cm a small fan (diameter 10.5 cm) was placed in a central position of the olfactometer to produce airflow (Fig 4.1, f) through the glass tubes and the experiment chamber. The airflow speed was around 0.5 m/s inside the test chamber.

All experiments were made at room temperature in the afternoon.

For the experiments, in one of the glass tubes a leaf of *Scrophularia nodosa* or *Verbascum nigrum* was placed, the other tube remained empty control. Both tubes were placed very closely to the gauze screen, in a way that they did not touch the gauze, so that no thigmotactic attitudes could adulterate the experiment. To draw the positively phototactic weevils to the gauze screen the olfactometer was placed with the back opening turned to a window and a black cardboard was wrapped around the experiment chamber.

To start the experiment one single test specimen was put into the experimental chamber and the chamber was closed with gauze (Fig 4.1, a). The weevils climbed up the gauze screen and possibly crossed the test sectors. When they arrived at the top, they dropped down and began to climb up again. The duration of stay was measured as time spent within a sector. It started when a weevil entered a test sector and was stopped when it left. For each specimen two times were taken, one for the crossing of a plant sector and one for the control sector. Each individual was used only once and was tested only for one plant flavour. 20 weevils per species were tested as a control and *Scrophularia* and 20 per species for control and *Verbascum*. As mentioned before *Cionus hortulanus* was tested twice, one population from *Verbascum* and one taken from *Scrophularia*.

After every experiment the gauze and the glass tubes were removed and cleaned. After every weevil the position of the control and the trial glass tube was swapped. The leaves were taken from plants cultured in the institute.

The data was analysed with SPSS 17.0 for Windows (Norusis 2008). At first the Gaussian distribution was tested by the Kolmogorov-Smirnov-test ($p = 0.05$) and for comparison of two groups a t-test (with Levene-test of variance equality, $p = 0.05$) or a U-test (exact significance, because $n \leq 30$, 2-sided) was performed, accordingly. Following comparisons were done for each weevil species: control vs. *Scrophularia*, control vs. *Verbascum* and *Scrophularia* vs. *Verbascum*.

Results

Tested species or populations showed significant response to the odour of their respective host plants and no significant reaction to the respective non-host plant (Fig. 4.2 and 4.3). In direct comparison species showed stronger reactions to the odour of their host plant than to the

odour of the respective other plant. However, there are two exceptions of these general results in the comparisons between the reactions to the two plant species *Verbascum* and *Scrophularia*: 1) In *C. hortulanus* - the only species living on both plants - the two populations differ in their reaction to their respective host plant. In the population from *Scrophularia* the preference of host plant over non-host plant is not significant, there is even a reaction to the non-host plant that is barely non-significant. The population from *Verbascum* has a very strong preference to its host plant, but there is also a significant reaction to the non-host plant. This means that both populations react to non-host plant material, but the preference for and bond to the host plant is stronger in the population from *Verbascum* (Fig 4.3). 2) The reaction of *C. scrophulariae* from *Scrophularia* to its host plant is not significantly stronger than to the non-host plant. However, since the reaction to the non-host plant does in their part not differ from control, the preference for *Scrophularia* is implicitly demonstrated (Fig 4.2).

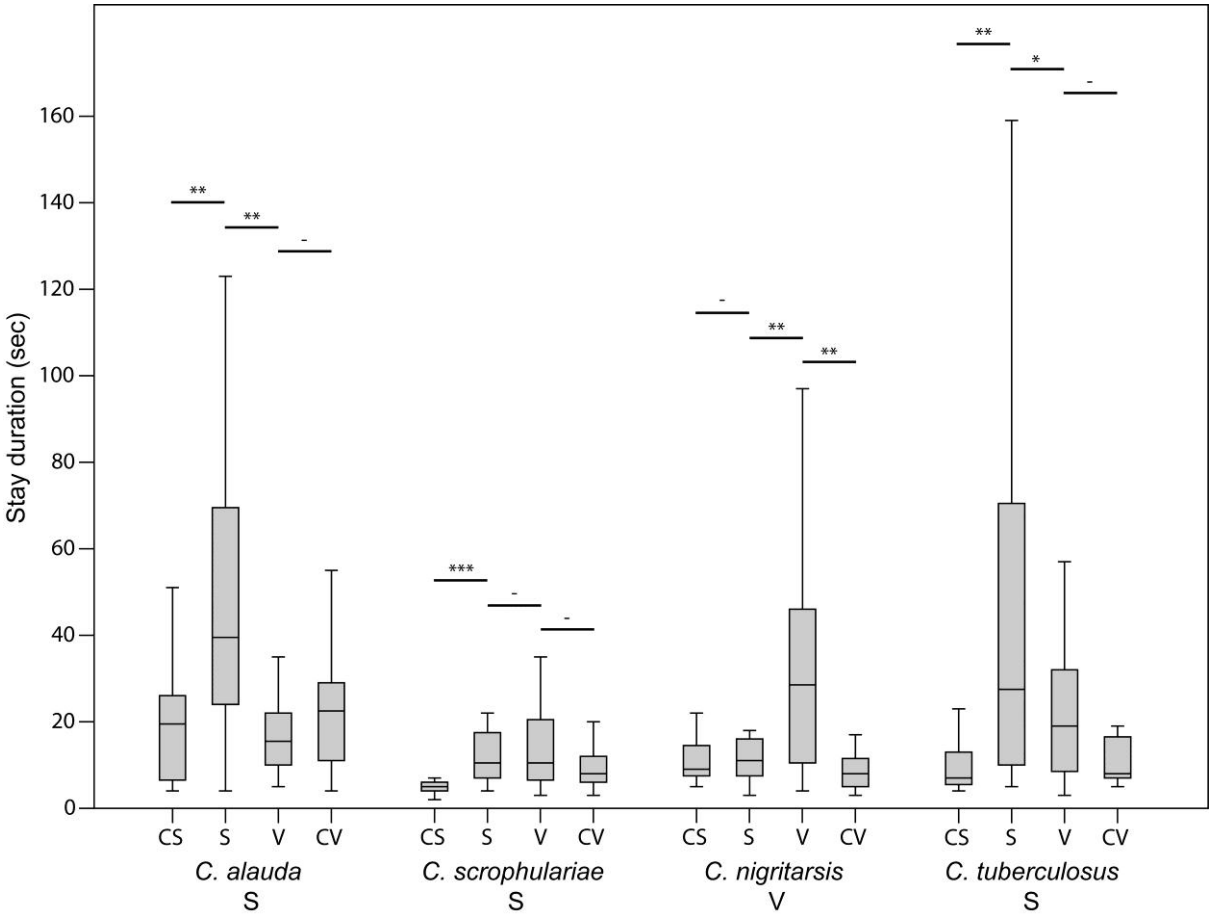

Figure 4.2: Duration of stay of four *Cionu*s species within plant odours and control in olfactometer tests (N = 20). Boxes show median and quartiles; outliers not shown. The horizontal bars indicate statistical comparison - no significant differences, * significant, ** very significant, *** highly significant differences. S = *Scrophularia*; V = *Verbascum*. The abbreviation under the species name stands for its host plant. CS are the controls of the experiments done with *Scrophularia*, CV are those of *Verbascum*.

Result summary: Despite some few statistic ambiguities every host plant use of *Cionus* spp. is linked with specific olfactory reaction and preference. Preference for *Verbascum* in spp. from *Verbascum* is generally strong. There are differences between the intraspecific situation in *C. hortulanus* and the situation in the other species: In *C. hortulanus* populations the olfactory host plant specificity is less powerful. With the results of these olfactory studies and the derived characteristics of host plant use we will discuss the interspecific and intraspecific host plant switches that occurred in Cionini evolution (Chapter 1).

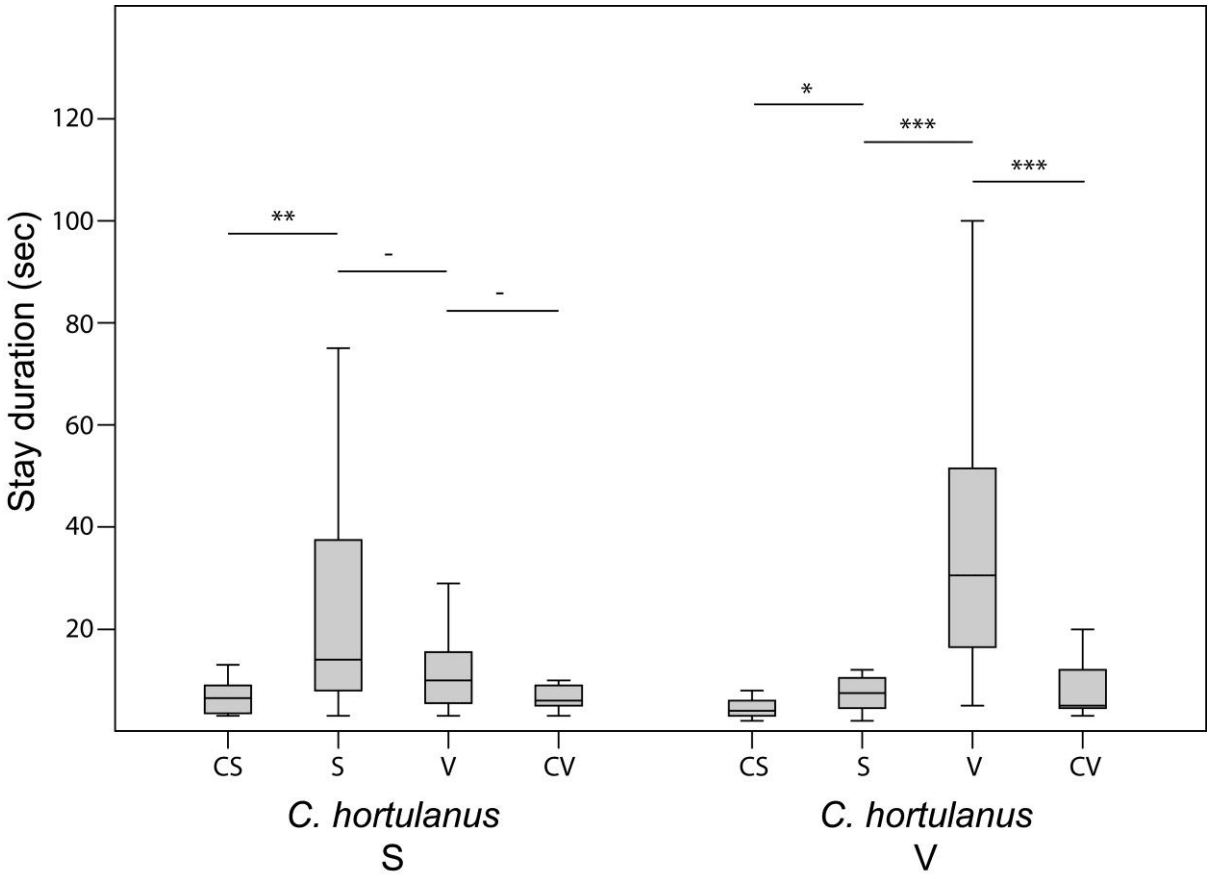

Figure 4.3: Duration of stay of two *Cionus hortulanus* populations within plant odors and control in olfactometer tests (N = 20). Boxes show median and quartiles; outliers not shown. The horizontal bars indicate statistical comparison - no significant differences, * significant, ** very significant, *** highly significant

Discussion

The results lead to the conclusion that the olfactory sense plays a major role in the recognition of the weevils' host plants. For each species, we recorded a positive olfactory response to its host plant.

