Inter- and Intrapopulation Variability in the Composition of Larval Defensive Secretions of Willow-Feeding Populations of the Leaf Beetle *Chrysomela lapponica*

Sven Geiselhardt[1] · Monika Hilker[1] · Frank Müller[1] · Mikhail V. Kozlov[2] · Elena L. Zvereva[2]

Abstract We explored the inter- and intrapopulation variability in the larval defensive chemistry of the leaf beetle *Chrysomela lapponica* with respect to the salicylic glycoside (SG) content of its host species. Secretions of larvae from three populations associated in nature with SG-poor willows contained nearly twice as many components and 40-fold higher concentrations of autogenously produced isobutyrates and 2-methylbutyrates than secretions of larvae from three populations associated with SG-rich willows, which in turn had 200-fold higher concentrations of host-derived salicylaldehyde. Reciprocal transfer experiments showed that the larvae from populations associated with SG-rich willows did not produce appreciable amounts of butyrates on either SG-rich or SG-poor willows, while populations feeding on several SG-poor willow species retained the ability for efficient sequestration of SGs, along with their ability to produce high amounts of isobutyrates and 2-methylbutyrates. Only the populations associated with SG-poor willows demonstrated among-family variation in the composition of defensive secretion and differential responses of individual families to willows with alternative SG levels, which can be seen as the prerequisites for shifting to novel hosts. These non-specialized populations show a dual defensive strategy, which corresponds to the ancestral state of this species, while populations that fully depend on host-derived toxins (feeding on SG-rich willows) or have lost the ability to produce salicylaldehyde (feeding on birches) are most deviant from the ancestral state. The results indicate that defensive strategies may differ between populations within a species, and suggest that this variation reduces extinction risks and maintains the high ecological diversity and wide distribution of *C. lapponica*.

Introduction

Phytophagous insects represent a large part of terrestrial biodiversity, and their high degree of specialization may be a key factor driving their extraordinary species richness. The need to cope with the plethora of toxic secondary metabolites of plants has significantly contributed to the specialization of phytophagous insects (Futuyma and Agrawal 2009).

Adaptation to specific plant metabolites may be a mixed blessing for the insect. On the one hand, this type of adaptation provides the chance to avoid competition with generalist herbivores. On the other, adaptation to specific plant compounds may limit the potential host range to those plant species that share a similar pattern of secondary metabolites. This view is supported by several molecular studies showing that the evolution of host plant affiliations matches secondary plant chemistry better than it matches plant phylogeny (Becerra 1997; Funk et al. 1995; Futuyma and McCafferty 1990). Thus, some authors have inferred that specialization could lead to an evolutionary dead end because it may result in the loss of genetic

✉ Elena L. Zvereva
elezve@utu.fi

[1] Institute of Biology, Dahlem Centre of Plant Sciences, Freie Universität Berlin, Haderslebener Str. 9, D-12163 Berlin, Germany

[2] Section of Ecology, Department of Biology, University of Turku, Turku FI-20014, Finland

diversity and consequently preclude adaptation to changing environments that might require a host shift (Kelley and Farrell 1998; Mayr 1963).

Leaf beetles of the subtribe Chrysomelina, which are generally highly specialized and produce defensive chemicals, represent a good model for elucidating the evolutionary history of host plant shifts and for exploring the underlying forces. Adult beetles of this taxon are defended by autogenously produced secretions, but the larval defensive system shows different degrees of host plant dependency (Pasteels et al. 1982). A host plant independent defense by *de novo* synthesized iridoid monoterpenes (e.g., chrysomelidial) is considered the ancestral state in the subtribe Chrysomelina (Termonia et al. 2001). Larvae of *Chrysomela* spp. and *Phratora vitellinae* have independently lost the ability to produce iridoid monoterpenes but instead have started to sequester salicylic glycosides (SGs; e.g., salicin and salicortin) from their hosts (Pasteels et al. 1983a, b; Pavan 1953). The SGs are hydrolyzed by a β-glucosidase and subsequently oxidized by a salicyl alcohol oxidase to provide salicylaldehyde (Brückmann et al. 2002; Michalski et al. 2008), the major compound of the larval defensive secretion (Pasteels et al. 1982). Finally, the common ancestor of the monophyletic *Chrysomela interrupta* species group has evolved a dual defensive strategy by retaining the ability to produce salicylaldehyde from sequestered plant SGs and additionally by developing the ability to produce esters of plant-derived alcohols with *de novo* synthesized carboxylic acids, i.e. iso- and 2-methylbutyric acid (Blum et al. 1972; Hilker and Schulz 1994; Schulz et al. 1997; Termonia et al. 2001).

In recent decades, *C. lapponica* (that belongs to the *interrupta* species group) has served as a model organism for a number of ecological and evolutionary studies. These studies have in particular explored among-population differences in genetic structure (Machkour-M'Rabet et al. 2008; Mardulyn et al. 2011) and host plant use (Gross et al. 2004a; Zvereva et al. 2010a), as well as factors that may have contributed to host plant shifts in *C. lapponica* (Gross et al. 2004b, 2007; Termonia and Pasteels 1999; Zvereva et al. 2010b). However, although larval defensive secretions are commonly recognized as important in the evolution of host plant use by this leaf beetle (Hilker and Schulz 1994; Kirsch et al. 2011; Termonia et al. 2001; Zvereva et al. 2010b), differences in the defensive chemistry between populations specialized on different host plants and originating from different geographic regions need further investigations. The complete lists of the components of larval defensive secretions are available only for the birch-feeding populations from Bavaria (Hilker and Schulz 1994) and Altai (Tolzin-Banasch et al. 2011), while only major compounds have been reported for a birch-feeding population from the Czech Republic and for a Finnish population associated with SG-rich willow, *S. myrsinifolia* (referred to as *S. borealis*: Gross and Hilker 1995; Gross et al. 2002). The secretion of larvae feeding on birches (leaves of which do not contain SGs) did not contain salicylaldehyde, which was the major defense component of larvae feeding on SG-rich willows (Gross and Hilker 1995; Hilker and Schulz 1994).

