Variation in defensive chemistry within a polyphagous Baikal population of *Chrysomela lapponica* (Coleoptera: Chrysomelidae): potential benefits in a multi-enemy world

Vitali Zverev, Mikhail V. Kozlov and Elena L. Zvereva

Department of Biology, University of Turku, Finland

Corresponding author: Elena L. Zvereva, elezve@utu.fi, tel. +358 2 3335492

Abstract Variation in anti-predator chemical defence is frequently observed in natural populations, but its adaptive significance remains debatable. Most populations of the chemically defended leaf beetle, *Chrysomela lapponica*, are specialized to their host plants, but some populations are polyphagous. We tested the hypothesis that the use of multiple host plants by a Baikal population of *C. lapponica* results in variation in the composition of its defensive secretions, leading to variation in defence effectiveness against different natural enemies. The secretions of larvae feeding on local host plants differed both in the origin of major components (sequestered or autogeneous) and in chemical profiles. This variation was at least partly associated with differences in the secondary chemistry among the five most abundant plant species used by this population. Larvae feeding on different hosts in nature suffered similar overall mortality from enemies, but the relative contributions of different enemy species (natural enemy profiles) varied among host plant species. Behavioural experiments with three predators and one parasitoid showed that this variation may result from idiosyncratic responses of the enemy species to the composition of the larval defences. These differences allow part of the polyphagous leaf beetle population to escape from the currently most abundant enemy on the host plant species that provides the best protection against this enemy. In this way, the within-population variability in chemical defence, associated with feeding on hosts differing in chemistry, can buffer prey populations against fluctuating pressures of specific enemies.

Keywords chemical defences, herbivorous insects, host plant chemistry, natural enemies, polyphagy.

Introduction

Organisms protect themselves against enemies in many ways, but chemical defences are among the most taxonomically and ecologically widespread defences and show striking diversity across species and higher taxa (Blum 1981; Ruxton et al. 2004; Eisner et al. 2007). Intensive studies of chemical defences in animals have revealed that defensive compounds often vary within populations, both in terms of their quantity and biochemical profiles (Blum 1981; Brower 1984; Pasteels et al. 1983a; Holloway et al. 1993); however, the ecological and evolutionary significance of this variation has received far less emphasis (Speed et al. 2012).

When prey with different biochemical profiles obtain similar levels of protection and pay similar costs, the among-individual variation in the composition of chemical defences have no effect on the survival of the individual within population (Pasteels et al. 1983a; Speed et al. 2012). However, surprisingly few studies have evaluated this prediction (Speed et al. 2012). Alternatively, several hypotheses (reviewed by Speed et al. 2012) suggest the importance of within-population variations in chemical defence profiles for survival of individuals when they are exposed to a diverse community of natural enemies. Some of these hypotheses are associated with herbivore feeding niche, because the amounts, concentrations and composition of defensive compounds frequently depend on the host plants used by a herbivore (Brower 1984; Hilker and Schulz 1994). Many herbivorous insects sequester plant allelochemicals for use in their own defence (Nishida 2002; Opitz and Müller 2009), and this ability is tightly linked with the evolution of their host-plant specialisation (Price et al. 1980; Bernays and Graham 1988).

The majority of phytophagous insect species feed on one or few closely related host plant species, whereas only 10% of species feed on plants from more than three different families (Bernays and Graham 1988). The predominance of specialised feeding habits

suggests that highly specialised species or populations of herbivores have an advantage when compared to polyphagous species or populations (Loxdale et al. 2011; Mooney et al. 2012). Studies on the evolution of feeding niche breadth in herbivorous insects have long been focused on the interactions between insects and their host plants (Ehrlich and Raven 1964; Futuyma and Moreno 1988). From this point of view, the benefits of specialisation are generally explained by a higher efficiency of food plant utilisation (physiologically and behaviourally) by individuals of specialised species than by individuals of polyphagous species (Dethier 1954; Fox and Morrow 1981). However, many cases remain unexplained where herbivores specialize on host plants that are suboptimal in terms of fitness returns (Dicke 2000).

During the past decades, the role of natural enemies in the evolution of host plant specialisation became increasingly appreciated (Price et al. 1980; Dicke 2000). In particular, the enemy-free space hypothesis states that specialist species are better adapted than generalists at using their host plant for defence from natural enemies (Bernays and Graham 1988; Dyer 1995; Singer and Stireman 2005). The greater effectiveness of chemical anti-predator defences in specialist insect herbivores compared to generalist ones has been recently confirmed by meta-analysis (Zvereva and Kozlov 2016). In general, studies, integrating the three trophic levels showed that specialists outperform generalists under diverse combinations of host plant quality and natural enemy effects (Mooney et al. 2012; Singer et al. 2014).

However, in spite of numerous benefits of specialisation, some herbivorous insect species have relatively wide feeding niches and their host plant range includes many species. This suggests that polyphagy as a feeding strategy has some advantages that at certain circumstances overweigh the benefits of diet specialisation (Logarzo et al. 2011; Muller et al. 2015). One of the advantages of polyphagy may be associated with its provision of variation

among herbivore individuals, in particular variation in chemical defence. This variation may be linked with host plant chemistry, because defensive compounds are frequently derived by herbivores from their host plants (Opitz and Müller 2009). Therefore, polyphagous populations of chemically defended herbivores may be more diverse in terms of defensive chemistry. However, the role of this variation in the protection of natural herbivore populations against their enemies remains unclear.

Leaf beetles (Coleoptera: Chrysomelidae) are popular objects in the studies of insect chemical defence. Some leaf beetle species synthesise their defensive secretions *de novo*, while others sequester secondary compounds of their host plants to produce their defences (Pasteels et al. 1990). For the subtribe Chrysomelina, *de novo* production of iridoid monoterpenes by larvae represents the ancestral state, whereas the sequestration of plant allelochemicals is evolutionarily derived (Pasteels et al. 1990). Among these beetles, the *Chrysomela interrupta* group is especially interesting because it has evolved a mixed defensive strategy involving both sequestration of plant chemicals and *de novo* production of defensive compounds (Termonia et al. 2001). *Chrysomela lapponica*, which belongs to this group, is one of most intensively studied leaf beetles with respect to both population ecology (e.g. Gross et al. 2004a, b; Zvereva et al. 1995; Zvereva et al. 2010a, b; Zvereva et al. 2016b) and chemical defence (e.g. Hilker and Schulz 1994; Tolzin-Banasch et al. 2011; Kirsch et al. 2011; Geiselhardt et al. 2015). Feeding on *Salix* species is an ancestral state for *C. lapponica*, but some populations of this species have shifted to *Betula* spp. (Mardulyn et al. 2011). In populations feeding on willows rich in salicylic glucosides (SGs), larvae sequester these compounds to produce salicylaldehyde and synthesise very small amounts of autogenous defensive compounds (Geiselhardt et al. 2015). In contrast, populations specialising on birches (lacking SGs) use as major defensive compounds iso- and 2-methylbutyric esters produced by esterification of *de novo* synthesised butyric acids by alcohols retrieved from