If we link the data presented here with the phylogeny of these weevils inferred from nuclear and mitochondrial genes (Chapter 1) (Fig 4.4) we can see some implications. Feeding on *Scrophularia* is apparently the ancestral state within the genus. *Cionus alauda* is the basal species, the next most basal is *C. scrophulariae*; both species show a strong specificity for their host plant *Scrophularia* in the odour tests (Fig 4.2). Then, in the tree there is a first host plant switch to *Verbascum*. Within the clade *C. nigritarsis* + *C. tuberculosus* there is a second switch back to *Scrophularia*. *C. tuberculosus* and *C. nigritarsis* are closely related, maybe even sister species. This cannot be said with certainty, because not all *Cionus* species were included in the analysis.

Cionus nigritarsis lives only on *Verbascum,* i.e. most probably represents the ancestral state after the first host plant switch. Similar to the spp. from *Scrophularia* discussed above *C. nigritarsis* significantly favours its host plant over the non-host plant (Fig 4.2). *C. tuberculosus* lives on *Scrophularia* after the second host plant switch. It also favours its host plant over *Verbascum*. Accordingly, both of these interspecific host plant switches are connected to change in (1) feeding and (2) olfactory behaviour, i.e. some major changes in life history. These major changes happened at least twice during *Cionus* evolution.

There are two more host plant switches within *C. hortulanus* that require separate discussion. These host plant switches are different from those discussed above: They are less strict, specimens from one host plant can be fed with and reared on the other host plant and vice versa (Chapter 3), and olfactory reactions are also less clear.

The *C. hortulanus* from *Verbascum* significantly favour their host plant over *Scrophularia*; the *C. hortulanus* from *Scrophularia* show no significant olfactory preference for either of the two plants, they only favour *Scrophularia* over the control (Fig 4.3). The explanation for this might be found in the lifecycle of the plants. *Scrophularia* is growing from spring to midsummer, *Verbascum* starts growing not until midsummer. For this reason of the chronological appearance of their potential host plants a host plant switch from *Verbascum* to *Scrophularia* in the course of the year is impossible. The opposite way, however, is well possible and might prolong the feeding period for the weevils. In this case the olfactory preferences that we recorded within *C. hortulanus* are maybe implied by the host plant the larvae are feeding on. If it was congenital the strong favour for one plant would implicate a phylogenetic differentiation of the populations living on *Scrophularia* and *Verbascum*. However, in the tree (Chapter 1) these populations are mixed (Fig 4.4), which means that no beginning speciation could be demonstrated so far. Accordingly, the intraspecific host plant

switches within *C. hortulanus* and respective implications are different from the interspecific switches discussed above.

In some identification keys of the genus *Cionus* there are some ambigouities about the apparent monophagy of the species. Wingelmüller (1937) and Freude et al. (1983) list occasional records for species from *Scrophularia* from *Verbascum*. This, however, might be artificial information; we have never seen a specimen of a *Scrophularia* species on *Verbascum*. We consider all species included here as monophagous (except *C. hortulanus*).

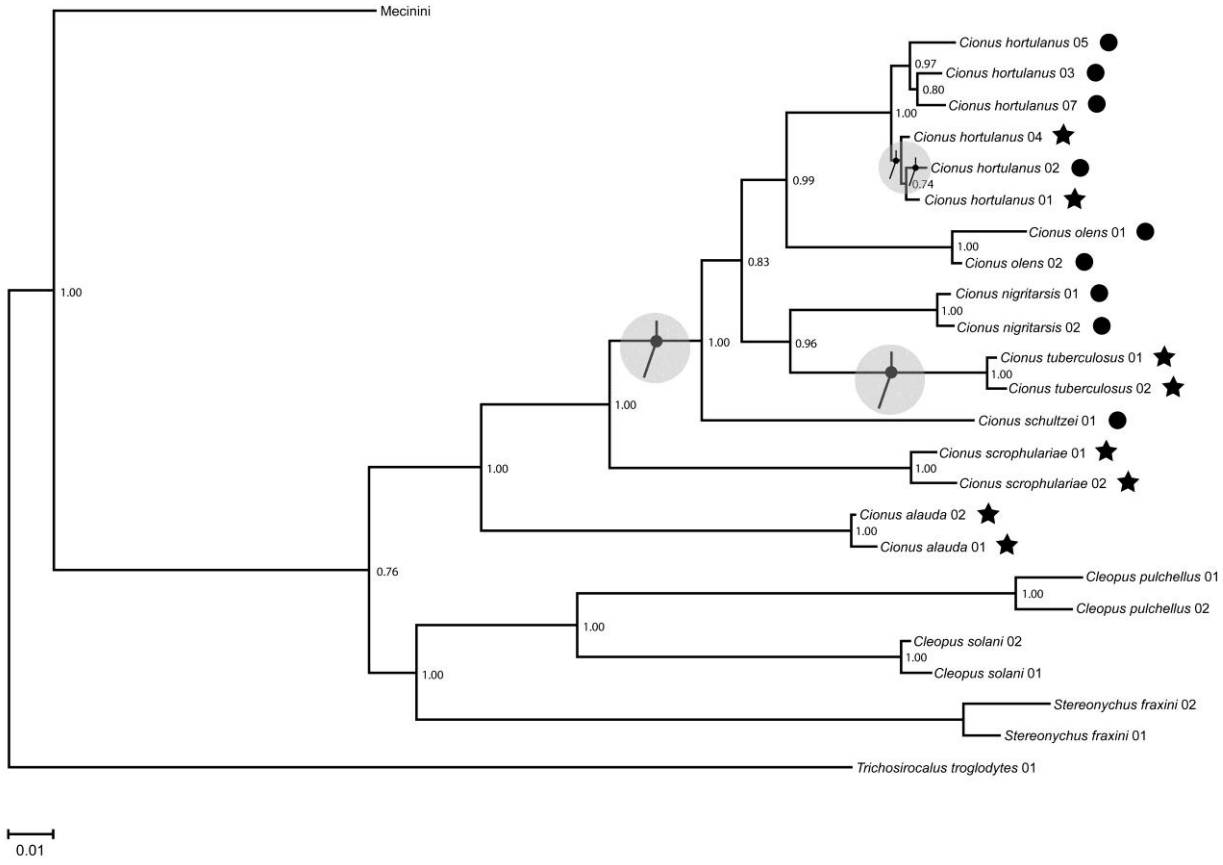

Figure 4.4: Part of the phylogenetic tree of the Mecininae (Chapter 1) (COI/II and EF1-α genes; Bayesian analysis). Given are the posterior probabilities of each node and the species host plants: star = *Scrophularia*; circle = *Verbascum*. The host plant switches are marked with grey-shaded switch sign.

The strong specificity for and adaption to host plants indicated by our data that switched twice in the *Cionus* tree is quite astonishing. Previous studies state that host plant switches in Curculionidae occur if the weevils are pre-adapted to the new hosts, which are often related to the old one, and that such a shift is frequently followed by a radiation scenario (Marvaldi et al. 2002, McKenna et al. 2009). Those radiations are so prominent, that the host plants can be used as a phylogenetic marker, for instance even within the same weevil subfamily (Mecininae) in the tribus Mecinini (Caldara 2001, Chapter 1). Here we can see that host plants

cannot be used as a marker for the Cionini which was already assumed (Chapter 1). Another phylogenetic marker which is sometimes an alternative to the host plant (Mitter et al. 1991, Sprick 1997, Dobler 2001, Kergoat et al. 2005) may play a bigger role here: the presence of secondary metabolites. However, the known common metabolites (aucubin and catalpol) are nearly the same for both plants (Chapter 2, Chapter 3). The above mentioned studies show that specialisation to one host plant must not be a dead end; because of the pre-adaptations the species might have on secondary compounds. It seems as if the genus *Cionus* is one of the groups which are pre-adapted to the plants chemistry, and as such a switch from one host plant to another, both containing the secondary compounds, might not be so problematic just as mentioned by Becerra (1997). Maybe the fact that *C. scrophulariae* has no significant preference for its host plant is a sign of such a pre-adaption. Additionally, Farrell & Sequeira (2004) showed that the host tissue can be more important than the host plant itself anyway. After all it seems very likely, that *Cionus* is one of these systems where host plant switches occur not only very commonly but also have no phylogenetic evidence.

The presence of secondary metabolites in both plant species involved in our tests also means that these, most notably aucubin and catalpol, cannot be the reasons for differences in olfactory preferences and therefore cannot be important parts of the olfactory reaction to host plants within *Cionus* in general. Which odours are the responsible ones for the obvious reactions and favours then? The answer may give the clue for understanding the adaptations of *Cionus* to their host plants. The question, however, has to remain open; we still have to get a better idea of the chemistry of the odours in order to find maybe other important secondary compounds of the host plants, which could have an important impact on the life of the weevils. Either the host plant switches are linked with a not known chemical component or it is a not yet understood evolutionary effect that selects for a strong linkage to the host plant, but also for the opportunity to switch it back and forth in the phylogeny – the genus *Cionus* seems to be a remarkable object for more extensive future studies on insect/host plant interactions, adaptations and speciation.

Chapter 5

Dispersal behaviour of two *Cionus* species CLAIRVILLE (Coleoptera, Curculionidae) taken from *Scrophularia nodosa* and released on *Verbascum nigrum*

Abstract

Cionus hortulanus is an oligophagous weevil species that uses plants of two different genera as hosts: *Verbascum* and *Scrophularia* species. Since all populations in a larger screen only used one of the host genera in the field, we tested here whether ecological specialization is occurring in this species. To this end, *C. hortulanus* were taken in the field from *S. nodosa*, then individually marked and were released in the field on *V. nigrum.* Then we monitored them for their dispersal ability and their propensity to colonize neighbouring *V. nigrum* plants. These data are compared to those of a second *Cionus* species, *C. tuberculosus* that is monophagous on *Scrophularia*, yet has the capacity to survive for several days on *V. nigrum*. But for *C. tuberculosus* dispersal could hardly be observed. For *C. hortulanus* the ability to establish and disperse throughout the whole habitat could be observed and perfectly fits with other dispersal studies, like the one of St. Pierre et al. (2005), for example. Furthermore, it was observed that the females seem to have another dispersal frequency as the males have. Another result is that there is growing evidence that *C. hortulanus* is one single species and not two cryptic ones on two different host plants.

Introduction

In Middle Europe the genus *Cionus* is represented by several species. These species either live on *Scrophularia* or on *Verbascum*. Only *C. hortulanus* (FOURCROY 1785) uses both genera as potential host plants and can locally be found on either one or the other host. The degree of host specialisation in this and other *Cionus* species, however, remains unclear: *C. tuberculosus* (SCOPOLI 1763), for example, has been described as being able to use both host

genera (Wingelmüller 1937, Freude et al. 1983) and this might possibly be the case in other *Cionus* species, too. In *C. hortulanus*, on the other hand, the ability to use both plant genera as hosts has been verified by us (Chapter 3). In addition, we observed that populations taken from different hosts also differ in their use of plant derived compounds - the iridoid glycosides aucubin and catalpol that the beetles sequester for their own defence (Chapter 2). Since these differing patterns cannot simply be explained by differences in the plant's iridoid glycoside content, the differences between beetles from either host could potentially indicate differing physiologies.

We thus set out here to test whether the dispersal behaviour of *C. hortulanus* provides further evidence for ecological specialisation on one of its two host plants. As a comparison *C. tuberculosus* was included in the study which should display a higher degree of specialisation for *S. nodosa* but might also be able to establish on *V. nigrum* regarding to the mentioned literature. The experimental design we chose is a mark-recapture study with beetles that were collected on *S. nodosa* and were then released on *V. nigrum* in the field. In the vicinity of the release site a large number of *V. nigrum* plants were growing while the next patch *of S. nodosa* was more than one kilometre away and separated by a small forest. This experimental design was chosen to monitor the beetles' dispersal and host choice behaviour and their propensity to establish on the *V. nigrum* plants.

Material and Methods

Eighty-four *Cionus hortulanus* and sixty *C. tuberculosus* weevils were collected in Sachsenwald (Schleswig-Holstein, Germany) from *Scrophularia nodosa* in May and June 2010. At this collecting site no *Verbascum nigrum* was available for the weevils at that time. They were held in a climatic chamber at 16°C and long day cycles (16h light) and fed with *S. nodosa*.