The dual defense strategy of the *interrupta* species group has been hypothesized to decrease the beetle's dependence on host plant chemistry and to allow specialized species to expand their host ranges or even shift to novel host plants, in particular to plants not containing SGs (Kuhn et al. 2004; Mardulyn et al. 2011; Termonia and Pasteels 1999; Termonia et al. 2001). The cited studies focus on host shifts from the Salicaceae in general to Betulaceae. However, willow species differ considerably in the chemistry of their foliage, in particular with respect to the SG content; some willows are almost completely lacking SGs (Julkunen-Tiitto 1989). Some populations of *C. lapponica* have evolved local adaptations to SG-poor or SG-rich willow species, whereas other populations remain non-specialized and exploit hosts with different SG levels (Zvereva et al. 2010a). This variation in the use of host plants, in combination with the detailed knowledge on both bottom-up and top-down factors affecting several populations of *C. lapponica* (Gross et al. 2004a, b; Hilker and Schulz 1994; Zvereva and Kozlov 2000; Zvereva and Rank 2003; Zvereva et al. 2002, 2010a, b), offers rich opportunities to study evolutionary mechanisms underlying host plant shifts in herbivores.

A prerequisite for the development of diverging traits of populations feeding on ancestral and novel host plants is genetic variation of the original population in traits that allow the use of a new host (Messina and Durham 2013; Tilmon et al. 1998). Both bottom-up and top-down forces may be involved in natural selection that leads to a host plant shift (Bernays and Graham 1988; Jaenike 1990; Joshi and Thompson 1995), and, therefore, variation in both host plant use and defense against natural enemies form the basis for this shift. The outcome of local selection depends on the genetic structure of the population; consequently, knowledge of the genetic variation (in relation to both life history and defensive traits) is necessary to determine which traits could be subjected to natural selection. Top-down factors have been hypothesized to contribute to a shift of some populations of *C. lapponica* to novel SG-poor hosts (Gross et al. 2004b; Zvereva et al. 2010b); however, the inter- and intrapopulation variability of this leaf beetle in the amount and composition of larval defensive secretions is poorly documented.

The aim of this study was to elucidate the variation in the larval defensive chemistry of *C. lapponica* and to discuss the ecological and evolutionary importance of this variation. We (1) compared the volume and composition of larval defensive secretions between populations feeding in nature on willows with different SGs levels, (2) evaluated the population potential for host plant shifts by investigating the plasticity of the

amount and composition of the secretions in response to transfer of larvae to a willow species with a contrasting SG level, and (3) explored the within-population variation in concentrations of major secretion components (both sequestered and autogenously produced).

Methods and Materials

The Study Object The leaf beetle *C. lapponica* L. is a univoltine species widely distributed in the Palaearctic region. Different populations feed on Salicaceae or Betulaceae. Adults hibernate in soil and start feeding and copulating on host plants soon after leaf flush. Females lay batches of 35–40 eggs on host plant leaves. Larvae feed for about 1 month and pupate on the host plants. When disturbed, larvae release droplets of defensive secretion from nine pairs of eversible glands on the dorsal side of the thorax and abdomen.

For this study, we used six populations of *C. lapponica* (Table 1) that differed in their primary host plants and in the presence of local adaptations to these hosts (i.e., demonstrated better fitness on the preferred host relative to other available hosts, according to the approach outlined by Kawecki and Ebert 2004). Three populations from Fennoscandia (Finland, Nikel, and Monche) feed in nature on SG-rich *S. myrsinifolia*, the Belarus population feeds primarily on SG-poor *S. caprea*, Ural and Baikal populations feed on several willow species containing traces to moderate amounts of SGs (Table 1). The Belarus, Ural, and Baikal populations are referred hereafter as populations associated with SG-poor willows.

Overwintered mated females from all populations were collected in spring 2004–2006 and transported to Apatity (Murmansk region, NW Russia). Females were kept individually in Petri dishes on leaves of the same willow species from which they were collected. Five egg batches per population (laid by different females) were selected, and emerged larvae from each batch (hereafter called a family) were divided into ten equal groups. Each group (2–4 larvae) was reared in a separate vial on one of the five individuals of two willow species differing in the SG level (altogether 50 vials per population): *S. myrsinifolia* (40–80 mg/g salicortin) and *S. caprea* (traces to 0.7 mg/g salicortin). Larvae of the Ural population, which feed in nature mostly on *S. glauca* (3.8 mg/g salicortin; all concentrations according to Julkunen-Tiitto 1989), and of the Monche population were additionally kept on *S. glauca* (Table S1).

Secretion Sampling and Analysis Droplets of secretions emitted by individual fourth instar larvae in response to disturbance were collected from all glands in calibrated glass capillaries, flame-sealed, and stored at −18 °C. Volumes of secretions were calculated from the length between the menisci of the secretion inside the capillary and the capillary diameter. The chemical composition of larval secretions was analyzed by gas chromatography coupled with mass spectrometry (GC/MS) on a Fisons GC model 8060 coupled to a Fisons MD 800 quadrupole MS (EI-mode at 70 eV). Each sample was dissolved in 10 µl dichloromethane with dodecane (100 ng/µl) as an internal standard (IS). The solution was mixed thoroughly by repeatedly aspirating and expelling the sample with the injection syringe at least five times before injection. An aliquote of 1 µl of a dissolved secretion sample was injected at 240 °C. Samples were separated on a 30-m DB5-ms capillary column (0.32-mm i.d., film thickness 0.25 µm, J. & W. Scientific, Folsom, CA, USA) with helium as the carrier gas. The temperature program started at 40 °C for 4 min and then increased to 280 °C at a rate of 10 °C/min. Eluted compounds were identified by comparing mass spectra and retention

Table 1 Characteristics of the studied populations of *Chrysomela lapponica*

Name	Latitude, N	Longitude, E	Preferred host plants (SG[1])	Local adaptation[2]
Finland	70°05′	27°51′	*Salix myrsinifolia* (high)	Yes
Nikel[3]	69°24′	30°13′	*S. myrsinifolia* (high)	Yes
Monche	67°52′	32°48′	*S. myrsinifolia* (high)	Yes
Ural	67°03′	63°34′	*S. glauca* (low to moderate) *S. dasyclados* (low to moderate) *S. phylicifolia* (low) *S. lanata* (low to moderate)	No
Baikal	51°30′	104°14′	*S. caprea* (low) *S. glauca* (low to moderate) *S. rorida* (low) *S. dasyclados* (low to moderate)	No
Belarus	54°30′	28°45′	*S. caprea* (low)	Yes

[1] Salicylic glycoside level in the foliage (Julkunen-Tiitto 1989, and E. Zvereva, unpublished data)

[2] According to Zvereva et al. (2010a) and unpublished

[3] The Nikel population was labelled as Kola population in earlier publications (Zvereva et al. 2010a, b)

indices with those of authentic samples (Hilker and Schulz 1994) or with mass spectra of our own library (based on authentic samples) and the NIST library (in MassLab 1.3, Fisons Instruments). Relative concentrations of components (peak area / 100 ng IS / μl secretion) were calculated by dividing the peak area of a component by the area of the IS and considering the dilution (see above) and volume of secretion per sample. All components with a total relative amount (sum of peak areas of all individuals / 100 ng IS) less than five were omitted from the data set, and the remaining 69 components were used for further analysis.