host plants (Schulz et al. 1997), and some of these birch-feeding populations have lost the ability to sequester their defences from host plants (Kirsch et al. 2011). These two types of populations demonstrate high levels of specialisation in host plant use, showing strong preference of their primary host plants (either SG-rich or SG-free) both in the field and in the laboratory experiments, and best performance when feeding on these primary host plants (Gross et al. 2004b; Zvereva et al. 2010a).

In contrast, some populations of *C. lapponica* from Siberia do not show any specialisation to host plant chemistry in terms of SGs content in their host plants. Beetles and larvae feed in nature on multiple plant species, do not show preference for any of local host species (Zverev and Zvereva, unpublished data) and perform similarly well on host plants differing in SG content (Zvereva et al. 2010a). Therefore, although these populations feed only on salicaceous species, they exhibit a more generalised mode of host plant use and can be considered as polyphagous relative to more specialised populations, which feed on either SG-rich or SG-free host plants. Larvae from polyphagous populations autogenously produce considerable amounts of esters when feeding on willows having very low concentrations of SGs, but when they are experimentally moved to host plants rich in SGs, they in addition sequester SGs to produce salicylaldehyde (Geiselhardt et al. 2015). Thus, these polyphagous populations demonstrate a high plasticity in their defensive strategy and potentially possess high within-population variation in chemical defences. Therefore, these populations offer an excellent opportunity to study variation in the effectiveness of chemical defences against a range of natural enemies and the influence of this variation on prey survival on different host plants.

In this study we asked whether composition of anti-predator chemical defence demonstrates within-population variability and whether this variability is associated with differences in chemistry among multiple host species used by polyphagous population of *C.*

lapponica. We also tested the hypothesis that variation in defensive chemistry will result in variation in natural enemy profiles on different natural host plants due to idiosyncratic responses of the enemy species to the composition of the larval defences. We addressed the following particular questions: (i) Are there differences in concentrations of major compounds and chemical profiles of defensive secretions between *C. lapponica* larvae naturally feeding on different host plants? (ii) Is this variation associated with variation in the secondary chemistry of host plants? (iii) Does the overall mortality of *C. lapponica* from natural enemies differ among host plant species? (iv) Do enemy species differentially respond to larval defensive secretions of differing compositions? (v) Do these differences result in variation in the relative contributions of different enemy species to the total mortality of *C. lapponica* among salicaceous host plant species?

Materials and methods

Study area

The study was conducted near the village of Mamai (51°27´N, 104°47´E), located 45 km east of the town of Baikalsk in Siberia, Russia, at altitudes of 450–500 m above sea level. The area is covered with mixed taiga forest, consisting mostly of *Pinus sibirica, Larix dahurica, Larix sibirica, Abies sibirica, Picea obovata* and *Populus suaveolens*. The data were collected along a small local road leading from Mamai to the Hamar-Daban Mountains. The shrubby vegetation at the roadsides included several willow species (listed in Fig. 1) and saplings of *P. suaveolens*, which all served as food for an abundant population of *C. lapponica*.

Distribution of *C. lapponica* among host plants

The host plant biomass in the study area and the density of *C. lapponica* on each host plant species were measured using the methods developed in an earlier study (Zvereva et al. 1995). On 7-8 August 2009, 23 July 2014 and 22-23 July 2015 we selected two (in 2014) or four (in 2009 and 2015) plots 2×25 m size (50 m^2) plots along a small road. For each individual of the potential host plants (Salicaceae) growing in the plot, we recorded its species, size class according to an approximate number of annual shoots and the number of *C. lapponica* larvae feeding on it. All records were conducted by the same person (ELZ) on warm sunny days.

To estimate the foliar biomass, we calculated the numbers of shoots in five randomly selected individuals of each of five size classes of each host species, and collected all leaves of 10 annual shoots from each of five bushes of each species; these leaves were dried at 80 °C for 24 hours and weighed to the nearest 1 mg. The median shoot numbers in each of five size classes were multiplied by an average shoot-specific foliar biomass to calculate the dry mass of the foliage of each host species within a plot (Zvereva et al. 1995).

The host-specific leaf beetle density was calculated from the total number of *C. lapponica* recorded on all individuals of a given plant species within a plot, divided by the total dry weight of foliage summed from all these plant individuals. The square-root transformed values were averaged across the plots, and the year-specific means were compared among host plant species by ANOVA.

Analysis of salicylates in host plants

SGs are important cues for host plant selection by many leaf beetles (e.g. Tahvanainen et al. 1985), and some leaf beetles, including some populations of *C. lapponica*, use them as

precursors for defence production (Pasteels et al. 1983b; Hilker and Schulz 1994). Therefore, we explored concentrations of SGs in five of the six salicaceous plant species found in our study area. *S. rhamnifolia* had a low abundance and we failed to find sufficient numbers of *C. lapponica* naturally feeding on this willow for secretion analysis; therefore, leaf samples were not collected for the analysis of SGs. Instead, we included in our analysis *S. glauca*, because in several proximate localities *C. lapponica* larvae were abundant on this willow species (Zvereva et al. 2010a). On 24 July 2014, we sampled two undamaged mature leaves from each of five individuals of each host species, placed them into sealed plastic bags containing silica gel for drying and preserved them in the same bags at room temperature.

Dry leaves were homogenised and 40-45 mg samples of leaf powder were extracted for 60 min with 70% aqueous acetone at room temperature with continuous stirring. The homogenate was centrifuged for 10 min at 2900 *g*; the pellet was extracted twice more and the combined extracts were concentrated to dryness in vacuo at 45 °C. The dry extracts were dissolved in 1 ml of water and purified by filtration (PTFE filter, 0.2 μm).