Before releasing the beetles, they were sexed by judging the length of their claws (Wingelmüller 1937, Freude et al. 1983). After that the weevils were individually marked with nail polish of different colours: blue, red, yellow and green. The tiny dots were placed with insect needles on four possible places of the weevils' elytra: left front, right front and left and right back. The minimum number of dots per weevil was one and the maximum was two. It had to be guaranteed that the weevils were able to fly even with these marks on their elytra,

therefore the amount of colour and the dimensions of the dots had to be as small as possible. The marks were not allowed to cover the edges of the elytra, to avoid that these stick together.

The weevils were released in July 2nd near Langenlehsten (Schleswig-Holstein, Germany) in the afternoon. At this site over 500 *V. nigrum* plants were growing and the one and only small population of *S. nodosa* was nearly 1km away and separated by a small forest. All beetles were released at the biggest *V. nigrum* plant. The plant's coordinates were taken by GPS (eTrex Vista HCx, Garmin). The weevils were searched for on the day after release and on the second, third, seventh, tenth, fifteenth, twenty-second and twenty-ninth day. July 2010 was very hot in that area with normally above 26 °C and up to 40 °C in the shadow, and mostly windless. Only on the twenty-second day it drizzled with a temperature of 22 °C.

Every *V. nigrum* plant was scanned for labelled specimens. The coordinates of the plants on which weevils were recovered and the distance to the release site were measured and saved by GPS. The plants in the surrounding area were scanned within 2 km; however, the density of *Verbascum* plants was highest in the test area. All other *Verbascum* plants were separated from the testing-field by (parts of) a forest with only one exception.

The recaptured weevils were left on the plant they were found on. The recovery was simplified by the behaviour of the weevils. They up the inflorescence axis and do not hide from predators; they only go underneath a leaf, if they want to hide from rain or from hot weather and insolation. To make sure that as many weevils as possible were recaptured only the same two persons experienced in collecting these beetles, Christian Baden and Viola Boxberger, carried out this survey.

Results

The recapture rates for *C. hortulanus* and *C. tuberculosus* can be seen in the tables 5.1-5.5. *C. hortulanus* specimens were found in high numbers in the days after the release (tables 5.1, 5.2 and 5.5). *C. tuberculosus,* in contrast, was not recaptured so many times and were not found in such a high rate on other specimens of *V. nigrum* than the release plant (tables 5.3 and 5.4).

If we look at *C. hortulanus* in detail we see that more than half of the weevils were still recovered on the release plant the next day. From there on the numbers of sightings on this plant declined continuously. The number of recaptured weevils from other *V. nigrum* plants was never higher than seven on a single day, though we found weevils on every day which

had been never found on other plants than the release site. The number of weevils that had dispersed to other plants rose up to twenty-three overall (table 5.1). The distance of the plants they were found on to the starting point was between 11 m and 16 m. The highest distance recorded was 63m between the current host-plant individual and the release plant (table 5.1). This is rather far, because this weevil must have flown over dozens of potential host plants to get there. Sex specific differences in dispersal can be supposed as nearly half of the recovered females left the release plant during the next month, but only a fourth of the males did (table 5.2).

Table 5.1 Numbers of *C. hortulanus* over time, which were recovered on the release plant or on other *V. nigrum* plants in the area. The mean distance to the release plant and the maximum distance to the starting point on that day are indicated in brackets the number of the previously recovered individuals per column is given. m = metres

Day	Recovered at starting point (new in list)	Moved from starting point (new in list)	Average distance to day zero	Maximum distance to starting point
0	84	-	-	-
1	45	5	12.6 m	13 m
2	28 (5)	6 (4)	11.5 m	21 m
3	17 (1)	7 (4)	16 m	22 m
7	16 (1)	1 (1)	3 m	3 m
10	7	6 (4)	7.8 m	16 m
15	5	6 (3)	15.3 m	65 m
22	1	5 (1)	11.6 m	19 m
29	0	3 (2)	7 m	7 m

Table 5.2 Dispersal of *C. hortulanus* weevils over the 29 days of the mark-recapture experiment release; sorted by distance categories and sex. m = metres

sex	Stayed on plant	Under 10 m	10-20 m	Over 20 m
Females	20	5	10	2
males	18	3	2	1

The dispersal of eight *C. hortulanus* individuals (table 5.5) allows some deductions. First, it seems as if there is no “perfect day” for leaving the present plant, but it seems to be a continuous event (see also table 5.1). Another point is that the flights do not seem to last for long if the next potential host plant is near – mostly dispersals distances were under 10m per day. It can be seen that the direction of the first dispersal flight is often determining the area the weevil is living in for the next few days. Another thing can be seen from table 5.5: dispersal occurred in all directions around the release plant and no preferred direction for

dispersal can be deduced. A special case is one male weevil which flew away from the starting plant and came back to the very same plant – an incidence which happened actually twice in *C. tuberculosus*, too.

Table 5.3 Numbers of *C. tuberculosus* over time which were recovered on the release plant or on other *V. nigrum* plants in the area. The mean distance to the release plant and the maximum distance to the starting point on that day are indicated in brackets the number of the previously recovered individuals per column is given. m = metres

Day	Not moved from starting point (new in list)	Moved from starting point (new in list)	Means of the distance to day zero	Maximum range to the starting point
0	60	-	-	-
1	18	-	-	-
2	13 (4)	-	-	-
3	8 (2)	1	21 m	21 m
7	5	2 (2)	13.5 m	23 m
10	4 (1)	3 (3)	6.7 m	9 m
15	1 (1)	-	-	-
22	1 (1)	-	-	-
29	0	1	7 m	7 m

Table 5.4 Dispersal of *C. tuberculosus* weevils over the 29 days of the mark-recapture experiment release; sorted by distance categories and sex. m = metres

sex	Stayed on plant	Under 10 m	10-20 m	Over 20 m
Females	16	4	-	-
males	6	1	-	2

C. tuberculosus vanished in a high number over the first day (table 5.3). Then their number declined continuously over the next days. Only about a tenth of the released weevils were recaptured on other plants in the area. The mean distance to the release plant and the maximum dispersal distance were similar to the values recorded for *C. hortulanus*. However, most of the recaptured *C. tuberculosus* weevils stayed on the release plant (table 5.4). Here the males had the tendency for dispersal over a longer distance than the females had.

On no plants (neither *Scrophularia* nor *Verbascum*) in the surrounding area (at least 1 km away) labelled weevils could be discovered. As we never observed partially removed markings on the elytra, and specimen could always be unambiguously identified, the loss of marked beetles can be neglected.

Table 5.5 Exemplary movement coordinates of 8 *C. hortulanus* individuals which were recovered several times moving from plant to plant. F = female, M = male, m = metres

♀/♂	**Day 1**	**Day 2**	**Day 3**	**Day 7**	**Day 10**	**Day 15**	**Day 22**	**Day 29**
F	0 m	0 m	-	W 3 m	SW 3 m	W 6 m	N 5 m	-
F	0 m	0 m	0 m	0 m	NE 4 m	N 7 m	NW 19m	-
F	0 m	0 m	-	0 m	-	SE 3 m	SE 10 m	N 7 m
F	0 m	0 m	0 m	0 m	0 m	0 m	NW 11m	-
M	0 m	0 m	0 m	0 m	-	0 m	0 m	N 7 m
F	-	0 m	0 m	0 m	SW 4 m	SW 7 m	-	-
M	-	SE 7 m	0 m	-	-	-	-	-
F	SE 12 m	SE 11 m	SW 22m	-	-	-	-	-

Discussion

Our knowledge about the ecology of *Cionus* increased further about some definite facts and some reasonable assumptions by the present paper. One fact is that *Cionus* is able to fly without any problems and does it, even without further need – because it disperses across potential host plants. The effect is that this genus seems to spread very quickly over the whole area of a potential habitat. The data implies this very strongly for *C. hortulanus* as can be seen from tables 5.1 and 5.5.

Some examples how the dispersal is taking place on an individual level are presented in table 5.5. We can see there that the beetles do not perform long flights and settle on a new plant for a long period but are moving repeatedly across the area. The direction of the wind cannot be the main factor of distribution, because it was mostly windless. A main direction of dispersal can neither be deduced for the whole period nor for single days.

For *C. tuberculosus* some conclusions can be drawn. The first one is very obvious: This species is able to live for some time on *Verbascum nigrum* which is usually a wrong host plant as all collectors we have talked with never found a specimen on this plant. In the laboratory we observed that this species may feed on *V. nigrum* but this never observed in the field. Older descriptions (Wingelmüller 1937, Freude et al. 1983) state that this species can in rare cases be found on *Verbascum* and we observed that this species is able to use this plant as a transitional host plant - otherwise it would not be possible for this species to survive so many summer days during our experiment without food. The number of recovered individuals decreased very fast (table 5.3). This can have several reasons: (1) The *C. tuberculosus* weevils could be adapted to the penumbra area where *S. nodosa* is normally growing. In this case this species might be hiding on the ground during the day. (2) This species might disperse very fast away from the false host plant. Hence, they were not found on the known *S. nodosa*

plants in the distant neighbourhood. If they dispersed they did a very long distance of several kilometres a day or (3) died. Maybe the theory that they first hide from the sun and had additionally a high mortality is most likely. No differences in the dispersal rate between males and females could be detected in this species (table 5.4).

In contrast, recapture rates in *C. hortulanus* were good both on the starting plant as well as after they dispersed over the whole test area (tables 5.1, 5.2 and 5.5). Over a quarter of all released individuals could be detected at any point in time as having dispersed to other plants (table 5.1). This dispersal rate seems to stay very constant over the month. The same phenomenon as in *C. tuberculosus* could be observed on the first day of the experiment: There is a decrease of marked individuals on the first day but not as strong as observed in *C. tuberculosus*. The most common dispersal distance on a single day is up to 20 m, within 15 days the longest observed distance is 65 m what is quite high considering that they have to fly over other potential host plants doing that. Over the course of the experiment the main dispersal range lay within a radius of 20 m around the release plant (tables 5.1 and 5.2). This corresponds to the results of a dispersal experiment done by St. Pierre et al. (2005) with the leaf beetle species *Chrysochus auratus* and with the weevil *Rhyssomatus lineaticollis* (St. Pierre & Hendrix 2003), whereas the dispersal of *C. tuberculosus* is similar to *Anonema laticlavia* (St Pierre et al. 2005). A different distribution rate of females and males can be assumed here, as 17 of 37 recovered females left the starting plant, but only 6 of 24 males did. Possibly the females distribute far better than the males. Sex specific differences in dispersal might be caused by differences in their behavior: Females could be deterred by the high density of individuals on the release plant since they have to find suitable oviposition sites. Males, on the other hand, might find this plant with its high density of potential mates attractive. The returning of a *C. tuberculosus* male and a *C. hortulanus* male to the release plant provides some evidence for this idea. A similar pattern as the females may show in our case was observed in other beetles (Herzig & Root 1996) where habitats with a high density of individuals have a stronger dispersal rate than others.

Two distribution studies were done with another species of the Mecininae: *Mecinus janthinus* (Anthony 2005, Wilson et al. 2006). This species has the reputation to have a slow distribution rate. The results of these two trails tested could not be more different: The first investigation described a distribution rate of just six metres a year (Anthony 2005). Whereas the second study of Wilson et al. (2006) observed for the same species a rate of three kilometres in four years. According to them the species is very good in flying. Consequently

we do not know where the ecological truth behind these two experiments lies. A comparison of that and our data for *Cionus* shows a clear indication that the here presented rate is more like the rate observed by Wilson et al. (2006). The *Cionus* genera are in no way as lethargic as *M. janthinus* according to Anthony (2005). The Curculionidae are described to be a very settled with normally no dispersal greater than 1kmper year (with exceptions) as mentioned by many authors before (Moriya & Horoyoshi 1998, Toepfer et al. 1999, Nigg et al. 2001). A distribution rate of nearly a kilometre a year seems more likely under good circumstances for distribution – as wind for example. Referring to our observations the spreading attitude of *Cionus* is qualifying this weevil for a potential biological control duty.