Data Analysis The similarities in the composition of defensive secretions among individuals and populations were examined by cluster analysis. For this analysis, we used only the data obtained from larvae fed with their native host species (Table S1). We calculated Bray-Curtis dissimilarity (BC) for all pairs of individuals, as follows:

$$BC(i, j) = \frac{\sum_{k=1}^{n} |y_{i,k} - y_{j,k}|}{\sum_{k=1}^{n} \left(y_{i,k} + y_{j,k} \right)}$$

where $y_{i,k}$ and $y_{j,k}$ are the relative concentrations of individual component k in individuals i and j, respectively (Bray and Curtis 1957). Prior to the analysis, relative concentrations were fourth root transformed. The unweighted pair-group average method (UPGMA) was used for linkage.

In order to determine how the SG level in the host plant species affects the volume and quantitative composition of larval secretions, we performed a multivariate two-way analysis of variance (MANOVA). Prior to this analysis, the relative amounts of six groups of compounds (salicylaldehyde, the sum of esters of isobutyric and 2-methylbutyric acid [henceforth referred to as butyrates], benzoates, free leaf alcohols, fatty alcohols, and fatty acid methyl esters) and the volumes of defensive secretions were transformed (as $\log_e[1 + \sqrt{x}]$) to meet the assumption of normality. We compared larval defensive secretion (i) between two groups of populations associated with SG-rich and SG-poor willows, and (ii) between larvae from each population that were fed in the laboratory on willows with different SG levels (SG-rich or SG-poor). Populations nested within each of two groups were considered as random factors (procedure MANOVA; SAS Institute 2009). We then separately analysed each of seven characteristics of larval defensive secretion using mixed model analysis of variance (ANOVA). In this analysis, the group of populations (naturally associated with SG-rich or SG-poor willows)

and the rearing host (SG-rich or SG-poor willow) were fixed factors, whereas the population nested within the group, the family (genotype) nested within the population, and their interactions with the rearing host were considered as random factors (procedure GLIMMIX; SAS Institute 2009). The significance of the random factors in this analysis was evaluated by calculating the likelihood ratio and testing it against χ^2 distribution (as described in Littell et al. 2006).

Results

Chemical Composition of Secretions In the larval defensive secretion of the investigated six populations of *C. lapponica* we recorded 69 individual components, which exceeded the threshold value, and identified 46 of these compounds (Table S1). The number of individual compounds recorded in a secretion collected from an individual larva varied from 4 to 51 (median value = 24). In general, the secretions contained mainly salicylaldehyde, esters of iso- and 2-methylbutyric acid (butyrates), and benzoic acid with plant-derived alcohols, free plant-derived alcohols, fatty alcohols, and fatty acid methyl esters (see Table S1 for details regarding individual compounds and their classification).

Inter-population Variability of Secretions of Larvae Fed with Their Native Host Plant Species The populations associated with SG-rich willows differed substantially from populations associated with SG-poor willows in the composition of their larval secretions, and individuals from these two groups of populations formed two separate clusters (Fig. 1). The three populations associated with SG-poor willows also clearly differed from each other, whereas no such differentiation was observed among the three populations feeding in nature on SG-rich willows (Fig. 1).

Populations associated with SG-poor willows (all samples combined within the population) had significantly more (ANOVA: $F_{1,4} = 13.6$, $P = 0.02$) components in the larval defensive secretions (Baikal: 58, Ural: 50, Belarus: 41) when compared with populations associated with SG-rich willows (Finland: 34, Monche: 29, Nikel: 27).