An HPLC-DAD system (La Chrom, Merck-Hitachi) with column XBridge C18 (100.0 × 2.1 mm i.d, a pore size 3.5 μm, Waters, Ireland) was used for analysis of salicylates. Two solvents were used for elution: (A) 0.1% aqueous formic acid solution, (B) 0.1% solution of formic acid in acetonitrile. Elution profile: 0-5 min 2% B in A; 5-55min, 2-20% B in A; 55-70 min, 20-50% B in A; 70-85 min, 50% B in A. Elution rate - 0.3 ml/min, and detection wavelength - 270 nm. UV spectra were recorded automatically at 200-420 nm on top of each peak. The injected volume of sample was 5 μL.

The UPLC-DAD-MS system consisted of a combined Acquity UPLC (Waters Corporation, Milford, MA, USA) and triple quadrupole mass-spectrometer (Xevo® TQ, Waters Corporation, Milford, MA, USA). An Acquity column UPLC® BEH Phenyl (2.1 × 100 mm, 1.7 μm, Waters Corporation, Wexford, Ireland) was used for the separation of

metabolites. Elution profile: 0-0.5 min 1% B in A; 0.5-5.0 min 0-30% B in A; 5.0-8.0 min, 30-90% B in A. Eluent flow rate was 0.5 ml/min. Two solvents (same as above) were used for elution of metabolites. The metabolites were detected at 190–500 nm using the negative ionisation mode. The ESI conditions were: capillary voltage 2.4 kV, desolvation temperature 500 °C, source temperature 150 °C, and desolvation and cone gas (N_2) 1000 and 100 l/h, respectively.

Salicylates were identified by comparing the UV and MS characteristics of metabolites with the parameters of the reference compound (salicin) and known compounds from different mass-spectrometry databases. Contents of salicin, salicortin and tremuloidin were determined based on the value of the major m/z fragment $[M-H+HCOOH]^-$ in their mass-spectra at 331.1. 469.1 and 435.1 Da, respectively. The calibration plot for recalculation of relative data into values in mg per 1 g of dry weight was prepared by UPLC-MS analysis of authentic salicin standards (Sigma-Aldrich) of known concentrations. Concentrations of SGs were compared among plant species using the Kruskal-Wallis test.

Secretion composition in larvae feeding on different host plants

Samples for the analysis of defensive secretions were taken in the field on 23-24 July 2014 from individual last instar *C. lapponica* larvae that were naturally feeding on five plant species; 10 larvae were collected per host species. Droplets of secretions emitted by each larva in response to disturbance were collected from all glands into calibrated glass capillaries which were then flame-sealed, transported to the laboratory and stored at –18 ºC. Volumes of secretions were calculated based on the lengths between the menisci of the secretions inside the capillary and the capillary diameter.

The secretions were analyzed by GC-MS on a Fisons GC model 8060 coupled to a Fisons MD 800 quadrupole MS (EI-mode at 70eV). Each sample was dissolved in 10 µl dichloromethane with dodecane (100 ng/µl) as an internal standard (IS). The solution was mixed thoroughly by aspirating and expelling the sample with the injection syringe at least five times before injection. An aliquote of 1 µl was injected at 240 ºC. Samples were separated on a 30-m DB5-ms capillary column (0.32-mm i.d., film thickness 0.25 µm, J. and W. Scientific, Folsom, CA, USA) with helium as the carrier gas. The temperature program started at 40 ºC for 4 min and then increased to 280 ºC at a rate of 10 °C/min. Eluted compounds were identified by comparing mass spectra and retention indices with those of authentic samples (Hilker and Schulz 1994) or with mass spectra of the library created during our previous study (Geiselhardt et al. 2015) and the NIST library (in MassLab 1.3, Fisons Instruments). Relative concentrations of components (peak area / 100 ng IS / µl secretions) 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 secretions per sample.

The variation in biochemical profiles of larval secretions among host plant species (after a ln(1+Öx) transformation) was explored by MANOVA. Only compounds with known defensive functions (Blum et al. 1972; Honda 1983; Hilker and Schulz 1994; Zvereva et al. 2016a) were included in this analysis: salicyaldehyde, 2-methybutyric acid, and various 2-methylbutyric and isobutyric esters (butyric esters hereafter) (Supporting Information, S1).

Mortality from predators

To estimate mortality of *C. lapponica* larvae from predation on different host plant species, we selected two to seven plants of *S. caprea, S. dasyclados* and *S. rorida* (growing singly at least five meters apart and not closer than 0.5 m from the nearest non-host neighbour)

naturally inhabited by *C. lapponica* larvae of the second or third instars. We failed to find individual plants of other host species (*S. rhamnifolia* and *P. suaveolens*) that met our selection criteria. On each of these plants, we selected a branch with a group of 10–25 larvae, and removed larvae from all other branches. We opted to use naturally formed groups of larvae instead of placing a certain number of them on the plant because larvae that are disturbed by handling lose part of their defensive secretions and tend to disperse, whereas undisturbed larvae usually feed in small groups. In this way, we provided natural conditions for enemies, which may be influenced by both the amount of defences and prey aggregation (Zvereva and Kozlov 2016). Larvae on each selected branch were counted at the start of the experiment (22 July 2015); the survivors were recorded on 27 July 2015.

The mortality of larvae was compared between host plant species using logistic regression and the events/trials syntax (procedure GLIMMIX; SAS Institute 2009): trial was the initial number of larvae and event was the number of larvae that disappeared (presumably died) by the end of the experiment. This experiment accounted only for larval mortality from predators, because mortality of *C. lapponica* from parasitoids can only be detected at the prepupal or pupal stages.

Mortality from parasitoids and pathogens

To estimate mortality from parasitoids we used a previously described method (Zvereva and Kozlov 2000; Zvereva et al. 2010b). On 7−8 August 2009, 25 July 2014 and 27 July 2015 we collected all prepupae and pupae from up to five individuals of each host species. Only plant individuals with five or more prepupae plus pupae were sampled; therefore, the numbers of sampled willow species and individuals differed among study years. The collected material was kept in the laboratory until adult beetles hatched. The insects were then sorted into the

following six categories: alive (i.e., the beetle hatched); killed by tachinid fly *Cleonice nitidiuscula* (Zett.) (Diptera, Tachinidae); killed by phorid flies *Megaselia breviseta* (Wood) (Diptera, Phoridae); killed by chalcid wasps *Schizonotus sieboldi* (Ratz.) (Hymenoptera; Chalcidae); dead with signs of infection (the last instar larvae that failed to pupate and were stuck onto the leaf; these often had visible fungal mycelia); and dead for unknown reasons. The mortality of larvae from parasitoids and pathogens was compared between host plant species using logistic regression and the events/trials syntax (procedure GLIMMIX; SAS Institute 2009). The mortality caused by different factors was compared between host plant species using Fisher's exact test.