That is maybe the first field observation of weevil's distribution on potential but - for the adult individuals - unknown host plants. The indication is that the establishment of this new released population is taking place is obvious. That these beetles are roughly found after a month is not surprising, they are over a year old at that moment and have done what their duty was – reproduction. In the Chapter 3 all weevils were collected from *Scrophularia*, too. The ones on the spring plant *Scrophularia* laid their eggs earlier than those transferred to the late summer plant *Verbascum*, and the prior became earlier senile, too. The guess that the reproduction is so exhausting that their lifespan shortens afterwards is not very unlikely. Another reason for the decreasing individual numbers is the very dry and hot summer – even the aboriginal weevils of the species *C. nigritarsis* and *C. hortulanus* got fewer.

In the context of the studies already performed with the genus *Cionus* we can make some further conclusions. (1) All previous indications that *C. hortulanus* is a single species and not two host races or cryptic species could be further corroborated here (Chapter 1, Chapter 3). (2) The guess that *Verbascum* is a possible host plant for all *C. hortulanus* populations could be confirmed (Chapter 3). (3) And that the establishment of *C. hortulanus* populations from *Scrophularia* on *Verbascum* is possible not only in laboratory (Chapter 3) but in the field is approved, too.

General Discussion

The different conclusions of my diverse experiments allow visualizing the host plant insect relationship of several genera of the Mecininae with a focus on the genus *Cionus* and an emphasis on the species *Cionus hortulanus.* The experiments will be discussed shortly in due consideration of previous experiments' results and then summarized comprehensively in a very short manner. Thus for a more detailed discussion of the single experiments have a look at each referring chapter. At the end an outlook is given for further experiments.

The phylogeny of the genera *Mecinus*, *Gymnetron*, *Rhinusa*, *Cionus*, *Cleopus* and *Stereonychus* containing mainly Middle European species reveals many new insights. Both on genera and on species level the knowledge of this weevil family enlarged caused by many new outcomes. Only these genera are analysed because they are all feeding on aucubin, catalpol or antirrhinoside (all iridoid glycosides) containing host plants (except *Stereonychus*, its host plant *Fraxinus* contains other iridoids) which is really conspicuous. The most prominent insight of the new phylogeny based on the nuclear EF1-α and the mitochondrial CO I/II genes is that the genus *Rhinusa* is not monophyletic (fig. 1.3) - contradictory to Caldara (2001), who made a phylogenetic tree based on 34 morphological characters. In my results the *Rhinusa* species living on *Verbascum* and *Scrophularia* are closer related to the genus *Gymnetron* living on *Veronica* than to the *Rhinusa* species developing on *Linaria*. The evidence is even stronger if I connect my results with the relationship of the host plant: *Verbascum*, *Scrophularia* and *Veronica* belong to the Scrophulariaceae whereas *Linaria* is member of the Plantaginaceae (Olmstad et al 2001, Albach et al. 2005). Consequently, in my phylogeny I have a *Gymnetron*/*Rhinusa* branch feeding on Scrophulariaceae and a *Rhinusa* branch on Plantaginaceae. This result is verisimilar because all branches of the tree are highly supported. And it is showing all hitherto concepts of the Mecinini phylogeny in another light and makes a new revision of the tribe essential.

Other insights from the new Mecinini phylogeny are 1) that the *Gymnetron* species living on *Plantago* should be placed in the genus *Mecinus*, just as Caldara (2001) suggested (fig. 1.3). 2) *Rhinusa tetrum* ab. *plagiellum* mentioned by Reitter (1916) is described as an aberration by GYLLENHAL, but my phylogeny clearly shows that it is an own species already described by ROSENSCHÖLD as *Rhinusa fuscescens*. 3) I cannot say if Caldara's phylogeny of the genera

from 2001 or 2008 is right because the resolution in the basal area is not good enough (fig. 1.3).

In relationship to their host plants the phylogeny of the Mecinini points out that mostly every cluster has its own host plant. Thus, every host plant switch lead to a radiation on the new plant. Only the genus *Mecinus* is mixes up its host plants as there are species on *Linaria* (*M. janthinus* and *M. heydeni*) and on *Plantago* (*M. pyraster*, *M. collaris* and several others) (fig. 1.3). After all, the host plants are a good phylogenetic marker for the tribe Mecinini.

The only problems within the Mecinini are two species clusters which could not be solved by my sequencing method (*R. netum* / *R. collinum* and *G. beccabungae* / *G. veronicae*). The reason for that might be that the time since speciation was not long enough to differentiate the selected genes enough, so far.

The phylogeny of the Cionini is different to the one of Caldara (2001) because the genus *Stereonychus* is in the present work a sister group to *Cleopus* and both together to *Cionus* (fig. 1.3). The host plant relationship in comparison to the phylogeny is a different case as in the phylogeny of the Mecinini. It seems clear that *Scrophularia* is the ancestral host plant of at least *Cionus*. A differentiation of the phylogeny caused by the host plants cannot be seen because after the basal species of *Cionus* the host plant (*Scrophularia* or *Verbascum*) use is mixed up in the tree (fig. 1.3). Consequently, there must have been many host plant switches in the history of this genus. Therefore, the host plants of the tribe Cionini cannot be used as a phylogenetic marker.

The knowledge of particular *Cionus* species increases, too: 1) The species *C. hortulanus* (the only one living on both host plants *Scrophularia* and *Verbascum*) seems to be a species without a cryptic speciation taking place. There is no strict dissociation of individuals taken from *Verbascum* and *Scrophularia* inside the phylogenetic tree (fig. 1.3). 2) *Cionus schultzei* is most obvious an distinct species and not only a race of *C. hortulanus* as indicated by Wingelmüller (1937).

These results will be the initial position for the discussion of the following four experiments. The first one is questioning if the Mecininae genera feeding on iridoid glycoside containing host plants are able to sequester these secondary plant compounds to defend themselves.

As it is described in chapter 2 the tested members of the Mecinini do not sequester IGs or to be precise they do not contain IGs in their adult life stage. What is remarkable because the

Cionini do sequester (table 2.2). But it matches quite perfectly with the ecological circumstances these tribes are living in and with their behaviour. The Mecinini have a very cryptic lifecycle as their larvae are living inside nearly every plant organs as stems, buds, seeds and root (Scherf 1964). Their overwintering takes place inside the plant as (in the most species) adults, too. Thus only the adults can be seen on the plants. Hence, their defence strategy seems to function without sequestered chemical compounds. Whereas the Cionini have prominent slug like larvae living ectophagously on their host plants covered by a viscous conspicuously secret. Even the pupation takes place outside the plants in amber coloured cocoons (Scherf 1964, Räther 1989). The larvae are not that immobile as it might sound here; indeed they are legless but because of their small larval abdominal legs they are able to move on the plant. That would be the third time in insects that this kind of abdominal legs evolved independently (Lepidoptera and Hymenoptera are the other two instances) (Prell 1925). The adults of the Cionini live ectophagously on the plants, more or less disguised depending on the host plant.

With that background information it is not surprising that the Cionini sequester and the Mecinini not (table 2.2). For the Cionini it seems to be a major key for its ectophagous lifecycle. The Mecinini do not require chemical defences because they are protected against predators by hiding inside the pants. That is a good correlation between chemical ecology, ecology and behaviour. It is a very interesting fact that the Mecinini do not sequester aucubin and catalpol because they are the first known specialist species on IG containing plants which are not sequestering these IGs at all.

Many already in the introduction mentioned publications described the benefits of an IG sequestration. There is an antimicrobial character against the entomopathogenic Bacteria *Bacillus thuringiensis* discovered for instance (Baden & Dobler 2009). For the leaf beetle genus *Longitarsus* a protection against chilopods (Baden & Dobler 2009) and ant species (Baden et al. 2011) is described, as well. Several Lepidoptera larvae are secured against wolf spiders (Theodoratus & Bowers 1999), spring spiders (Stromeyer et al. 1998), ants (de la Fuente et al. 1995, Dyer & Bowers 1996), the *Podisus maculiventris* bugs (Bowers & Stamp 1997), many other invertebrate predators (e.g. Nishida and Fukami 1989, Stamp 1992, Nishida 1995, Nieminen et al. 2003, Opitz et al. 2010) and vertebrate predators as well (e.g. Bowers, 1980, 1981; Bowers and Farley, 1990). Thus it is verisimilar that the Cionini gain an antimicrobial effect and a protection against predators what might be the main adaptation for their ectophagous lifestyle.

In comparison to the leaf beetle genus *Longitarsus* the concentrations of the sequestered IGs are quite high. The highest concentration of IGs in *Cionus* measured is 6.94 % of their dry weight (DW) for *C. hortulanus* (table 2.2) and for *Longitarsus* 2.23 % of the DW in *L. nigrofasciatus* (Willinger & Dobler 2001). These high amounts of sequestered IGs in *Cionus* are striking. Additionally, the affinity towards catalpol is quite high which is not uncommon and can be found in the genus *Longitarsus* (Willinger & Dobler 2001) as well as in several Lepidoptera species (e.g. Bowers and Puttick 1986, Belofsky et al. 1989, Bowers and Collinge 1992).

Another interesting result is that the tested *Cleopus* species do only sequester catalpol and no aucubin. One outstanding aspect is, that *C. pulchellus* from *Scrophularia* sequesters up to 5-8 times more catalpol than *C. solani* living on *Verbascum*. In the genus *Cionus* the opposite seems to be normal: species on *Scrophularia* sequester more aucubin and the ones on *Verbascum* more catalpol. Only the species *C. scrophulariae* and one population of *C. nigritarsis* are exceptions of that rule. This "rule" is even observable in one single species, *C. hortulanus*, the only species living on both plant genera. I will have a closer look at this species within the further three experiments later on.

Within the *Cionus* data are some remarkable details. Beginning with the weevils from *Scrophularia* the first noticeable species is *C. scrophulariae* because it sequesters more catalpol than aucubin as mentioned (with the quite high concentration of 3.13 % DW). Another astonishing result can be found by the comparison of the *C. alauda* populations (table 2.2): The IG amount of population 1 is much higher than of population 2 – although they were collected in nearby areas in the same month, only a year later. The similar time of the year is very important as the plants have a changing IG amount over the year (Bowers et al. 1992, Bowers & Stamp 1993, Fuchs & Bowers 2004). *C. nigritarsis* from *V. nigrum* is a special case because in one population the concentration of aucubin is higher than the catalpol one - that is remarkable for a species from *Verbascum* (table 2.2). The species with the most adapted lifecycle is *C. olens* has the lowest concentration of aucubin of all tested *Verbascum* feeding species and a normal catalpol concentration (table 2.2). This might be a hint for a better chemical adaptation, as well. Within this species occurs an abnormally which we already know similar for *C. alauda*: the two populations were collected at the same time in locations only a short distance away from each other – though the difference in the IG concentration is four times. Maybe the concentrations inside the plants are quite diverse even

in such small distances; to answer such questions it is essential to do experiments with weevils only on one single plant whose IG concentrations is known.

Again *C. hortulanus* is a special case. The two populations from *Verbascum* have a higher concentration of catalpol and the two from *Scrophularia* a higher aucubin one (table 2.2). The IG concentration of the *Scrophularia* feeding populations are low but normal for weevils living on *Scrophularia*, whereas the populations from *Verbascum* are not only very divers in their catalpol concentrations (1.62 % and 6.14 %) but also up to thirty-six times higher than the concentrations in the *Scrophularia* feeding populations - for aucubin it is only twice as high. Because all populations were collected in Schleswig-Holstein (Germany) the differences are enormous.