The larval defensive secretion of each population associated with SG-poor willows contained individual components that have not been found in populations associated with SG-rich willows. The number of these unique components was highest in the Baikal population (nine), whereas larval secretions of Ural and Belarus populations each contained a single unique component (Table S1). Four components (isoamyl 2-methylbutyrate,
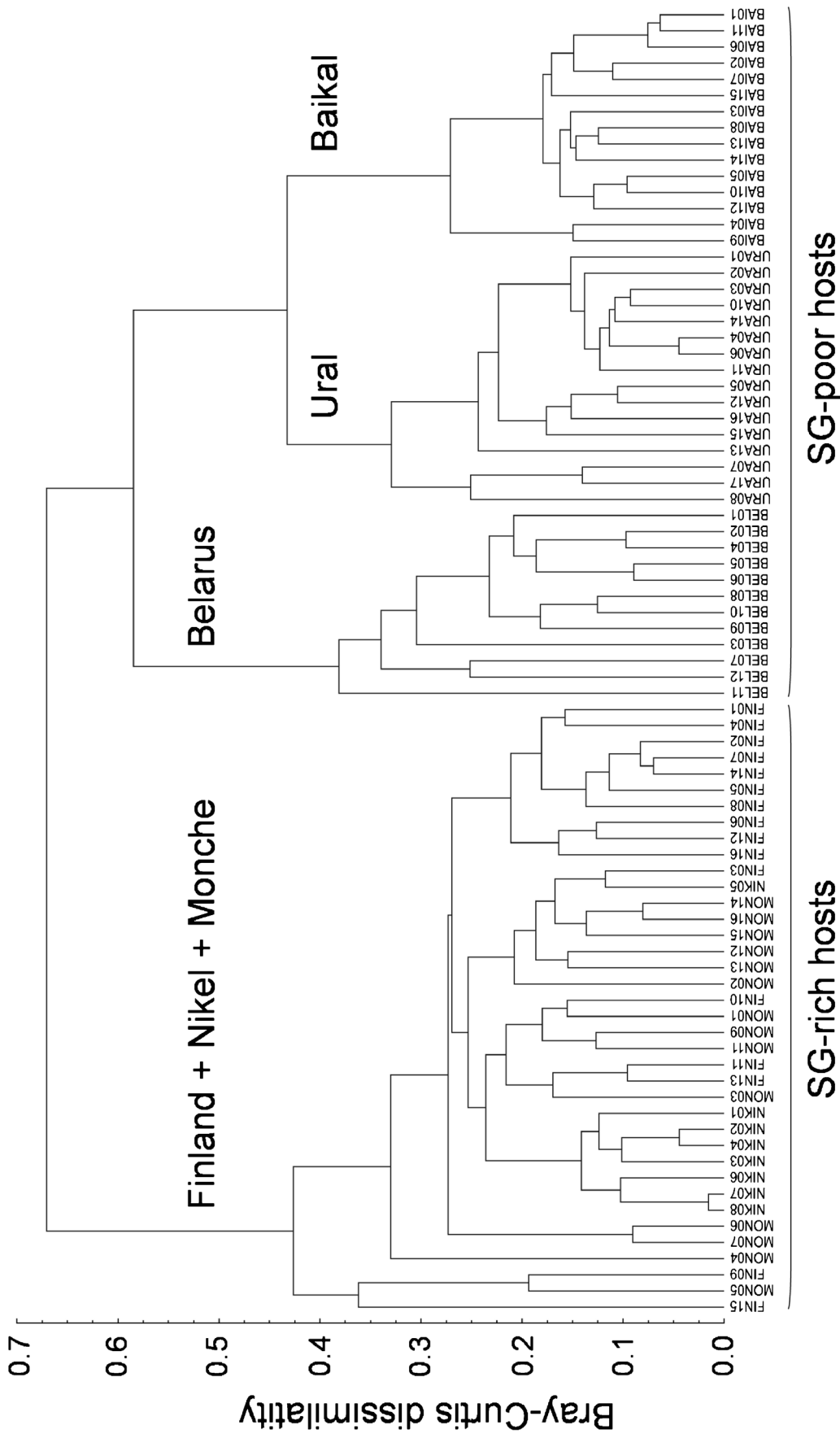
Fig. 1 UPGMA-dendrogram of a cluster analysis based on the Bray-Curtis dissimilarity of individual defensive secretions of *Chrysomela lapponica* larvae from six geographic populations (Bai – Baikal; Bel – Belarus; Ura – Ural; Fin – Finland; Nik – Nikel; Mon – Monche) fed with their native host plants. The codes of individuals match the codes in Table S1
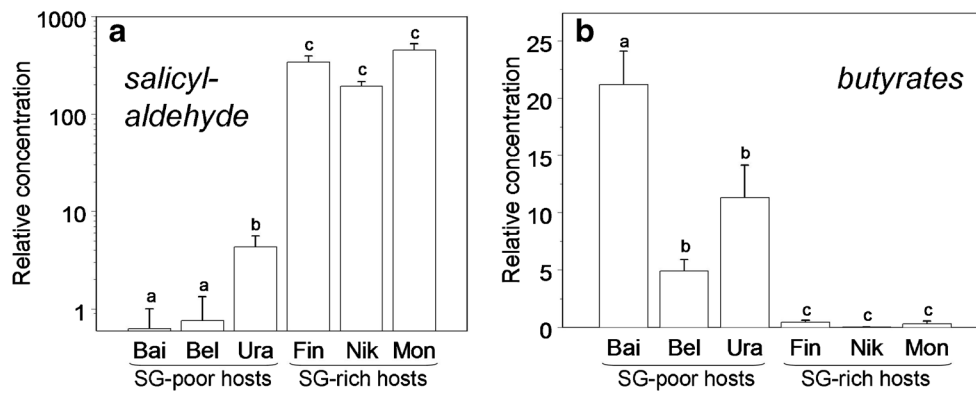
Fig. 2 Mean (±SE) relative amounts (peak area/100 ng IS/μl secretion) of (**a**) plant-derived salicylaldehyde and (**b**) autogenously produced butyrates in larval defensive secretions of *Chrysomela lapponica* from six populations (Bai – Baikal, $N=15$; Bel – Belarus, $N=12$; Ura – Ural, $N=16$; Fin – Finland, $N=16$; Nik – Nikel, $N=8$; Mon – Monche, $N=14$) fed with their native host plants. Values labelled by *different letters* differ at $P<0.05$ (Tukey test)

(Z)-3-hexenyl butyrate, 8-(isobutyryloxy)- and 8-(2-methylbutyryloxy)-linalool) were shared by all populations associated with SG-poor willows but were absent in all populations associated with SG-rich willows, which in turn shared the single component (geraniol) missing in all populations associated with SG-poor willows. No population-specific components were found in any population associated with SG-rich hosts.

The MANOVA of the characteristics of defensive secretions (i.e., volume and relative amounts of salicylaldehyde, butyrates, benzoates, free leaf alcohols, fatty alcohols, and fatty acid methyl esters) confirmed the significant differences between the two groups of populations (naturally feeding on SG-poor and SG-rich hosts). When these groups of populations were analyzed separately, significant differences among populations were found only for those associated with SG-poor willows (MANOVA: $F_{2,56}=7.72$, $P=0.001$). These differences were associated primarily with significant variation in the relative amounts of salicylaldehyde (Fig. 2a, Table 3).

The relative amounts of salicylaldehyde in the defensive secretions of larvae from populations associated with SG-rich willows were, on average, 200 times higher than the concentrations found in the secretions of larvae from populations associated with SG-poor willows (Fig. 2a). The relative concentrations of butyrates showed an opposite pattern: butyrate levels were, on average, 40 times higher in populations associated with SG-poor willows than in populations associated with SG-rich willows (Fig. 2b). Neither the secretion volumes nor the relative concentrations of benzoates differed between these two groups of populations (Fig. 3a, d).

Inter-population Variability of Secretions of Larvae Fed with Non-native Host Plant Species The larvae from populations associated with SG-rich and SG-poor willows

differed in their responses to willows with alternative SG levels (Table 2). The explored characteristics of the secretions showed specific responses to SG levels in host plants fed to larvae ($P<0.001$ for the main effect and all interactions), thus justifying the separate analyses of factors affecting individual characteristics of a secretion.

Larvae from populations associated in nature with SG-rich willows showed a 3-fold decrease in the volume of their secretions and a 30-fold decrease in the concentrations of benzoates when fed with SG-poor willows, while populations associated with SG-poor willows showed equally high volumes of secretions and concentrations of benzoates regardless of the SG contents of their hosts (Fig. 3a, d). The concentrations of salicylaldehyde depended only on the SG level in the willow that was fed to the larvae (Table 3). Comparison of secretions from larvae originating from SG-poor and SG-rich plants revealed that SG concentrations in larval secretions were equally high when they were fed with SG-rich plants and equally low when fed with SG-poor species (Fig. 3b). In contrast, the concentrations of butyrates were independent of the SG level in the willow species fed to the larva (Table 3, Fig. 3c).