Experiments with natural enemies

We studied whether defence effectiveness was influenced by the composition of larval secretions by conducting experiments with several of the natural enemies of *C. lapponica* that were abundant in our study area: specialist parasitoid fly *M. breviseta*, specialist predator larva of syrphid fly *Parasyrphus nigritarsis* Zett. (Diptera, Syrphidae), and two generalist predators: wood ant *Formica polyctena* Forst. (Hymenoptera, Formicidae) and pentatomid bug *Rhacognathus punctatus* (L.) (Hemiptera, Pentatomidae). In all experiments, we compared the responses of enemies to larvae with the greatest contrast in the composition of their defensive secretions, i.e. larvae collected from or reared on either *S. caprea* (major components of secretions are autogenously produced butyric esters) or *P. suaveolens* (major component of secretions is salicylaldehyde sequestered from host plant SGs).

We determined whether secretion composition influenced prey selection by a predator using the methods described by Gross et al. (2004a) and Zvereva et al. (2010b). Experiments with syrphid fly larvae were conducted on 30-31 July 2015 in Petri dishes (85 mm in

diameter, with the bottoms covered with wet filter paper) in a laboratory environment. One fly larva was placed into each dish and offered two small pieces of filter paper that had been soaked with secretions of larvae reared on either *S. caprea* or *P. suaveolens*. The larva's first choice and the number of attacks on each piece of paper were recorded during 10 min of uninterrupted observations. Nine syrphid larvae starved for 24 h participated in this experiment; seven of them were used twice (after another 24 h of food deprivation) for a total of 16 tests. Neither of these two characteristics differed between the first and second rounds of the experiment (first choice: $c^2 = 0.13$, d.f. = 1, $P = 0.72$; number of attacks: $F_{1,28} = 0.01$, $P = 0.93$), and therefore the data were combined for the further analyses.

Experiments with pentatomid bugs were also conducted in the laboratory in Petri dishes, each containing one bug (adult or last instar nymph), which had been food deprived for 24 h, and one *C. lapponica* larvae from each of *S. caprea* and *P. suaveolens*. During two sessions (22 and 31 July 2015), the predatory behaviour of 13 bugs was observed until an attack and beginning of feeding, and the selection of prey was recorded. In addition, a small trial was conducted with five bugs to test whether the secretion itself was attractive; the method was the same as used in the experiments with the syrphid fly larvae.

Experiments with wood ants were conducted in the field on 24 and 28 July 2014 at a site where no *C. lapponica* was found within 100 m from the ant hill; therefore, the ants from this nest were naïve with respect to either type of secretion. Before start of observation we tested whether the ants were interested in proteinaceous food by offering non-defended prey (a medium sized fly). Last instar *C. lapponica* larvae of about same size were used immediately after they had been collected from their host plants (10 larvae from *S. caprea* and 11 larvae from *P. suaveolens*). Two kinds of prey were placed, in turn, on the ground near the ant trails (each prey on a different patch), and ant behaviour was observed as

described in Zvereva et al. (2016a). We recorded the number of ants repelled from the prey until the larva was attacked.

Field experiments with parasitoid *M. breviseta* flies were conducted as described in Zvereva and Rank (2004). On 23 and 24 July 2014 and on 23 and 26 July 2015 five bushes of *S. caprea* were selected at least 10 m apart, and 3 sticky traps with cotton balls were attached to leaves of each plant. Cotton balls were soaked with the following substances: water (control), water with added secretions collected from 3−4 last instar larvae of *C. lapponica* feeding on *S. caprea*, and water with an added drop of synthetic salicylaldehyde. We had to use the pure chemical instead of larval secretions due to a shortage of larvae feeding on *P. suaveolens* in nature. Our previous studies showed that the phorids of the Kola Peninsula are similarly attracted to synthetic salicylaldehyde and to larval secretions containing salicylaldehyde (Zvereva et al. 2010b; and unpublished). After one hour of exposure, we recorded the number of phorid flies that landed on the sticky resin on each trap.

Results

Distribution of *C. lapponica* among host plants

In our study site larvae of *C. lapponica* were found feeding on five salicaceous species. The density of larvae was highest on *S. caprea* followed by *S. rorida* (Fig. 1): larvae were found on almost every individual plant of these species. The distribution of *C. lapponica* among these host species varied among the study years (year × host interaction: $F_{7,24} = 3.72$, $P = 0.007$). Larvae were found on all five host species in 2009, on four hosts in 2014 and on three hosts in 2015.

Secondary chemistry of host plants

Salicin, salicortin and tremuloidin were the major SGs, and their foliar concentrations varied considerably among the studied species (Fig. 2). Among the glucosides sequestered for defence by *C. lapponica* larvae, salicin had the highest concentrations in *S. dasyclados* and *S. glauca*, whereas salicortin was present mostly in *P. suaveolens* (Fig. 2A, B); the salicortin concentrations were several orders of magnitude higher in poplar than in willow species. Concentrations of tremuloidin were highest in *S. glauca*, and this compound was absent from *S. caprea* and *P. suaveolens* (Fig. 2C). Differences in the concentrations of SGs between *P. suaveolens* and the four willow species ($\chi^2 = 12.3$, d.f. = 1, $P = 0.0004$) represented the greatest part of among-species variation in chemistry.

Chemistry of larval secretions

The biochemical profiles of larval secretions (Online Resource 1) were considerably influenced by host plant species, as indicated by the significant interaction between host plant and a particular chemical compound in the MANOVA test ($F_{68, 748} = 12.86$, $P < 0.0001$). This interaction remained significant when the analysis was restricted to larvae feeding on willows and to autogenous components of secretions ($F_{48, 460} = 5.79$, $P < 0.0001$). Concentrations of both sequestered salicylaldehyde and *de novo* synthesised components, such as 2-methylbutyric acid and major esters (2-phenyethil isobutyrate and 2-phenylethil 2 methylbutyrate), varied among larvae reared on different host plant species, although the sum of all butyric esters showed no significant variation (Fig. 3). The concentrations of salicylaldehyde in larval secretions was highest when larvae fed on *P. suaveolens* (Fig. 3C), but did not vary among larvae feeding on different willow species ($F_{3, 35} = 1.12$, $P = 0.36$).