Nevertheless, the most stunning matter about the correlation between the plants and the sequestration of the weevils is that the tested plants *V. nigrum* and *S. nodosa* possess nearly equivalent concentrations of aucubin and catalpol (table 2.2). The question if the kind of sequestration is determined in the phylogeny is obvious. The phylogeny of the Cionini done in this study shows together with the sequestration data that there is not a particular point of host plant or sequestration change in the history of the Cionini, because the host plant switches occurred several times. This means that the shifts concerning the kind of sequestration must have evolved several times. One can only say that the tested species of the genus *Cleopus* do only sequester catalpol - therefore a phylogenetic incidence of sequestration type can be seen only at the genus level inside the Cionini.

For *C. hortulanus* the phylogeny shows that there seems to be no separation between populations from *Scrophularia* and *Verbascum* as mentioned before (fig. 1.3). Therefore an evolving ability of different sequestration types can be declined here, too. Or the differentiation in this species is so young that it is not possible to detect it with my experiments yet. Thus, the explanation of the different sequestration rates must be in the plant-insect interactions because that a special metabolism in the beetles occurred several times independently seems to be unlikely.

To answer this problem a further experiment with *C. hortulanus* was performed in which the plant-insect interactions were studied regarding the question if the different sequestration rates are induced by the weevil or the plant (Chapter 3). Therefore *C. hortulanus* females were taken from a population feeding on *Scrophularia* and were reared on separated *Scrophularia* and *Verbascum* plants. During that time samples from every life stage (except eggs) of the

beetles were collected together with plant samples. Their IG content was analysed by using a HPLC-MS.

The dataset showed many interesting details. The first and most obvious result is that the proportions of the aucubin-catalpol concentration in the individuals are the same as I observed on populations and species level: individuals on *Scrophularia* contained more aucubin and on *Verbascum* more catalpol (table 3.1, fig. 3.1 & fig. 3.2). The new data gave me much more insights into this issue I would never have gained without looking at the individual's level. The females seem to sequester not more IGs than the males, for example. Such different sequestration rates between the sexes are reported in other studies (Opitz & Müller 2009).

Though I measured a very high range of IG concentrations in the individuals of the offspring generation, the range was not higher than the ones I found on populations' or species' level in the previous experiment (Chapter 2). So there must be a difference in the ability to sequester IGs at the individual's level – not only at a species' level. That is a striking result for itself as many publications published the concentrations of sequestered IGs only for a few individuals or populations but our results clearly show that a few examples cannot stand for whole species. This means that the difference inside a single species is maybe too high to compare the exact sequestered concentrations between species.

After all, one problem still remains: the plants' IG concentrations do not explain the ones in the weevils (table 3.1, fig. 3.1 & fig. 3.2). Catalpol is more effectively sequestered in several cases not only in beetles (Willinger & Dobler 2001) but in other insects like Lepidoptera (Bowers & Puttick 1986, Belofsky et al. 1989, Bowers & Collinge 1992), too. In these publications are already some approaches to interpret this effective sequestration of catalpol: 1) Maybe the insects are able to transfer other IGs into catalpol (Bowers & Puttick 1986) - these could be derivatives of catalpol (Stermitz et al. 1986) – but others than aucubin (Bowers & Collinge 1992); 2) They just sequester catalpol much more efficient than aucubin (Stermitz et al. 1986).

What do all these instances tell us about the species *C. hortulanus*? The first definite thing is that populations taken from *Scrophularia* are able to perform their lifecycle on *Verbascum* as well. Hence, this is an additional hint that this species do not seem to perform a cryptic speciation. Another hint we get is that the concentration of aucubin in the plant might be responsible for the concentration in the weevil. Because one *Scrophularia* plant had a higher amount of aucubin in the beginning and a lower one at the end of the experiment – just as the

weevils had. That might be evidence that the so far detected amounts of IGs in other publications are more likely a mirror of the plants they were feeding on than the potential sequestration ability of the insects. Particularly, as the sequestration rates in *Cionus* and here for *C. hortulanus* especially are very diverse for the different individuals. And it is clear now that no differentiation of the weevils' genetic equipment is responsible for the differing sequestration rates – as all weevils were taken from the same population. The conclusion is that the differences are caused by the plants. The plants' concentrations of aucubin and catalpol cannot be the reason as mentioned; maybe there is a catalpol derivatives occurring in *Verbascum* but not in *Scrophularia*? This hypothetical derivatives could be the precursor for catalpol in the weevils' metabolism, what is the case in caterpillars of *E. anicia* or in sawfly larvae on *P. lanceolata* (Gardner and Stermitz, 1988; Opitz et al., 2010). A second possibility is that the *Verbascum* plant transforms aucubin into the more active catalpol while it is consumed and *Scrophularia* does not. That could be a possibility as aucubin is the precursor for catalpol in the plants' metabolism (Damtoft 1994).

After all, every *C. hortulanus* individual seems to be able to live on both potential host plant genera. But there are some ecological questions which are coming up instantly. One is if the weevils do have a favour host plant? To answer this question I performed an olfactorian test in which I tested most of the *Cionus* species occurring in Northern Germany (Chapter 4).

The result of this experiment indicates that the olfactory sense plays a major role in the recognition of the host plants, as we recorded for each species a positive response to their host plant. Linked with the established phylogeny of the Cionini some implications become clear (fig. 4.4). The first one is that both the basal species *C. alauda* and *C. scrophulariae* have a strong boundary towards their host plant *Scrophularia*. And then after at least two host plant switches in the phylogeny of *Cionus* (Fig 4.4) *C. tuberculosus* as well as *C. nigritarsis* favour their host plants *Scrophularia* respectively *Verbascum* (fig. 4.2). Both switches must have been connected with a change in 1) feeding and 2) olfactory behaviour - and that even for assumedly sister species.

In the literature are some ambiguities about the monophagy of the *Cionus* species. There are occasional records listed of species normally from *Scrophularia* to be found on *Verbascum* in Wingemüller (1937) and Freude et al. (1983). Maybe these are artificial information as I have never seen a specimen of a *Scrophularia* feeding species on *Verbascum* and the strong linkage and adaptation to host plants indicated by my data is quite astonishing. Previous studies indicate that host plant switches within the Curculionidae occur if species are pre-adapted to

the new host. These new hosts have normally something in common with the old one, like secondary metabolites – such a shift is often followed by a radiation scenario (Marvaldi et al. 2002, McKenna et al. 2009). Those radiations are often prominent enough to use them as phylogenetic marker even within the Mecininae (Caldara 2001 or the present work within the Mecinini). That is not possible for the Cionini (fig. 1.3). Therefore secondary metabolites of the host plant are possible phylogenetic markers (Mitter et al. 1991, Sprick 1997, Dobler 2001, Kergoat et al. 2005). As we can learn in the mentioned studies specialisation must not be a dead-end but can even lead to new host plants. Maybe such a pre-adaptation to the IGs inside the plants was the key for the radiation of *Cionus* mixed on two host plants. Such a host plant switch might not be very problematic if the plants contain the same secondary metabolites (Becerra 1997). Maybe it is a cue for pre-adaptation that *C. scrophulariae* has no significant preference for its host plant in comparison to *Verbascum*.

There are two more host plant switches in the phylogeny of *Cionus* which require a separate discussion and these belong to the species *C. hortulanus*. These host plant switches are different to those above because this species uses as mentioned before both host plants. Therefore the boundary is less strict and the individuals can be fed and reared on both plants as we know from other experiments (Chapter 3). Their linkage to their population's host plants is less clear: *C. hortulanus* from *Verbascum* favours this plant as well as *Scrophularia* over the control, but in comparison *Verbascum* is the favourite. Whereas the population from *Scrophularia* shows in the comparison no favouritism (fig. 4.3). A possible explanation might be found in the lifecycle of the plants. *Scrophularia* is growing in the first half of the summer and *Verbascum* in the second half. Hence, a host plant switch during the season from *Verbascum* to *Scrophularia* is highly unlikely. In contrast a switch from *Scrophularia* to *Verbascum* during a year is very well possible. As the olfactorian data do not show a strong division between the two populations it is no sign for a beginning speciation in this species - just as the phylogenetic and the chemical ecology data are not. We have to keep in mind that the whole discussions of these *C. hortulanus* host plant switches have not the same status as between species.

Is it really possible for *C. hortulanus* from *Scrophularia* to live on *Verbascum* in the field? Or is it only possible under constant conditions in the laboratory? Have the odour reactions towards *Verbascum* any ecological matter? What is about the information of *C. tuberculosus* on *Verbascum* by Wingelmüller (1937) and Freude et al. (1983)? Are *Cionus* species relatively immobile or are they able to spread? As such is contrarily discussed for *Mecinus*

(Anthony 2005, Wilson et al. 2006). To answer these questions I performed a dispersal experiment with *C. hortulanus* and *C. tuberculosus* from *Scrophularia nodosa* on *Verbascum nigrum* (Chapter 5).

The first and obvious answer is that both *Cionus* species are able to fly. The effect is that they seem to spread within a potential habitat during a season. Dispersal tests were performed for *Mecinus janthinus* before by Anthony (2005) and Wilson et al. (2006). Our present results are more familiar with those of Wilson et al. (2006) as the weevils are by no means as immobile as mentioned for *Mecinus* by Anthony (2005). After all, the Curculionidae are described to be very immobile with usually no greater dispersal than 1km per year (Moriya & Horoyoshi 1998, Toepfer et al. 1999, Nigg et al. 2001). That seems very possible for *Cionus* within a whole season, too.

C. tuberculosus should be discussed first. The first indication is obvious: The species is able to tolerate *V. nigrum* for a while even it is usually the wrong host plant. Now, it seems plausible that it is possible to find a *C. tuberculosus* on *V. nigrum* as mentioned by Wingelmüller (1937) and Freude et al. (1983). The number of rediscovered individuals decreased very fast - this can have many reasons (table 5.3): 1) The species was hiding on the ground in the shadows. 2) They may have dispersed very quickly and very far – even several kilometres. 3) They just died.

For the key species *C. hortulanus* the data shows another situation because the individuals' recovering was good (table 5.1). Within a month over a quarter of the specimens could be detected on other plants than the release plant. The dispersal rate itself seems to be quite constant. The most usual dispersal distance during a single day is up to 20 m - for the ones which moved at all. The farthest detected dispersal was up to 65 m within 15 days that is quite remarkable considering that the individual had to fly over dozens of potential host plants to do so. Anyway the main dispersal was within a 20 m radius around the release plant. This result fits perfectly with the data of St Pierre et al (2005) which were collected with the leaf beetle species *Chrysochus auratus* and those data collected with *Rhyssoamtus lineaticollis* (St Pierre & Hendrix 2003) as well. The dispersal of *C. tuberculosus* is more like *Anonema laticlavia* one (St Pierre et al. 2005). A different dispersal rate of males and females can be assumed for *C. hortulanus* as 46 % of the recovered females left the release plant but only 25 % of the males did (table 5.2). That overcrowded habitats do have a higher dispersal rate than others has already been discovered for the Coleoptera by Herzig and Root (1996).

In context with the already mentioned experiments concerning *Cionus hortulanus* I can make some further conclusions: The first is that in this experiment can no indications for a specification event be detected. Furthermore, it is now clear that *C. hortulanus* from *Scrophularia* is able to switch to *Verbascum* as host plant and to find the plant in particular.

This experiment is maybe the first field observation of weevils' distribution potential on for the individuals unknown plants. That this potential is existent is now obvious. That any individual is hardly found after four weeks is not surprising as they were adults of the former year and according to this species old. Additionally, the weather during these four weeks was very hot and dry.