Intra-population Variability of Secretions A significant (or marginally significant) family effect indicates the presence of genetic variation with respect to the volume of secretion and to the concentrations of fatty acid methyl esters (Table 3). The concentrations of salicylaldehyde in larval secretions did not vary among families; however, statistically significant interaction between family and SG level of the host (Table 3) indicates that families differ in their abilities to sequester salicylaldehyde from SG-poor and SG-rich willows. Interactions between beetle family and SG level in host plant also were
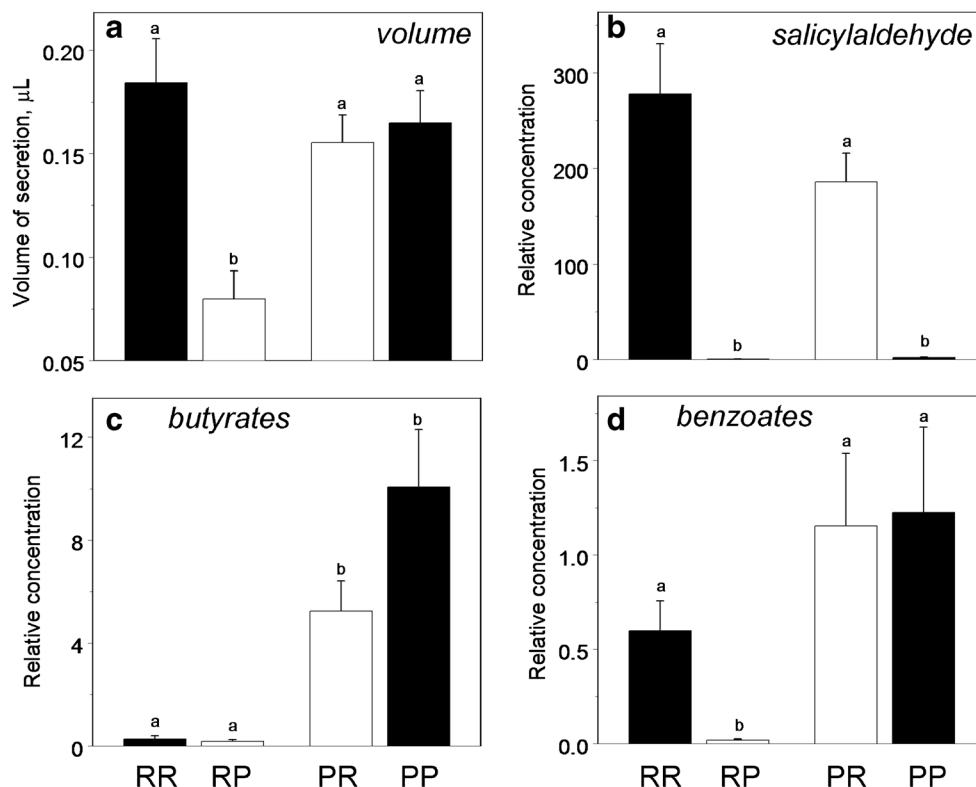
Fig. 3 Mean (±SE) volume (**a**) and relative concentrations (peak area/100 ng IS/μl secretion) of salicylaldehyde (**b**), butyrates (**c**) and benzoates (**d**) in larval defensive secretions of *Chrysomela lapponica* dependent upon the level of salicylic glycoside (SG) in the naturally preferred host plant and in the host plant fed to larvae in laboratory experiments. RR – larvae from populations associated with SG-rich willows fed with SG-rich willows ($N=7$ families); RP – larvae from

populations associated with SG-rich willows fed with SG-poor willows ($N=8$ families); PR – larvae from populations associated with SG-poor willows fed with SG-rich willows ($N=14$ families); PP – larvae from populations associated with SG-poor willows fed with SG-poor willows ($N=18$ families). *Filled bars*: larvae fed with their native hosts; *empty bars*: larvae fed with alternative hosts. Values labelled by *different letters* differ at $P<0.05$ (Tukey test)

significant for relative concentrations of fatty alcohols (Table 3). The significance of all these effects was due exclusively to variation within SG-poor populations (data not shown).

Table 2 Sources of variation in characteristics[a] of defensive secretion of *Chrysomela lapponica* larvae from six populations fed in the laboratory experiment on willows that differ in the level of salicylic glycosides (SG) (repeated measures MANOVA, tests of hypotheses for between subjects effects)

Source of variation	d.f.	Mean square	F	P
SG level in native host (SG-poor vs. SG-rich)	1	3.40	9.20	0.003
SG level in rearing host[b] (SG-poor vs. SG-rich)	1	34.86	94.36	<0.001
SG level in native host × SG level in rearing host	1	2.46	6.67	0.011
Error	180	0.37		

[a] Characteristics included in the analysis: volume and relative amounts of salicylaldehyde, butyrates, benzoates, free leaf alcohols, fatty alcohols and fatty acid methyl esters

[b] Host species fed to larvae in the laboratory experiment

Discussion

Inter-population Variability of Larval Secretions on Native Host Plants Our study demonstrated a striking difference in quantitative and qualitative compositions of larval defensive secretions between populations of *C. lapponica* associated in nature with SG-poor and SG-rich willows.

Larvae from populations associated with SG-poor willows produce a more diverse secretion (41–58 components) when compared to larvae from populations associated with SG-rich willows (27–34 components). Similarly, a high chemical diversity (69 components) was reported in larval secretions of a Bavarian population feeding on SG-free birches (Hilker and Schulz 1994). This difference between populations feeding on SG-poor/SG-free and SG-rich hosts is most likely associated with a higher ability of the larvae feeding on SG-poor/SG-free hosts to esterify *de novo* produced carboxylic acids with numerous plant-derived and *de novo* produced alcohols, thereby releasing a highly diverse secretion. This diversity of components may prevent adaptation of natural enemies to these secretions and/or exploitation of secretion compounds as kairomones.