Major *de novo* components (2-phenyethil isobutyrate and 2-phenylethil 2 methylbutyrate) were also at their highest concentrations in secretions of larvae feeding on *P. suaveolens* (Fig. 3C, D), but variation in concentrations of these compounds remained significant when the analyses were restricted to larvae feeding on willows ($F_{3, 35} = 3.56$, $P = 0.02$ and $F_{3, 35} = 4.43$, $P = 0.01$, respectively).

Mortality from natural enemies

In our study site we observed following potential predators: wood ants, various bugs and spiders, ladybird larvae, syrphid larvae and insectivorous birds. Among these, we directly recorded syrphid fly *P. nigritarsis* larvae, pentatomid bug *R. punctatus* and an unidentified ladybird larva preying on *C. lapponica* larvae.

Predation rates on experimental plants did not differ between *S. caprea* and *S. dasyclados*, but were significantly higher on *S. rorida* (Fig. 4A; $F_{2,8} = 7.03$, $P = 0.017$). Total mortality from parasitism and pathogens did not differ among the different host plants (Fig. 4B; 2009: $F_{1,10} = 0.07$, $P = 0.80$; 2014: $F_{2,13} = 0.29$, $P = 0.75$), but the relative contributions of individual mortality factors varied among the host plant species in both 2009 (Fig. 5A; Fisher's exact test, $P = 0.046$) and 2014 (Fig. 5B; Fisher's exact test, $P = 0.003$).

Larvae feeding on *S. caprea* (the only host species that was sampled during all study years) demonstrated pronounced among-year variations in mortality from both parasitoids ($F_{2,26} = 33.5$, $P < 0.0001$) and pathogens ($F_{2,26} = 12.0$, $P = 0.0002$). This variation was significant for all groups of parasitoids: phorid flies ($F_{2,26} = 37.5$, $P < 0.0001$), chalcidid wasps ($F_{2,26} = 12.0$, $P = 0.0002$) and tachinid flies ($F_{2,26} = 3.73$, $P = 0.04$).

Behaviour of natural enemies

Larvae of the syrphid fly, *P. nigritarsis*, actively searched for prey and attacked pieces of filter paper soaked with prey defensive secretions, striking the paper and scratching its surface with their mouthparts. The filter paper moistened with water, which covered the bottom of the Petri dish, was never attacked by syrphid larvae. The first attack occurred at similar rates on papers with prey secretions of different composition, and the numbers of attacks during a 10-min session were similar for these two types of secretions (Table 1).

A similar experiment showed that the predatory bug *R. punctatus* was not attracted to filter papers with defensive secretions of either prey in any of five replicates. The larvae differing in secretion composition in a dual choice test were attacked at similar rates (Table 1); all attacked larvae were killed and fed upon. Observations made during this experiment indicated that bugs searched for their prey based on the prey's fresh faeces and that prey movement stimulated the attack. In most cases, a bug attacked larvae by slowly approaching it from behind (especially in the case when the bug followed faecal traces of the prey), or it attacked from the side, keeping its rostrum stretched forward along the surface and penetrating the prey from the non-defended ventral side of the abdomen. This behaviour allowed the bugs to avoid contact with the defensive secretions of the prey, which were frequently released from dorsally located glands as the predator approached. In the single observed case when a bug contacted a secretion, the bug retreated and cleaned the secretion from its body; after that, the bug immediately resumed attacking the prey.

In the experiment with *F. polyctena* ants, the control prey was immediately taken by the first ant that found it. When *C. lapponica* larvae were offered, one to ten ants retreated upon contact with larval secretions before one of ants attacked (bit) the prey; after that, the prey was carried by the ants in the direction of the ant nest. The number of ants repelled by a

single prey was greater for larvae feeding in nature on *P. suaveolens* than for larvae feeding on *S. caprea* (Table 1).

The fly parasitoid *M. breviseta* landed more frequently on the sticky traps with salicylaldehyde than on traps with secretions of *C. lapponica* feeding on *S. caprea* (Table 1), while no flies were found on the control traps with water.

Discussion

Within-population variation in defensive chemistry

Our records show that the population of *C. lapponica* in the Baikal area has a relatively broad diet and feeds naturally on several plant species. Although the host plants all belong to the family Salicaceae, they differ substantially in their secondary chemistry, as we demonstrated by the analysis of salicylic glucosides.

We have found that the studied population of *C. lapponica* demonstrates considerable variation in both the total concentration of defensive compounds and the relative concentrations of different components of larval secretions, i.e. in their biochemical profiles. In our previous experiments (Geiselhardt et al. 2015) sibling larvae produced secretion of different composition when fed on two host plants substantially differing in chemistry. Therefore we conclude that variation found in our study is at least partly explained by variations in the chemistry of the host plant foliage. The greatest difference in biochemistry has been found between *P. suaveolens* and willows: poplar contains high concentrations of SGs (primarily salicortin), whereas the concentrations of SGs in all the studied willow species are low. Consequently, *C. lapponica* larvae that feed on willows synthesise butyric acid *de novo* to produce a variety of esters using plant-derived alcohols, as do *C. lapponica*

populations that feed on birch (Hilker and Schultz 1994; Tolzin-Banasch et al. 2011), but produce no or only trace amounts of salicylaldehyde. By contrast, larvae from the same population feeding on poplar, in addition to autogenous compounds, sequester SGs to produce large amounts of salicylaldehyde. Thus, in contrast to the highly specialised populations of *C. lapponica*, which have either lost the ability to sequester host SGs (Kirsch et al. 2011) or produce very low amounts of autogenous compounds (Geisehardt et al. 2015), the defensive strategy of the non-specialised Baikal population is plastic and ranges from sequestration to *de novo* synthesis, depending on the secondary chemistry of the host plant. Earlier, we demonstrated experimentally that in this population, larvae from the same brood produce primarily host-plant–derived salicylaldehyde when fed on SG-rich plants and considerable amounts of autogenous components when fed on SG-poor plants (Geiselhardt et al. 2015). Our current study confirms that both these defensive strategies are used in nature by the non-specialised Baikal population of *C. lapponica*.