After all, the experiments done with the mentioned genera of the Mecininae perform a picture of an interesting weevil group with specific relationships towards their host plants. Two different groups can be turned out very easily: the two tribes of the Mecinini and Cionini. The former have a cryptic lifestyle within their host plants at the larval stages (Scherf 1964) and the adults of tested species contain no sequestered aucubin or catalpol. Therefore they must have other options in their metabolism to cope with those toxins. Maybe a weevil with such a cryptic lifestyle is in no need of sequestered compounds to protect itself. The most interesting fact about that is that they are the first known specialised species on IG containing plants which do not sequester aucubin or catalpol. The relationship of the Mecinini towards their host plants is not only because of their sundry possibilities to live inside as well as outside (the adults) the plant a special one. Also because of the radiation they performed every time they occurred on a new host plant. This can be seen in their phylogeny (fig. 1.3). The only exceptions are the *Mecinus* species living on *Linaria*. All others are sorted very neatly into their clades and genera (with the mentioned problem that *Rhinusa* seems to be not monophyletic). This shows a strong boundary between these weevils and their host plants with maybe a hypothetic pre-adaptation to their host plants before. And it shows that the Mecinini had another kind of radiation than the Cionini, whereas the Mecinini performed a radiation after each occurrence on a new host plant the Cionini radiated mixed up on two host plants – both with result of mostly monophagous species.

The Cionini do not have this strong sorting of their species after their host plants. As one can see in the phylogeny there must have been multiple host plant switches during their evolution. They do have other kinds of relationships towards their host plants: In contrast to the Mecinini they do sequester the IGs aucubin and catalpol (*Cionus*) respectively only catalpol (*Cleopus*). The ratios of the sequestered IGs are with a few exceptions up to the weevil's host plant:

Cionus weevils living on *Scrophularia* contain a higher concentration of aucubin than catalpol and beetles taken from *Verbascum* more catalpol than aucubin (table 2.2). The reason for that is not clear yet, but it cannot be the concentrations of aucubin and catalpol in the plants or differences in the metabolism of the *Cionus* species: Even within one species taken from one population I observed these mentioned results. The several hypotheses for different sequestration patterns are mentioned before. The olfactory tests showed that there is a strong linkage between *Cionus* species and their host plants. This result is very fascinating as there have been many host plant switches within the genus and that might have been easier and more likely without such a strong favouritism. But even in the species *C. hortulanus* I was able to observe the mentioned differences of IG sequestration. To test if the *C. hortulanus* taken from *Scrophularia* are able to live on *Verbascum* in the field, too (I tested it under close to laboratory conditions before, see chapter 3), I conducted a dispersal experiment. This experiment showed that the species is able to live on both plants without any problems. This is a further proof that 1) *C. hortulanus* is single species and 2) that the different patterns in IG sequestration are more likely caused by differences between the host plants.

Those differences in the secondary metabolite concentrations of all IGs and their derivatives in *Verbascum* and *Scrophularia* species have to be precisely investigated in future. To investigate if the different patterns in IG sequestration are caused by catalpol derivatives (of which noteworthy concentrations only occur in *Verbascum*) and which could be precursors for catalpol in the weevil's metabolism. To make sure if the weevils are able to modify aucubin or other IGs to catalpol, it would be useful to run tests with radioactive labelled substances. The advantage of this method is that the labelled substances can be fed to the beetles and it can afterwards be observed if catalpol is produced by the weevils based on plant's precursors. We would therefore gain an insight into the weevils' metabolism and could most likely resolve the problem of the different sequestration pattern. But before we could do this we need the mentioned knowledge of the IGs existing in *Scrophularia* and *Verbascum* with a special attention to the ones only existing in *Verbascum*. Furthermore we would be able to investigate how the Mecinini cope with the ingested IGs. As they are the first known specialists feeding on aucubin and catalpol containing plants without sequestering those IGs it would be very interesting and important for this subject to better understand their metabolism. What are they doing with those IGs? Are IGs getting metabolized or simply excreted? And are those possibilities only to be found in the Mecinini or in other insects, too?

Furthermore we should investigate where in the weevil's body the IGs aucubin and catalpol are stored and what functions they fulfill. And as these IGs are present in larvae, pupae and adults of *Cionus* and *Cleopus*, it is of interest if there are differences between different life stages in the use of these IGs. Are the IGs presented to potential predators, to avoid an attack? Or are they used as agents against bacteria? Maybe the most crucial role of the sequestered IGs is the protection of the larvae, as those are very exposed and conspicuously coloured.

There are many questions left and many possibilities given to further work on with the Mecininae. But the level of knowledge is quite good by now. So far there were only few publications about the Mecininae and most are several decades old. By now we do not only have a very good idea of their phylogeny, but also of their patterns of sequestration, ecological traits and their plant-insect-interactions. Considering the multitude of sequestration patterns and differences in their ecological traits the weevils of the Mecininae are predestined to be the model organisms for future IG sequestration research. With the studies presented here a very good basis for further investigations is given, as the spectrum of available information about these weevils has now grown considerably.

References

Adler, L. S., Schmitt, J. & Bowers, M.D. (1995). Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). *Oecologia* 101: 75-85

Albach, D. C., Meudt, H. M. & Oxelmann, B. (2005). Piecing together the „new" Plantaginaceae. *American Journal of Botany* 92: 297-315

Anthony, A. (2005). Toadflax, Fire, *Mecinus janthinus* and compensatory growth. Master of Science Thesis in Land Resources and Environmental Science, Bozeman Montana: Montana State University.

Baden, C.U. & Dobler, S. (2009). Potential benefits of iridoidglycoside sequestration in *Longitarsus melanocephalus* (Coleoptera, Chrysomelidae). *Basic and Applied Ecology*. 10: 27-33

Baden, C. U., Geier, T., Franke, S. & Dobler, S. (submitted). Sequestered iridoid glycosides - highly effective deterrents against ant predators?

Bartholomaeus, A. & Ahokas, J. (1995). Inhibition of P-450 by Aucubin: is the biological activity of Aucubin due its glutaraldehyde-like aglucon? *Toxicology Letters* 80: 75-83

Barton, E. B. (2008). Phenotypic plasticity in seedling defense strategies: compensatory growth and chemical induction. *Oikos* 117: 917-925

Becerra, J. X. (1997). Insects on Plants: Macroevolutionary Chemical Trends in Host Use. *Science* 276: 253-256

Belofsky, G., Bowers, M. D., Janzen, S. & Stermitz, F. (1989). Iridoid glycosides of *Aureolaria flava* and their sequestration by *Euphydryas phaeton* butterflies. *Phytochemistry* 28: 1601-1604

Beninger, C. W., Cloutier, R. R. & Grodzinski, B. (2008). The Iridoid Glucoside, Antirrhinoside, from *Antirrhinum majus* L. has Differential Effects on Two Generalist Insect Herbivores. *Journal of Chemical Ecology* 34: 591-600

Beninger, C. W., Cloutier, R. R., Monteiro, M. A. & Grodzinski, B. (2007). The Distribution of Two Major Iridoids in Different Organs of *Antirrhinum majus* L. at Selected Stages of Development. *Journal of Chemical Ecology* 33: 731-747

Biere, A., Marak, H. B. & van Damme, J. M. M. (2004). Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? *Oecologia* 140: 430-441

Boros, C. A., & Stermitz, F. R. (1991). Iridoids. An updated review, Part II. *Journal of Natural Products* 54: 1173-1246

Boros, C. A., Stermitz, F. R. & McFarland, N. (1991). Processing of irdoid glycoside antirrhinoside from *Maurandya antirrhinoflora* (Scrophulariaceae) by *Meris paradoxa* (Geometridae) and *Lepipolys* species. *Journal of Chemical Ecology* 17: 1123-1133

Bowers, M. D. (1980). Unpalatability as a defense strategy of *Euphydryas phaeton* (Lepidoptera: Nymphalidae). *Evolution* 34: 586-600

Bowers, M. D. (1981). Unpalatability as a defense strategy of western checkerspot butterflies (*Euphydryas scudder*, Nymphalidae). *Evolution* 35: 367-375

Bowers, M. D. (1983). The role of iridoid glycosides in host-plant specificity of checkerspot butterflies. *Journal of Chemical Ecology* 9: 475-493

Bowers, M. D. (1984). Iridoid glycosides and host-plant specificity in larvae of the buckeye butterfly, *Junonia coenia* (Nymphalidae). *Journal of Chemical Ecology* 10: 1567-1577

Bowers, M. D. & Puttick, G. M. (1986). Fate of ingested iridoid glycosides in lepidopteran herbivores. *Journal of Chemical Ecology* 12: 169-178

Bowers, M.D. (1988). Chemistry and coevolution: iridoid glycosides, plants, and herbivorous insects. In: Spencer KC (ed) Chemical mediation of coevolution. Academic Press, San Diego pp. 133-165

Bowers, M. D. & Puttick, G. M. (1988). Response of generalist and specialist insects to qualitative allelochemical variation. *Journal of Chemical Ecology* 14: 319-334

Bowers, M. D. & Puttick, G. M. (1989). Iridoid glycosides and insect feeding preferences: gypsy moth (*Lymantria dispar*, Lymantriidae) and buckeyes (*Junonia coenia*, Nymphalidae). *Ecological Entomology* 14: 247-256

Bowers, M.D. & Farley, S. (1990). The behaviour of gray jays, *Perisoreus canadensis*, towards palatable and unpalatable Lepidoptera. *Animal Behav*iour 39, 699-705

Bowers, M. D. (1991). Iridoid glycosides. In: Rosenthal, G. A., Berenbaum, M. R. (Eds.), Herbivores: Their Interactions with Plant Secondary Metabolites, vol. I. Academic Press, San Diego, pp. 251-295

Bowers, M.D. (1992). The evolution of unpalatability and the cost of chemical defense in insects. pp 216-244. In: Roitberg BD, Isman MB (Eds.) Insect Chemical Ecology. An Evolutionary Approach. Chapman and Hall, New York

Bowers, M. D. & Stamp, N. E. (1992). Chemical variation within and between individuals of *Plantago lanceolata* (Plantaginaceae). *Journal of Chemical Ecology* 18: 985-995

Bowers, M. D. & Collinge, S. K. (1992). Fate of iridoid glycosides in different life stages of the buckeye, *Junonia coenia* (Lepidoptera: Nymphalidae). *Journal of Chemical Ecology* 18: 817-831

Bowers, M. D., Collinge, S. K., Gamble, S. E. & Schmitt, J. (1992). Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia* 91: 201-207

Bowers, M. D. & Stamp, N. E. (1993). Effects of plant age, genotype, and herbivory on *Plantago* performance and chemistry. *Ecology* 74: 1778-1791

Bowers, M. D., Bockvar, K. & Collinge, K. (1993). Iridoid glycosides of *Chelone glabra* (Scrophulariaceae) and their sequestration by larvae of a sawfly, *Tenthredo grandis* (Tenthredinidae). *Journal of Chemical Ecology* 19: 815-823

Bowers, M. D. & Stamp, N. E. (1997a). Fate of host-plant iridoid glycosides in lepidopteran larvae of Nymphalidae and Arctiidae. *Journal of Chemical Ecology* 12: 2955-2965

Bowers, M. D. & Stamp, N. E. (1997b). Effect of hostplant genotype and predators on iridoid glycoside content of pupae of a specialist insect herbivore, *Junonia coenia* (Nymphalidae). *Biochemical Systematics and Ecology* 25: 571-580

Bowers, M. D. (2003). Hostplant suitability and defensive chemistry of the Catalpa sphinx, *Ceratomia catalpae*. *Journal of Chemical Ecology* 29: 2359-2367

Brockerhoff, E. G., Withers, T. M., Kay, M. & Falds, W. (1999). Impact of the Defoliator *Cleopus japonicus* (Coleoptera, Curculionidae) on *Buddleja davidii* in the laboratory. *Proceedings of the Fifty Second New Zealand Plant Protection Conference.* 113-118

Caldara, R. (2001). Phylogenetic analysis and higher classification of the tribe Mecinini (Coleoptera, Curculionidae, Curculioninae). *Koleopterologische Rundschau* 71: 171-203