Table 3 Among-population and among-family variation in individual characteristics of defensive secretions of *Chrysomela lapponica* larvae fed in laboratory experiments on willows that differ in levels of salicylic glycosides (SG) (mixed model ANOVA; F / P for fixed factors and χ^2 / P for random factors)

Factor	Source of variation	Volume of secretion	Relative concentrations[b]					
			Salicylaldehyde	Free leaf alcohols	Fatty alcohols	Fatty acid methyl esters	Benzoates	Butyrates
Fixed	SG level in native host (SG-poor vs. SG-rich)	1.68 / 0.37	0.06 / 0.83	0.06 / 0.82	18.5 / 0.02	0.13 / 0.75	1.30 / 0.33	12.4 / 0.03
	SG level in rearing host[a] (SG-poor vs. SG-rich)	9.06 / 0.06	556.4 / <0.001	0.91 / 0.41	0.01 / 0.94	33.5 / 0.007	6.69 / 0.08	1.33 / 0.33
	SG level in native host × SG level in rearing host	12.0 / 0.04	7.52 / 0.06	0.03 / 0.88	0.03 / 0.87	17.0 / 0.02	3.53 / 0.15	1.76 / 0.28
Random	Population (SG level in native host)	2.46 / 0.12	5.12 / 0.02	0.25 / 0.62	0.56 / 0.45	2.56 / 0.11	1.16 / 0.28	1.63 / 0.20
	Family (Population)	3.64 / 0.06	2.59 / 0.11	0.01 / 0.94	0.55 / 0.46	4.98 / 0.03	0.89 / 0.34	1.85 / 0.17
	SG level in rearing host × Population (SG level in native host)	2.32 / 0.13	4.19 / 0.04	13.1 / <0.001	0.54 / 0.46	3.17 / 0.08	1.73 / 0.19	3.83 / 0.05
	SG level in rearing host × Family (Population)	1.84 / 0.18	6.21 / 0.01	1.59 / 0.21	6.77 / 0.009	0.14 / 0.71	0.54 / 0.46	0.13 / 0.72

[a] Host species fed to larvae in the laboratory experiment

[b] Peak area/100 ng IS/μl secretion

The major component of the larval defensive secretions from the three Fennoscandian populations of *C. lapponica* specialized on SG-rich *S. myrsinifolia* is salicylaldehyde, which is derived from SGs sequestered from their host plants. Salicylaldehyde is a strong repellent for a number of generalist enemies (Denno et al. 1990; Lundvall et al. 1998; Zvereva et al. 2010b). The populations associated with SG-poor host plants show low concentrations of salicylaldehyde in the larval secretion, but concentrations of autogenously produced butyrates are, on average, 40-fold higher compared to populations associated with SG-rich hosts. Esters of iso- and 2-methylbutyric also acid repel natural enemies (Blum et al. 1972; Hilker and Schulz 1994). Consistent with this, secretions from larvae fed with SG-poor willow (*S. caprea*) repel generalist predators (wood ants) (Zvereva et al. 2010b). Thus, autogenously produced butyrates provide protection from natural enemies in populations of *C. lapponica* feeding on SG-poor willows and can be considered as major defensive compounds in these populations as well as in birch-feeding populations of *C. lapponica* from Bavaria, the Czech Republic, and the Altai region (Gross and Hilker 1995; Hilker and Schulz 1994; Tolzin-Banasch et al. 2011). The role of benzoates in the defensive secretion of *C. lapponica* larvae is unknown so far, but benzyl benzoate is known to repel ants (Novak and Pflanzer 1982).

Total concentrations of defensive compounds in the secretions are lower in populations naturally feeding on SG-poor hosts when compared to SG-rich hosts (Fig. 2). This difference may partly explain the lower efficiency of secretions containing primarily butyrates in repelling generalist enemies when compared to secretions containing primarily salicylaldehyde (Zvereva et al. 2010b).

Effects of Host Plant Change on Characteristics of Defensive Secretion One of the most important findings of the study is the among population differences in the responses to host plants with alternative SG levels. The efficiency of larval defense in populations naturally feeding on SG-poor willows is likely to increase when larvae are fed with a SG-rich host, because these larvae produce the same volume of secretions, and their secretions contain the same concentrations of butyrates and benzoates as were produced on the native host, in addition to large amounts of salicylaldehyde. In contrast, the efficiency of larval defense in populations that are specialized on SG-rich willows is likely to decrease greatly when larvae are fed with a SG-poor host because these larvae produce smaller volumes of secretions, and their secretions contain lower concentrations of defensive compounds than the secretions they produce on the native host. Similarly, larvae from birch-specialized populations produce low amounts of secretions and are not able to release appreciable amounts of salicylaldehyde when forced to feed on willows (Gross et al. 2004a; Hilker and Schulz 1994), due to loss of the enzyme necessary to produce salicylaldehyde (Kirsch et al. 2011). This reduction in the efficiency of chemical defenses in both types of highly specialized populations, observed when they were fed with a host plant with alternative SG levels, decreases the likelihood of shifting back to ancestral hosts. In contrast, populations with a low level of

specialization could even benefit from a shift to a SG-rich host due to an increase in production and efficiency of larval defensive secretion.

Within-population Variation in the Composition of Secretions Defensive toxins often are variable within populations, both in terms of their total quantity and their chemical constituents. The persistence of this diversity can be adaptive and, therefore, maintained by natural selection (Speed et al. 2012).

The significant (or nearly significant) among-family variation in several characteristics of larval defensive secretions that we discovered in populations naturally associated with SG-poor willows suggests that this variation is at least partly heritable. Significant heritability of *de novo* synthesized secretion components was reported previously in adults of the leaf beetle *Oreina gloriosa* (Eggenberger and Rowell-Rahier 1992) and of the confused flour beetle, *Tribolium confusum* (Yezerski et al. 2000). In line with these studies, we found that the families of *C. lapponica* differ in the abilities to sequester or produce *de novo* some of the components of the secretions on alternative host plants, as indicated by a significant effect of family by host plant interactions. These interactions demonstrate that genotypes of *C. lapponica* differentially respond to willow species contrasting in SG level, i.e., genetic variation with respect to larval defensive chemistry, required for specialization on either a SG-rich or a SG-poor host plants, exists in populations naturally associated with SG-poor willows.

Within-population variation in larval defensive secretions assures population survival under the variable pressure of different groups of natural enemies, which may use different components of defensive secretion as chemical cues to detect their prey (Jones et al. 1986). This may be especially true for *C. lapponica*, because a number of both parasitoid and predatory species use larval secretions of this species to locate prey (Gross et al. 2004b; Zvereva and Rank 2004).

Evolution of Defense Strategy in *Chrysomela lapponica* Earlier studies concluded that a dual defense strategy—when larval secretions contain both autogenous and sequestered compounds—is the ancestral state for *C. lapponica* (Termonia and Pasteels 1999; Termonia et al. 2001). We suggest that the dual defense strategy evolved in non-specialized populations that were able to use several willow species with low to moderate concentrations of SGs, and that the variation among host plants with respect to SG content could have favored the evolution of this strategy. The Baikal and Ural populations, which possess the dual defense strategy and lack the local adaptations to either SG-rich or SG-poor willows (Zvereva et al. 2010a), are likely to retain the ancestral state of *C. lapponica*.