The variation in the composition of larval secretions is not limited to differences in the origin (sequestered or synthesised *de novo*) of defensive compounds. We found that biochemical profiles of secretions produced *de novo* by larvae feeding on SG-poor willows also vary depending on the willow species. The synthesis of butyric esters, which are the main defensive constituents in this type of secretion, involves esterification of *de novo* synthesised iso- and 2-methylbutyric acids by alcohols taken from the host plant (Schulz et al. 1997; Termonia et al. 2001); therefore, this variation may be explained by differences in the alcohol profiles between willow species. However, in our study the concentration of 2-methylbutyric acid also differs considerably between the host plants. These results are in line with a study of another leaf beetle, *Oreina speciosa*, which also demonstrated a host plant influence on the within-population variation in the profiles of defensive compounds (cardenolides) synthesised *de novo* by the beetles (Triponez et al. 2007).

To conclude, the larval defensive secretions in the Baikal population demonstrate considerable variation in chemical profiles, which is associated with differences in the chemical profiles of the multiple naturally used host plants. An important question is whether the variation in the larval defensive secretions among host species results in the differences in larval mortality from natural enemies.

Mortality due to natural enemies

Our study uncovered no differences in overall mortality of *C. lapponica* from natural enemies on different host plants species, indicating that none of the host plants used by the Baikal population of this leaf beetle species provides survival advantages for individuals. At the same time, we found differences among the host plant species with respect to the relative contribution of mortality factors, i.e. the enemy profiles of the larvae.

Our experiments with individual enemy species allowed us to uncover the reasons behind the observed differences in enemy profiles among the host plants. In these experiments, we compared two types of larval secretions that most contrasted in their origins and chemistries. The four studied enemy species showed different responses to two types of prey: larvae fed on *S. caprea* lacking SGs and thus producing only autogenous secretions, and larvae fed on SG-rich *P. suaveolens* and thus producing primarily sequestered salicylaldehyde.

The four studied species of predators and parasitoids do not cover the entire variety of natural enemies, but illustrate all three possible types of responses to prey defences: (i) the higher avoidance of salicylaldehyde secretions compared to butyrate secretions (ants), potentially leading to greater prey survival on SG-rich host plants; (ii) attraction to salicylaldehyde secretions (phorid flies), potentially leading to greater prey survival on SG-

poor host plants; and (iii) no preference (syrphid fly, bug *R. punctatus*), resulting in equal prey survival on SG-rich and SG-poor host plants. Furthermore, preliminary observations on the predation of great tits on *C. lapponica* larvae indicate that these birds likely belong to the first group (unpublished data). Similarly, salicylaldehyde may provide defence against some infections (Gross et al. 2002). In contrast, tachinid flies demonstrate lower parasitism rates on SG-rich host plants (Zvereva and Rank 2003) and thus belong to the second group. Many species of predatory bugs overcome prey chemical defences independently of their chemistry (Zvereva and Kozlov 2016) and thus belong to the third group.

Phorid parasitoids are an important mortality factor for *C. lapponica*, causing up to 80% mortality in the Kola population (Zvereva and Kozlov 2000) and up to 40% in the current study. Phorid flies use salicylaldehyde as a cue in their host search in the SG-specialised populations of *C. lapponica* (Gross et al. 2004a; Zvereva and Rank 2004), but the higher attractiveness of salicylaldehyde for phorids in the Baikal population of *C. lapponica*, compared to the more widespread autogenous secretions, is somewhat surprising. This finding may indicate that the ancestral population of *C. lapponica* in the Baikal area mostly used SG-rich host plants, and only later shifted to SG-poor hosts, as did several birch-specialised populations (Mardulyn et al. 2011). It is likely that the local phorid parasitoid population retains the ability to use as a search cue the component of secretions (salicylaldehyde) previously produced by a major part of the prey population. Consequently, the high attractiveness of salicylaldehyde for parasitoids may explain why only 4% of the local population feeds on poplar, although the overall concentration of defensive compounds in the larval secretions is highest on this host (Fig. 3), and the SGs in the host plants do not impose negative effects on larval development (Zvereva et al. 2010a).

Similarly to the phorid parasitoids, the specialist predatory syrphid larvae use defensive secretions as a search cue (Köpf et al. 1997; Gross et al. 2004a), and salicyaldehyde

was found to be stronger signal for these predators than autogenous defence of *C. lapponica* from birch population (Gross et al. 2004a). However, the predatory syrphid larvae showed a similar attraction to either type of defence in the Baikal population of *C. lapponica*. Since our experiment followed the design of the experiments by Gross et al. (2004a), we explain this discrepancy by an adaptation of the Baikal population of syrphids to use various defensive compounds present in the local prey population, whereas the experiment by Gross et al. (2004a) involved predators from a locality where the prey population sequestered salicylaldehyde.

Wood ants are important predators of herbivorous insects, including chemically defended leaf beetle larvae (Zvereva et al. 2016a). Previous experiments on ant predators in other populations of *C. lapponica* showed that wood ants were more strongly repelled by larvae with sequestered secretions than with autogenous secretions (Zvereva et al. 2010b), and the current study supported this finding. Although both kinds of defences were ultimately overcome due to collective efforts and all larvae offered near the ant trail were killed, the differences in the numbers of ants repelled before the first attack attempt may lead to differences in the probability of prey survival when the ant density is low (Zvereva et al. 2016a).

Many bug species are severe predators of herbivorous insects, including chemically defended species (Traugott and Stamp 1996; Boevé and Müller 2005). In our study, *R. punctatus* bugs did not show any aversion for either type of defence, nor were they attracted to defensive secretions. In line with other studies on true bugs (Rank and Smiley 1994; Boevé and Müller 2005), our observations show that bugs, due to their morphology and behaviour patterns, avoid contact with secretions released by their prey. Consequently, the chemical defences of herbivorous insects generally do not provide effective protection against predatory bugs (Zvereva and Kozlov 2016).