Caldara, R. (2008). Revisione Delle Specie Paleartiche Del Genere *Gymnetron* (Insecta, Coleoptera: Curculionidae). *ALDROVANDIA* 4: 27-103

Caldara, R., Sassi, D. & Tosevski, I. (2010). Phylogeny of the weevil genus *Rhinusa* STEPHENS based on adult morphological characters and host plant information (Coleoptera: Curculionidae). *Zootaxa* 2627: 39-56

Camara, M. D. (1997). Physiological mechanisms underlying the costs of chemical defence in *Junonia coenia* HÜBNER (Nymphalidae): A gravimetric and quantitative genetic analysis. *Evolutionary Ecology* 11: 451-469

Cerda, H., Fernandez, G., Lopez, A. & Varga, J. (1999). Olfactory attraction of the sugar cane weevil (Coleoptera: Curculionidae) to host plant odors and its aggregation pheromone. *Florida Entomologist* 82: 103-112

Cunningham, P. (1980). Biology of *Cionus hortulanus* (Fourc.) (Col, Curculionidae). *Entomologist's Monthly Magazine* 115: 243-244

Damtoft, S. (1994). Biosynthesis of catalpol. *Phytochemistry* 35: 1187-1189

Darrow, K. & Bowers, M. D. (1999). Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. *Journal of Chemical Ecology* 25: 1427-1440

Davini, E., Iavarone, C., Trogolo, C. Aureli, P. & Pasolini, B. (1986). The quantitative isolation and antimicrobial activity of the aglycone of aucubin. *Phytochemistry* 25: 2420-2422

De La Fuente, M.-A., Dyer, L. A. & Bowers, M. D. (1995). The iridoid glycoside, catalpol, as a deterrent to the predator *Camponotus floridanus* (Formicidae). *Chemoecology* 5/6: 13-18

Dickason, E. A. (1968). Observations on the Biology of *Gymnaetron pascuorum* (Gyll.) (Coleoptera, Curculionidae). *The Coleopterists' Bulletin* 22: 11-15

Dimmock, G. (1882). The Cocoons of *Cionus Scrophulariae*. *Psyche.* 3: 411-413

Dobler, S. (2001). Evolutionary aspects of defense by recycled plant compounds in herbivorous insects. *Basic and Applied Ecology* 2: 15-26

Dobler, S., Petschenka, G. & Pankoke, H. C. (2011). Coping with toxic plant compounds – the insect's perspective on iridoid glycosides and cardenolides. *Phytochemistry* (accepted)

Dyer, L. A. & Bowers, M. D. (1996). The importance of sequestered iridoid glycosides as a defense against an ant predator. *Journal of Chemical Ecology* 22: 1527- 1539

El-Naggar, L. J. & Beal, J. L. (1980). Iridoids. A Review. *Journal of Natural Products*. 43: 649-706

Fabre, J. H. (1922). The Life Of The Weevil – The works of J. H. Fabre. Hodder and Stoughton, London, 278 Seiten

Farrell, B. & Sequeira, A. S. (2004). Evolutionary Rates in the adaptive Radiation of Beetles on Plants. *Evolution* 58: 1984-2001

Franke, A., Rimpler, H. & Schneider, D. (1987). Iridoid glycosides in butterfly *Euphydryas cynthia* (Lepidoptera, Nymphalidae). *Phytochemistry* 26: 103-106

Franz, N. M. & Valente, R. M. (2005). Evolutionary trends in derelomine flower weevils (Coleoptera: Curculionidae): from associations to homology. *Invertebrate Systematics* 19: 499-530

Franz, N. M. & Engel, M. S. (2010). Can higher-level phylogenies of weevils explain their evolutionary success? A critical review. *Systematic Entomology*. 35: 597-606

Freude, H., Harde, K. W. & Lohse, G. A. (1983). Die Käfer Mitteleuropas. Band 11. Goecke & Evers, Krefeld, 342 Seiten

Fuchs, A. & Bowers, M. D. (2004). Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *Journal of Chemical Ecology* 30: 1723-1741

Gardner, D. R. & Stermitz, F. R. (1988). Host plant utilization and iridoid glycoside sequestration by *Euphydryas anicia* (Lepidoptera, Nymphalidae). *Journal of Chemical Ecology* 14: 2147-2168

Hamid, M. N., Perry, J. N., Powell, W. & Rennolls, K. (1999). The effect of spatial scale on interactions between two weevils and their food plant. *Acta Oecologica* 20: 537-549

Handjieva, N. V., Ilieva, E. I., Spassov, S. L. & Popov, S. S. (1993). Iridoid Glycosides from *Linaria* Species. *Tetrahedron* 49: 9261-9266

Harvey, J. A., van Nouhuys, S. & Biere, A. (2005). Effects of quantitative Variation in Allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *Journal of Chemical Ecology* 31: 287-302

Hernandez-Vera, G., Mirtovic, M., Jovic, J., Tosevski, I., Caldara, R., Gassmann, A. & Emerson, B. C. (2010). Host-associated genetic differentiation in a seed parasitic weevil *Rhinusa antirrhini* (Coleoptera: Curculionidae) revealed by mitochondrial and nuclear sequence data. *Molecular Ecology* 19: 2286-2300

Herzig, A. & Root, R. B. (1996). Colonization of host patches following long-distance dispersal by a goldenrod beetle, *Trirhabda virgate*. *Ecological Entomology* 21: 331-344

Høgedal, B. D. & Mølgaard, P. (2000). HPLC analysis of the seasonal and diurnal variation of iridoids in cultivars of *Antirrhinum majus*. *Biochemical Systematics and Ecology* 28: 949-962

Huelsenbeck, J. P. & Ronquist F. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17(8): 754–755

Hundsdoerfer, A. K., Rheinheimer, J. & Wink, M. (2009). Towards the phylogeny of the Curculionoidea (Coleoptera): Reconstructions from mitochondrial and nuclear ribosomal DNA sequences. *Zoologischer Anzeiger* 248: 9-31

Ishiguro, K., Yamaki, M. and Takagi, S. (1982). Studies on the iridoid related compounds. I. On antimicrobial activity of aucubigenin and certain iridoid aglycones. *Yakugaku Zasshi* 102, 755–759

Ishiguro, K., Yamaki, M. & Takagi, S. (1983). Studies on iridoid-related compounds, II. The structure and antimicrobial activity of aglucones of galioside and gardenoside. *Journal of Natural Products* 46: 532-536

Jamieson, M. A. & Bowers, M. D. (2010). Iridoid Glycoside Variation in the Invasive Plant Dalmatian Toadflax, *Linaria dalmatica* (Plantaginacae), and Sequestration by the Biological Control Agent, *Calophasia lunula*. *Journal of Chemical Ecology* 36: 70-79

Jensen, S. R., Albach, D. C., Ohno, T. & Grayer, R. J. (2005). *Veronica*: Iridoids and cornoside as chemosystematic markers. *Biochemical Systematics and Ecology* 33: 1031-1047

Jeong, H.-J., Koo, H.-N., Na, H.-J., Kim, M.-S., Hong, S.-H., Eom, J.-W., Kim, K.-S., Shin, T.-Y. & Kim, H.-M. (2002). Inhibition of TNF-α and IL-6 production by Aucubin through Blockade of NF-κB activation in RBL-2H3 mast cells. *Cytokine* 18: 252-259

Kaplan, M. A. C. & Gottlieb, O. R. (1982). Iridoids as Systematic Markers in Dicotyledons. *Biochemical Systematics and Ecology* 10: 329-347

Kergoat, G. J., Delobel, A., Fediere, G., Ru, B. L. & Silvain, J.-F. (2005). Both host–plant phylogeny and chemistry have shaped the African seed-beetle radiation. *Molecular Phylogenetics and Evolution* 35: 602-611

Kim, D.-H., Kim, B.-R., Kim, J.-Y. & Jeong Y.-C. (2000). Mechanism of covalent adduct formation of aucubin to proteins. *Toxicology Letters* 114: 181-188

Klockars, G. K., Bowers, M. D. & Cooney, B. (1993). Leaf variation in iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and oviposition of the buckeye, *Junonia coenia* (Nymphalidae). *Chemoecology* 4: 72-78

Kölsch, G. & Pedersen, V. B. (2008). Molecular phylogeny of reed beetles (Col., Chrysomelidae, Donaciinae): The signature of ecological specialization and geographical isolation. *Molecular Phylogenetics and Evolution* 48: 936-962

Konno, K., Hirayama, C. & Shinbo, H. (1996). Glycine in digestive juice: a strategy of herbivorous insects against chemical defense of host plants. *Journal of Insect Physiology* 43: 217-224

Konno, K., Yasui, H., Hirayama, C. & Shinbo, H. (1998). Glycine protects against strong proteindenaturing activity of oleuropein, a phenolic compound in privet leaves *Journal of Chemical Ecology* 24: 735-751

Konno, K., Hirayama, C., Yasui, H. & Nakamura, M. (1999). Enzymatic activation of oleuropein: A crosslinker used as a chemical defense in the privet tree. *Proceedings of the National Academy of Science* 96: 9159-9164

Lampert, E. & Bowers, M. D. (2010). Host plant influences on iridoid glycoside sequestration of generalist and specialist caterpillars. *Journal of Chemical Ecology* 36, 1101-1104

Liu, J., He, Q.-J., Zou, W., Wang, H.-X., Bao, Y.-M., Liu, Y.-X. & An, L.-J. (2006). Catalpol increases hippocampal neuroplasticity and up-regulates PKC and BDNF in the aged rats. *Brain Research.* 1123: 68-79

Lopez-Vaamonde, C., Godfray, H. C. J. & Cook, J. M. (2003). Evolutionary dynamics of host-plant use in a genus of leaf-mining moth. *Evolution* 57: 1804-1821

Marak, H. B., Biere, A. & van Damme, J. M. M. (2002a). Two herbivore-deterrent iridoid glycosides reduce the in-vitro growth of a specialist but not of a generalist pathogenic fungus of *Plantago lanceolata* L. *Chemoecology* 12: 185-192

Marak, H. B., Biere, A. & van Damme, J. M. M. (2002b). Systemic, genotype-specific induction of two herbivore-deterrent iridoid glycosides in *Plantago lanceolata* L. in response to fungal infection by *Diaporthe adunca* (ROB.) NIESSEL. *Journal of Chemical Ecology* 28: 2429-2448

Marvaldi, A. E., Sequeira, A. S., O'Brien, C. W. & Farrell, B. D. (2002). Molecular and Morphological Phylogenetics of Weevils (Coleoptera, Curculionoidea): Do Niche Shifts Accompany Diversication? *Systematic Biology* 51: 761-785

McKenna, D. D., Sequeira, A. S., Marvaldi, A. E. & Farrell, B. D. (2009). Temporal lags and overlap in the diversification of weevils and flowering plants. *PNAS* 7083-7088

McNeill, M. R., Withers, T. M. & Goldson, S. L. (2005). Potential non-target impact of *Microctonus aethiopoides* LOAN (Hymenoptera: Braconidae) on *Cleopus japonicus* WINGELMÜLLER (Coleoptera: Curculionidae), a biocontrol agent for putative release to control the butterfly bush *Buddleja davidii* FRANCHET in New Zealand. *Australian Journal of Entomology* 44: 201-207

Mead, E. W., Foderaro, T. A., Gardner, D. R. & Stermitz, F. R. (1993). Iridoid glycoside sequestration by *Thessalia leanira* (Lepidoptera: Nymphalidae) feeding on *Castilleja integra* (Scrophulariaceae). *Journal of Chemical Ecology* 19: 1155-1166

Mitter, C., Farrell, B. & Futuyma, D. J. (1991). Phylogenetic Studies of Insect-Plant Interactions: Insights into the Genesis of Diversity. *Tree* 6: 290-293

Moriya, S. & Horoyoshi, S. (1998). Flight and locomotion activity of the sweet potato weevil (Coleoptera, Brentidae) in relation to adult age, mating status and starvation. *Journal of Economic Entomology* 91: 439-443

Newman, D. A. & Thomson, J. D. (2005). Interactions among nectar robbing, floral herbivory and ant protection in *Linaria vulgaris*. *OIKOS* 110: 497-506

Nieminen, M., Suomi, J., Van Nouhys, S., Sauri, P. & Riekkola, M. L. (2003). Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *Journal of Chemical Ecology* 29, 823-844

Nigg, H. N., Simpson, S. E., Ramos, L. E., Tomerlin, T., Harrison, J. M. & Cuyler, N. (2001). Distribution and movement of adult *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in a Florida citrus grove. *Florida Entomologist* 84: 641-651

Nishida, R. & Fukami, H. (1989). Host plant iridoid-based chemical defense of an aphid, *Acyrthosiphon nipponicus*, against ladybird beetles. *Journal of Chemical Ecology* 15, 1837-1845

Nishida, R. (1995). Sequestration of plant secondary compounds by butterflies and moths. Chemoecology 5/6, 127-138.