The presence of genetic variation in characteristics of defensive secretions (Table 3) in non-specialized populations

creates a prerequisite for specialization on different host plants. The heritability of the autogenous production of secretion components lends support to the hypothesis (Gross et al. 2004a; Termonia and Pasteels 1999) that top-down effects may be important selection factors in the evolution of this leaf beetle. One direction of specialization is a shift from willows with low to moderate SG content to Betulaceae, accompanied by the loss of the ability to produce salicylaldehyde (Hilker and Schulz 1994; Kirsch et al. 2011). Another direction is a shift to willows with extremely high concentrations of SGs, resulting in an increase in the production of salicylaldehyde, accompanied by a dramatic decrease in the ability for autogenous production of defensive compounds.

Populations specialized on SG-rich willows synthesise low amounts of butyrates, and they also are unable to release considerable volumes of secretion when transferred to SG-poor willows. The defensive abilities of these transferred larvae are, therefore, low: in field experiments, they were attacked and killed by wood ants immediately upon encounter, while larvae that fed on SG-rich willow repelled up to 15 ants, and the first attack occurred only when secretions were totally exhausted (Zvereva et al. 2010b). The absence of intra-population variation in the production of butyrates in these specialized populations indicates that they have lost (or nearly lost) the genetic basis for the selection for high butyrate production, which would be required to compensate for the loss of salicylaldehyde if a shift to SG-poor hosts occurs.

A specific example of specialization on a SG-poor host is presented by the Belarus population of *C. lapponica*. This population feeds in nature almost exclusively on SG-poor *S. caprea*, although SG-rich *S. myrsinifolia* is growing in the same habitats (Zvereva et al. 2010a). Nevertheless, larvae of the Belarus population survive well and produce large amounts of salicylaldehyde (along with considerable amounts of butyrates) when forced to feed on *S. myrsinifolia*. In this case, local adaptation to a SG-poor host, which was presumably driven by interspecific competition (Zvereva et al. 2010b), is reversible and, in contrast to populations specialized to birch, this population can potentially shift to willow species containing high amounts of SGs. Intriguingly, larvae from the Belarus population repelled wood ants more efficiently when fed on SG-rich *S. myrsinifolia* than they did when fed on SG-poor *S. caprea* (Zvereva et al. 2010b) even though, in nature, the population retains its fidelity to *S. caprea*. This example fits perfectly to the theoretical model (Speed et al. 2012) suggesting that the costs of competition may drive animals to use otherwise suboptimal resources, and it demonstrates clearly that better protection from natural enemies is not always a driving force in host plant shifts.

The populations of *C. lapponica* associated with either birch or SG-rich willows have lost the ability to produce either salicylaldehyde or butyrates and, therefore, deviated substantially from the ancestral state (i.e., dual defense). In line with

conclusions by Fox and Morrow (1981), our results indicate that host plant specialization and defensive strategies may both differ between populations of a single insect species, and they may reflect specific local conditions. The benefits of specialization (Loxdale et al. 2011) may be numerous, but populations that fully depend on a host-derived defensive strategy or that have lost the ability to produce salicylaldehyde from plant-derived SGs are likely to have a higher risk of extinction than are populations that rely on a dual defensive strategy. However, *C. lapponica* in general enjoys benefits of both specialism and generalism due to high variability between populations in characteristics of defensive secretions and their dependence on host plants. This among-population variation is likely to reduce the extinction risk of a species and to maintain high ecological diversity and wide distribution of *C. lapponica*.

References

Becerra JX (1997) Insects on plants: macroevolutionary chemical trends in host use. Science 276:253–256

Bernays EA, Graham M (1988) On the evolution of host specificity in phytophagous arthropods. Ecology 69:886–892

Blum MS, Brand JM, Wallace JB, Fales HM (1972) Chemical characterization of defensive secretion of a chrysomelid larva. Life Sci 11:525–531

Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 27:325–349

Brückmann M, Termonia A, Pasteels JM, Hartmann T (2002) Characterization of an extracellular salicyl alcohol oxidase from larval defensive secretions of Chrysomela populi and Phratora vitellinae (Chrysomelina). Insect Biochem Mol Biol 32:1517–1523

Denno RF, Larsson S, Olmstead KL (1990) Role of enemy free space and plant quality in host plant selection by willow beetles. Ecology 71:124–137

Eggenberger F, Rowell-Rahier M (1992) Genetic component of variation in chemical defense of Oreina gloriosa (Coleoptera, Chrysomelidae). J Chem Ecol 18:1375–1404

Fox LR, Morrow PA (1981) Specialization: species property or local phenomenon? Science 211:887–893

Funk DJ, Futuyma DJ, Orti G, Meyer A (1995) A history of host associations and evolutionary diversification for Ophraella (Coleoptera, Chrysomelidae) – new evidence from mitochondrial DNA. Evolution 49:1008–1017

Futuyma DJ, Agrawal AA (2009) Macroevolution and the biological diversity of plants and herbivores. Proc Natl Acad Sci U S A 106:18054–18061

Futuyma DJ, McCafferty SS (1990) Phylogeny and the evolution of host plant associations in the leaf beetle genus Ophraella (Coleoptera, Chrysomelidae). Evolution 44:1885–1913

Gross J, Hilker M (1995) Chemoecological studies of the exocrine glandular larval secretions of two chrysomelid species (Coleoptera): Phaedon cochleariae and Chrysomela lapponica. Chemoecology 5:185–189

Gross J, Podsiadlowski L, Hilker M (2002) Antimicrobial activity of exocrine glandular secretion of Chrysomela larvae. J Chem Ecol 28:317–331

Gross J, Fatouros NE, Hilker M (2004a) The significance of bottom-up effects for host plant specialization in Chrysomela leaf beetles. Oikos 105:368–376

Gross J, Fatouros NE, Neuvonen S, Hilker M (2004b) The importance of specialist natural enemies for Chrysomela lapponica in pioneering a new host plant. Ecol Entomol 29:584–593

Gross J, Fatouros NE, Neuvonen S, Hilker M (2007) The role of competitors for Chrysomela lapponica, a north Eurasian willow pest, in pioneering a new host plant. J Pest Sci 80:139–143

Hilker M, Schulz S (1994) Composition of larval secretion of Chrysomela lapponica (Coleoptera, Chrysomelidae) and its dependence on host-plant. J Chem Ecol 20:1075–1093

Jaenike J (1990) Host specialization in phytophagous insects. Annu Rev Ecol Syst 21:243–273