Overall, the different invertebrate enemies of *C. lapponica* demonstrated specific reactions to the composition of the defensive secretions of their prey. This variation in responses may have important consequences for prey populations with a high diversity of defences in the face of a set of enemies with unpredictable abundances (Speed et al. 2012). Natural enemy abundance varies between years, and different enemies usually do not fluctuate in synchrony. For example, the mortality from three parasitoid species in the Kola population of *C. lapponica* showed no correlation during six years of observations (Zvereva and Kozlov 2000). In our current study, the mortality caused by all three studied parasitoid species and by pathogens demonstrated significant among-year variation, and the mortality from different enemies peaked in different years. Therefore, in the years when a certain enemy species is abundant, a part of the prey population will escape from this enemy by feeding on the host plant which provides the most effective defence against that particular enemy. Consequently, different host plants will assure the best protection of *C. lapponica* larvae in different years, depending on which enemy is currently most abundant. For example, in years when predation by ants (and presumably by insectivorous birds) is high, prey population will survive better on SG-rich host plants, because these predators have stronger aversion to salicyaldehyde-containing secretions than to butyrate-containing secretions. In contrast, in years when phorid or tachinid parasitoids reach high abundance, survival of *C. lapponica* population will be higher on SG-poor host plants, because these parasitoids attack hosts with salicyaldehyde secretion more frequently (Zvereva and Rank 2003). This suggestion is indirectly supported by the among-year variations observed in the distribution of the last instar larvae among the host plants (Fig. 1), which may be explained by the among-year variations in the relative abundances of the natural enemy species.

Thus, among-individual variation in chemical defence associated with using different host plants may buffer population against periodically increasing pressure of a specific

enemy. In this way, a polyphagous population may escape from top-down density regulation, avoiding a density decline following an increase in enemy pressure. This plastic defensive strategy differs from anti-predator strategies of specialised populations, which assure either defences against generalist enemies at the cost of increased vulnerability to specialist enemies (populations feeding on SG-rich willows), or avoidance of specialist enemies at the cost of increased vulnerability to generalist enemies (populations feeding on birches or SG-poor willows: Gross et al. 2004a; Zvereva et al. 2010b). All these strategies provide protection against natural enemies for the population, but the effectiveness of each strategy may depend on the local diversity of natural enemies and on the relative abundance of specialist and generalist enemies. It was suggested that specialisation of *C. lapponica* on SG-rich willows has evolved to escape from generalist enemies (Pasteels et al. 1990), whereas the subsequent shift of some populations to SG-free birches could be driven by specialist enemies (Gross et al. 2004a). In line with these hypotheses, we suggest that high within-population variation of chemical defences in a polyphagous population, associated with the use of host plants differing in secondary chemistry, is maintained by similar pressures from specialist and generalist enemies.

Specialized populations of *C. lapponica* may appear more vulnerable to density increase of a specific enemy and thus show less stable population dynamics, sometimes suffering dramatic population declines. In line with this suggestion, population of *C. lapponica* in the Kola Peninsula, which is specialized on SG-rich willows, shows extreme fluctuations in population densities (Zvereva et al. 2016b). Similarly, many European populations of *C. lapponica*, which are specialized on birches, are now almost extinct after several outbreaks that have occurred in the beginning of 1990s (Gross, Hilker, Warchalovski, personal communications). In contrast, the density of the Baikal population of *C. lapponica* remains stable during many years (Didorenko, personal communication; and our study).

These observations are in agreement with the conclusion that populations of polyphagous species are less variable in density than populations of species with a narrow feeding niche (MacArthur 1955; Redfearn and Pimm 1988; but see Forsman et al. 2015), and with the suggestion that this relationship may be linked with parasitism and predation (Redfearn and Pimm 1988).

Variations in chemical defence within prey populations may, potentially, provide a considerable advantage for these populations in the face of a diverse complex of natural enemies (Speed et al. 2012). In particular, some experimental studies have demonstrated that prey populations with polymorphic chemical defences suffer less from bird predation than do monomorphic populations (Skelhorn and Rowe 2005; Barnett et al. 2014). Although the variation in chemical defence profiles may be genetically based (Eggenberger and Rowell-Rahier 1992; Holloway et al. 1993), our current study showed that this variation in herbivorous insects at least partly depends on the host-plant species, even when the defensive chemicals are synthesised *de novo*. Thus, one of the advantages of polyphagy may appear in providing chemical diversity among individuals within a population, which, similarly to other forms of polymorphism (Karpestam et al. 2016), may afford protection against natural enemies for individuals and populations, promoting ecological success of a polyphagous populations in a multi-enemy world.

Acknowledgements We thank G. Soderman, J. Sorvari, J. Pohjoismäki and H. Disney for identification of natural enemies, V. Chepinoga for identification of plants, V. Ossipov and R. Julkunen-Tiitto for analyzing plant samples, M. Hilker, F. Müller and B. Helbig for providing facilities and help with the analysis of larval secretions, S. Didorenko, J. Gross, M. Hilker, and A. Warchalowski for sharing with us their unpublished observations, and three anonymous reviewers for inspiring comments to an earlier draft of the manuscript. The study

was supported by the Academy of Finland through research grant to E. L. Zvereva (project 268124) and travel grants to E. L. Zvereva and V. Zverev, and by EU Erasmus+ through mobility grant to V. Zverev.

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Table 1 Behavioural responses of natural enemies to larvae of *Chrysomela lapponica* possessing sequestered defence (secretion containing mainly salicylaldehyde) and autogeneous defence (secretion containing mainly butyrates)

Species	Order, family	Feeding habit	Speciali-sation	Measured character[a]	Main component of secretions		Statistics and d.f.	*P*
					Salicylaldehyde	Butyrates		
Parasyrphus nigritarsis Zett.	Diptera, Syrphidae	Predator	Specialist	Number of attacks on filter paper with secretions during 10 min	2.31 ± 0.44	1.69 ± 0.35	Paired $t_{15} = 0.97$	0.35
				First choice of filter paper with secretions	7	9	$\chi^2_1 = 0.06$	0.81
Rhacognathus punctatus (L.)	Hemiptera, Pentatomidae	Predator	Generalist	First choice of live prey	7	6	$\chi^2_1 = 0.15$	0.69
Formica polyctena Forst.	Hymenoptera, Formicidae	Predator	Generalist	Number of repelled ants	3.73 ± 1.13	1.50 ± 0.77	$F_{1,20} = 4.22$	0.05
Megaselia breviseta (Wood)	Diptera, Phoridae	Parasitoid	Specialist	Number of attracted flies	1.56 ± 0.44	0.25 ± 0.13	Paired $t_8 = 2.53$	0.04

[a] Means ± S.E. for measured responses; numbers of events for the first choice data.