Norusis, M. J. (2008). SPSS 17.0 guide to data analysis, Prentice Hall, Upper Saddle River, New Jersey, 672 Seiten

Oberprieler, R. G., Marvaldi, A. E. & Anderson, R. S. (2007). Weevils, weevils, weevils everywhere. *Zootaxa* 1668: 491-520

Olmstead, R. G., DePamphilis, C. W., Wolfe, A. D., Young, N. D., Elisons, W. J. & Reeves, P. A. (2001). Disintegration of the Scrophulariaceae. *American Journal of Botany* 88(2): 348-361

Opitz, S. E. W. & Müller, C. (2009). Plant chemistry and insect sequestration. *Chemoecology*, 19, 117-154

Opitz, S. E. W., Jensen, S. R. & Müller, C. (2010). Sequestration of Glucosinolates and Iridoid Glucosides in Sawfly Species of the Genus *Athalia* and Their Role in Defense Against Ants. *Journal of Chemical Ecology* 36, 148-157

Pardo, F., Perich, F., Torres, R. & Delle Monache, F. (1998). Phytotoxic iridoid glucosides from the roots of *Verbascum thapsus*. *Journal of Chemical Ecology* 24: 645-653

Pereyra, P. C. & Bowers, M. D. (1988). Iridoid glycosides as oviposition stimulants for the buckeye butterfly, *Junonia coenia* (Nymphalidae). *Journal of Chemical Ecology* 14: 917-928

Peters, R. S. & Abraham, R. (2009). A wind tunnel olfactometer of novel desgin: Testing the response to substrate volatiles on a vertical gauze screen. *Entomologische Mitteilungen aus dem zoologischen Museum Hamburg* 15: 127-133

Prell, H. (1925). 4. Zur Biologie der Blattschaber (Cionini). I. Die Enstehung der larvalen Gallerthülle und des Puppenkokons. *Zoologischer Anzeiger* 62: 3-48

Prudic, K. L., Oliver, J. C. & Bowers, M. D. (2005). Soil nutrient effects on oviposition preference, larval performance, and chemical defense of a specialist insect herbivore. *Oecologia* 143: 578-587

Pungitore, C. R., Ayub, M. J., Garcia, M., Borowski, E. J., Sosa, M. E., Ciuffo, G., Giordano, O. S. & Tonn, C. E. (2004). Iridoids As Allelochemicals and DNA Polymerase Inhibitors. *Journal of Natural Products* 67: 357-361

Räther, M. (1989). Notes on four weevils in the tribe Cionini (Coleoptera: Curculionidae) associated with *Scrophularia nodosa* L. (Scrophulariaceae). Part I: Biology and ecology of the weevils. *Bonner zoologische Beiträge* 40: 109-121

Rayor, L. S. & Munson, S. (2002). Larval feeding experience influences adult predator acceptance of chemically defended prey. *Entomologia Experimentalis et Applicata* 104: 193-201

Reitter, E. (1916). Fauna Germanica. Die Käfer des deutschen Reiches. K. G. Lutz Verlag, Stuttgart 231 pages

Rheinheimer, J. (2002). Die artenreichste Tierfamilie der Welt. *Biologie in unserer Zeit* 32: 236-243

Rombouts, J. E. & Links, J. (1956). The chemical nature of the antibacterial substance present in *Aucuba japonica* THUNBG. *Experimentia* XII/2: 78-80

Ronquist, F. & Huelsenbeck, J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioniformatics* 19: 1572-1574

Rønsted, N., Franzyk, H., Mølgaard, P., Jaroszewski, J. W. & Jensen, S. R. (2003). Chemotaxonomy and evolution of *Plantago* L. *Plant Systematics and Evolution* 242: 63-82

Rønsted, N., Göbel, E., Franzyk, H., Jensen, S. R. & Olsen, C. E. (2000). Chemotaxaonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. *Phytochemistry* 55: 337-348

Scherf, H. (1964). Die Entwicklungsstadien der mitteleuropäischen Curculioniden (Morphologie, Bionomie, Ökologie). *Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft* 506: 1-335

Sesterhenn, K, Distl, M. & Wink, M. (2007). Occurrence of iridoid glycosides in in vitro cultures and intact plants of *Scophularia nodosa* L.. *Plant Cell Reports* 26: 365-371

Smith, J. M. (1959). Notes on Insects, Especially *Gymnaetron* ssp. (Coleoptera: Curculionidae), Associated with Toadflax, *Linaria vulgaris* Mill. (Scrophulariaceae), in North America. *The Canadian Entomologist* 41: 116-121

Sprick, P. (1997). Beiträge zur Ökologie phytophager Käfer (Col., Chrysomelidae, Curculionoidea) II. Plausibilitätsprüfung von Wirtspflanzenangaben bei phytophagen Käfern unter besonderer Berücksichtigung sekundärer Pflanzeninhaltsstoffe. *Mitteilungen der Arbeitsgemeinschaft Rheinischer Koleopterologen* 7: 73-104

Stamp, N. E. (1992). Relative susceptibility to predation of two species of caterpillar on plantain. *Oecologia* 92: 124-129

Stamp, N. E. & Bowers, M. D. (1996). Consequences for plantain chemistry and growth when herbivores are attacked by predators. *Ecology* 77: 535-549

Stamp, N. E. & Bowers, M. D. (2000). Do enemies of herbivores influence plant growth and chemistry? Evidence from a seminatural experiment. *Journal of Chemical Ecology* 26: 2367-2386

Stephenson, A. G. (1982). Iridoid glycosides in the nectar of *Catalpa speciosa* are unpalatable to nectar thieves. *Journal of Chemical Ecology* 8: 1025-1034

Stermitz, F. R., Gardner, D. R., Odendaal, F. J. & Ehrlich, P. R. (1986). *Euphydryas anicia* (Lepidoptera: Nymphalidae) utilization of iridoid glycosides from *Castilleja* and *Besseya* (Scrophulariaceae) host plants. *Journal of Chemical Ecology* 12: 1459-1468

Stermitz, F. R. (1988). Iridoid glycosides and aglycones as chiral synthons, bioactive compounds, and lepidopteran defenses. pp. 397–402. In: Cutler, H. G. (Ed.), Biological Active Natural Products, Symposium. American Chemical Society, Washington D.C.

Stermitz, F. R., Gardner, D. R. & McFarland, N. (1988). Iridoid Glyocisde Sequestration By Two Penstemon-Feeding Geometrid Larvae. *Journal of Chemical Ecology* 14: 435-441

St. Pierre, M. J. & Hendrix, S. D. (2003). Movement patterns of *Rhyssomatus lineaticollis* SAY (Coleoptera: Curculionidae) within and among *Asclepias syriaca* (Asclepiadaceae) patches in a fragmented landscape. *Ecological Entomology* 28: 579-586

St. Pierre, M. J., Hendrix, S. D. & Lewis, C. K. (2005). Dispersal ability and host plant characteristics influence spatial population structure of monophagous beetles. *Ecological Entomology* 30: 105-115

Strohmeyer, H. H., Stamp, N. E., Jarzomski, C. M. & Bowers, M. D. (1998). Prey species and prey diet affect growth of invertebrate predators. *Ecological Entomology* 23: 68-79

Taskova, R. M., Peev, D., & Handjieva, N. (2002a). Iridoid glucosides of the genus *Veronica* s.l. and their systematic significance. *Plant Systematics and Evolution* 231: 1-17

Taskova, R. M., Gotfredsen, C. H. & Jensen, S. R. (2006). Chemotaxonomy of Veronicaceae and its allies in the Plantaginaceae. *Phytochemistry* 67: 286-301

Taskova, R. M., Evstatieva, L., Handjieva, N. & Popov S. (2002b). Iridoid Patterns of Genus *Plantago* L. and their systematic significance. *Zeitschrift für Naturforschung* 57c: 42-50

Theodoratus, D.H. & Bowers, M. D. (1999). Effects of sequestered iridoid glycosides on prey choice of the prairie wolf spider, *Lycosa carolinensis. Journal of Chemical Ecology* 25: 283–295

Toepfer, S., Gu, H. & Dorn, S. (1999). Spring colonisation of orchards by *Anthonomus pomorum* from adjacent forest borders. *Entomologia Experimentalis et Applicata* 93: 131-139

Tristram, J. N. (1978). The peritrophic membrane and cocoon ribbons in larvae of *Cionus scrophulariae*. *Journal of Insect Physiology* 24: 391-398

Tunset, K., Nilssen, A. C. & Andersen J. (1993). Primary attraction in host recognition of coniferous bark beetles and bark weevils (Col., Scolytidae and Curculionidae). *Journal of Applied Entomology* 115: 155-169

Urban, C. (1930a). Beiträge zur Naturgeschichte einiger Rüsselkäfer. III. *Entomologische Blätter* 26: 97-104

Urban, C. (1930b). Beiträge zur Naturgeschichte einiger Rüsselkäfer. III. *Entomologische Blätter* 26: 171-176

Van der Sluis, W. G., van der Nat, J. M. & Labadie, R. P. (1983). Thin layer chromatographic bioassay of iridoid and secoiridoid glucosides with a fungitoxic aglucone moiety using β-glucosidase and the fungus *Penicillium expansum* as a test organism. *Journal of Chromatography* 259: 522-526

Wahlberg, N. (2001). The phylogenetics and biochemistry of host-plant specialization in Melitaeine butterflies (Lepidoptera: Nymphalidae). *Evolution* 55: 522-537

Wieffering, J. H. (1966). Aucubinartige Glucoside (Pseudokinane) und verwandte Heteroside als systematsiche Merkmale. *Phytochemistry* 5: 1053-1064

Willinger, G. & Dobler, S. (2001). Selective sequestration of iridoid glycosides from their host plant in *Longitarsus* flea beetles. *Biochemical Systematics and Ecology*. 29: 335-346

Wilson, L. M., Sing, S. E., Piper, G. L., Hansen, R .W., De Clerck-Floate, R., MacKinnon, D. K. & Randall, C. (2005). Biology and Biological Control of Dalmatian and Yellow Toadflax. USDA Forest Service, FHTET – 05 – 13

Wingelmüller, A. (1937). Monographie der paläarktischen Arten der Tribus Cionini. *Koleopterologische Rundschau* 23: 143-221

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64: 3-19

Withers, T., Richardson, B., Kimberley, M., Moore, J., Kay, M. & Jones, D. (2003). Can population modeling predict potential impacts of biocotrol? A case study using *Cleopus japonicus* on *Buddleja davidii*. *Proceedings of the XI International Symposium on Biological Control of Weeds* 57-62

Wurst, S., Van Dam, N. M., Monroy, F., Biere, A. & Van der Putten, W. H. (2008). Intraspecific Variation in Plant Defense Alters Effects of Root Herbivores on Leaf Chemistry and Aboveground Herbivore Damage. *Journal of Chemical Ecology* 34: 1360-1367

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