Jones CG, Hess TA, Whitman DW, Silk PJ, Blum MS (1986) Idiosyncratic variation in chemical defenses among individual generalist grasshoppers. J Chem Ecol 12:749–761

Joshi A, Thompson JN (1995) Trade-offs and the evolution of host specialization. Evol Ecol 9:82–92

Julkunen-Tiitto R (1989) Phenolic constituents of Salix: a chemotaxonomic survey of further Finnish species. Phytochemistry 28:2115–2125

Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. Ecol Lett 7:1225–1241

Kelley ST, Farrell BD (1998) Is specialization a dead end? The phylogeny of host use in Dendroctonus bark beetles (Scolitidae). Evolution 52:1731–1743

Kirsch R, Vogel H, Muck A, Reichwald K, Pasteels JM, Boland W (2011) Host plant shifts affect a major defense enzyme in Chrysomela lapponica. Proc Natl Acad Sci U S A 108:4897–4901

Kuhn J, Pettersson EM, Feld BK, Burse A, Termonia A, Pasteels JM, Boland W (2004) Selective transport systems mediate sequestration of plant glucosides in leaf beetles: a molecular basis for adaptation and evolution. Proc Natl Acad Sci U S A 101:13808–13813

Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O (2006) SAS for mixed models, 2nd edn. SAS Institute Inc., Cary

Loxdale HD, Lushai G, Harvey JA (2011) The evolutionary improbability of 'generalism' in nature, with special reference to insects. Biol J Linn Soc 103:1–18

Lundvall P, Neuvonen S, Halonen M (1998) Interspecific differences in the susceptibility of adult leaf beetles (Col., Chrysomelidae) to predation by willow warblers (Phylloscopus trochilus). Rep Kevo Subarctic Res Station 22:19–24

Machkour-M'Rabet S, Mardulyn P, Pasteels JM (2008) Genetic differentiation among European samples of the arctic-alpine leaf beetle, Chrysomela lapponica. Entomol Exp Appl 129:181–188

Mardulyn P, Othmezouri N, Mikhailov YE, Pasteels JM (2011) Conflicting mitochondrial and nuclear phylogeographic signals and evolution of host-plant shifts in the boreo-montane leaf beetle Chrysomela lapponica. Mol Phylogenet Evol 61:686–696

Mayr E (1963) Animal Species and Evolution. Harvard Univ Press, Cambridge

Messina FJ, Durham SL (2013) Adaptation to a novel host by a seed beetle (Coleoptera: Chrysomelidae: Bruchinae): effect of source population. Environ Entomol 42:733–742

Michalski C, Mohagheghi H, Nimtz M, Pasteels J, Ober D (2008) Salicyl alcohol oxidase of the chemical defensive secretion of two chrysomelid leaf beetles: molecular and functional characterization of two

new members of the glucose-methanol-choline oxidoreductase gene family. J Biol Chem 283:19219–19228

Novak D, Pflanzer F (1982) Versuche mit einigen neuen Repellents gegen Pharaoameisen. Angew Parasitol 23:47–48

Pasteels JM, Braekman JC, Daloze D, Ottinger R (1982) Chemical defence in chrysomelid larvae and adults. Tetrahedron 38: 1891–1897

Pasteels JM, Gregoire JC, Rowell-Rahier M (1983a) The chemical ecology of defense in arthropods. Annu Rev Entomol 28:263–289

Pasteels JM, Rowell-Rahier M, Braekman JC, Dupont A (1983b) Salicin from host plant as precursor of salicylaldehyde in defensive secretion of chrysomeline larvae. Physiol Entomol 8:307–314

Pavan M (1953) Antibiotics and insecticides of animal origin. I. The active principle of the larva of *Melasoma populi*. Arch Zool Ital 38:157–184

SAS Institute (2009) SAS/Stat. User's Guide, Version 9.2. SAS Institute, Cary SAS Institute (2009) SAS/Stat. User's Guide, Version 9.2. SAS Institute, Cary

Schulz S, Gross J, Hilker M (1997) Origin of the defensive secretion of the leaf beetle *Chrysomela lapponica*. Tetrahedron 53:9203–9212

Speed MP, Ruxton GD, Mappes J, Sherratt TN (2012) Why are defensive toxins so variable? An evolutionary perspective. Biol Rev 87: 874–884

Termonia A, Pasteels JM (1999) Larval chemical defense and evolution of host shifts in *Chrysomela* leaf beetles. Chemoecology 9:13–23

Termonia A, Hsiao TH, Pasteels JM, Milinkovitch MC (2001) Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. Proc Natl Acad Sci U S A 98:3909–3914

Tilmon KJ, Wood TK, Pesek JD (1998) Genetic variation in performance traits and the potential for host shifts in *Enchenopa* treehoppers (Homoptera: Membracidae). Ann Entomol Soc Am 91:397–403

Tolzin-Banasch K, Dagvadorj E, Sammer U, Kunert M, Kirsch R, Ploss K, Pasteels JM, Boland W (2011) Glucose and glucose esters in the larval secretion of *Chrysomela lapponica*; selectivity of the glucoside import system from host plant leaves. J Chem Ecol 37:195–204

Yezerski A, Gilmor TP, Stevens L (2000) Variation in the production and distribution of substituted benzoquinone compounds among genetic strains of the confused flour beetle, *Tribolium confusum*. Physiol Biochem Zool 73:192–199

Zvereva EL, Kozlov MV (2000) Natural enemies of the leaf beetle, *Melasoma lapponica*, in the impact zone of a nickel-copper smelter: density dependence and effects of air pollution. J Appl Ecol 37:298–308

Zvereva EL, Rank NE (2003) Host-plant effects on parasitoid attack on the leaf beetle, *Chrysomela lapponica*. Oecologia 135:258–267

Zvereva EL, Rank NE (2004) Fly parasitoid *Megaselia opacicornis* uses defensive secretions of the leaf beetle *Chrysomela lapponica* to locate its host. Oecologia 140:516–522

Zvereva EL, Kozlov MV, Kruglova OY (2002) Color polymorphism in relation to population dynamics of the leaf beetle *Chrysomela lapponica*. Evol Ecol 16:523–539

Zvereva EL, Kozlov MV, Hilker M (2010a) Evolutionary variation on a theme: host plant specialization in five geographical populations of the leaf beetle *Chrysomela lapponica*. Popul Ecol 52:389–396

Zvereva EL, Kruglova OY, Kozlov MV (2010b) Drivers of host plant shifts in the leaf beetle *Chrysomela lapponica*: natural enemies or competition? Ecol Entomol 35:611–622