Figure captions

Fig. 1. Density of *Chrysomela lapponica* on different host plant species in different study years. Sca – *Salix caprea*, Sro – *S. rorida*, Sda – *S. dasyclados*, Srh – *S. rhamnifolia*, Sgl – *S. glauca*, Psu – *Populus suaveolens*. The means (±SE) are based on the values obtained from 50 m^2 plots (four plots in 2009 and 2015, two plots in 2014).

Fig. 2. Concentrations of salicylic glucosides in host plants of *Chrysomela lapponica*. Values are means (±SE) based on five plant individuals. Among-species variation is analysed by Kruskal-Wallis test. For abbreviations of plant names see Fig. 1..

Fig. 3. Relative concentrations (peak area/100 ng Internal Standard/µl secretion) of major defensive compounds in secretions of *Chrysomela lapponica* naturally feeding on different host plant species. Butyrates include also isobutyrates and 2-methylbutyrates. Values are means (±SE) each based on ten larvae; values marked with different letters significantly differ from each other (Duncan test). Among-species variation is analysed by ANOVA. For abbreviations of plant names see Fig. 1.

Fig. 4. Mortality of *Chrysomela lapponica* naturally inhabiting different host plant species. A: mortality of the last instar larvae due to predators over a five day interval in 2015; B: total mortality due to parasitoids in 2009 and 2014. Values are means (±SE) based on plant individuals (plant numbers are shown in parentheses). For abbreviations of plant names see Fig. 1.

Fig. 5. Mortality of *Chrysomela lapponica* naturally inhabiting different host plant species due to different parasitoid species and pathogens in 2009 (A) and 2014 (B). Values are means (±SE) based on plant individuals (plant numbers are shown in parentheses). For abbreviations of plant names see Fig. 1.

Online resource: Table S1 Composition of the larval defensive secretions of *Chrysomela lapponica* from Baikal population (relative amount = peak area/100 ng Internal Standard) feeding in nature on five host plant species.

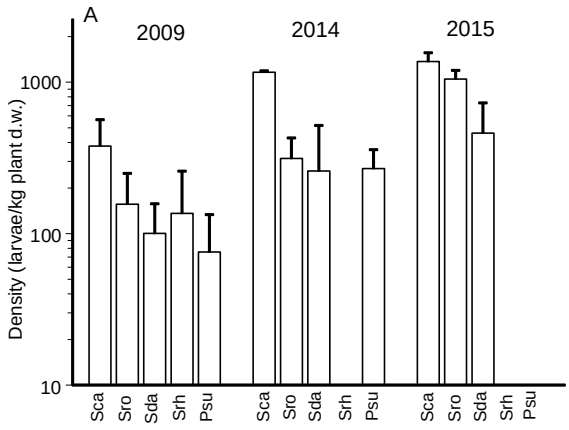
A
2009
2014
2015
1000
100
10
Density (larvae/kg plant d.w.)
Sca
Sro
Sda
Srh
Psu
Sca
Sro
Sda
Srh
Psu
Sca
Sro
Sda
Srh
Psu

Fig. 1

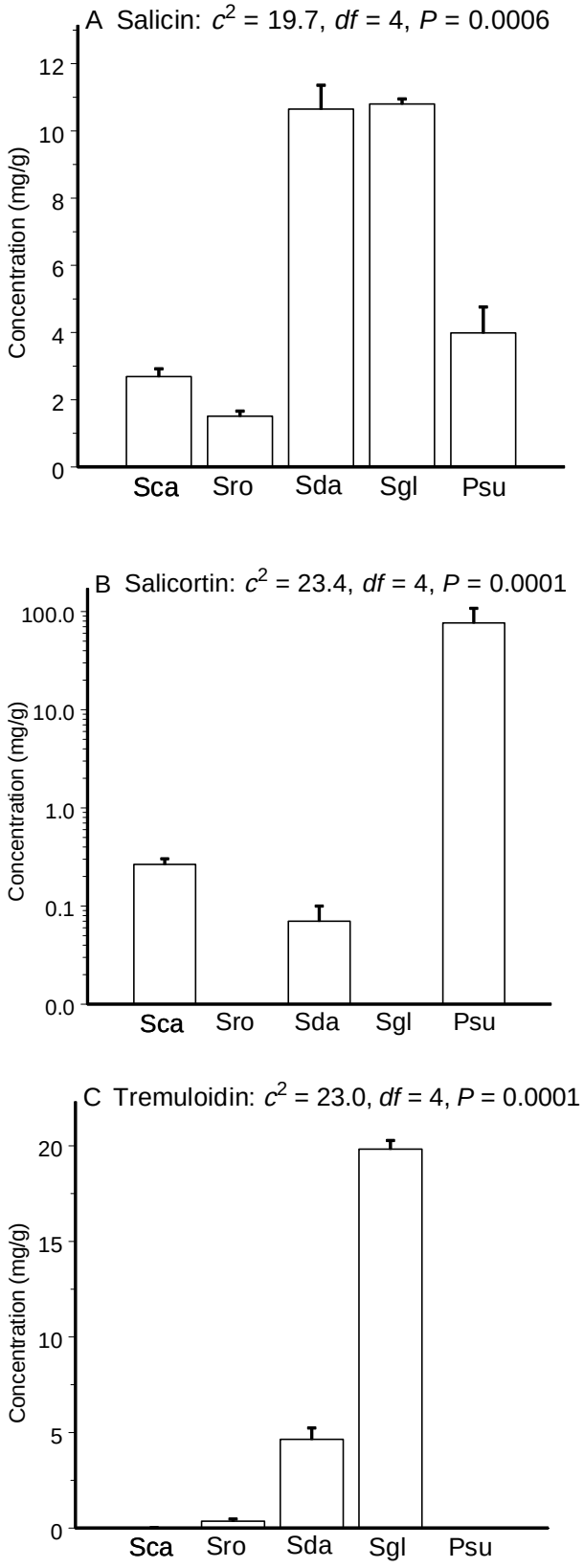
A Salicin: c^2 = 19.7, *df* = 4, *P* = 0.0006
Concentration (mg/g)
12
10
8
6
4
2
0
Sca
Sro
Sda
Sgl
Psu
B Salicortin: c^2 = 23.4, *df* = 4, *P* = 0.0001
Concentration (mg/g)
100.0
10.0
1.0
0.1
0.0
Sca
Sro
Sda
Sgl
Psu
C Tremuloidin: c^2 = 23.0, *df* = 4, *P* = 0.0001
Concentration (mg/g)
20
15
10
5
0
Sca
Sro
Sda
Sgl
Psu

Fig. 2

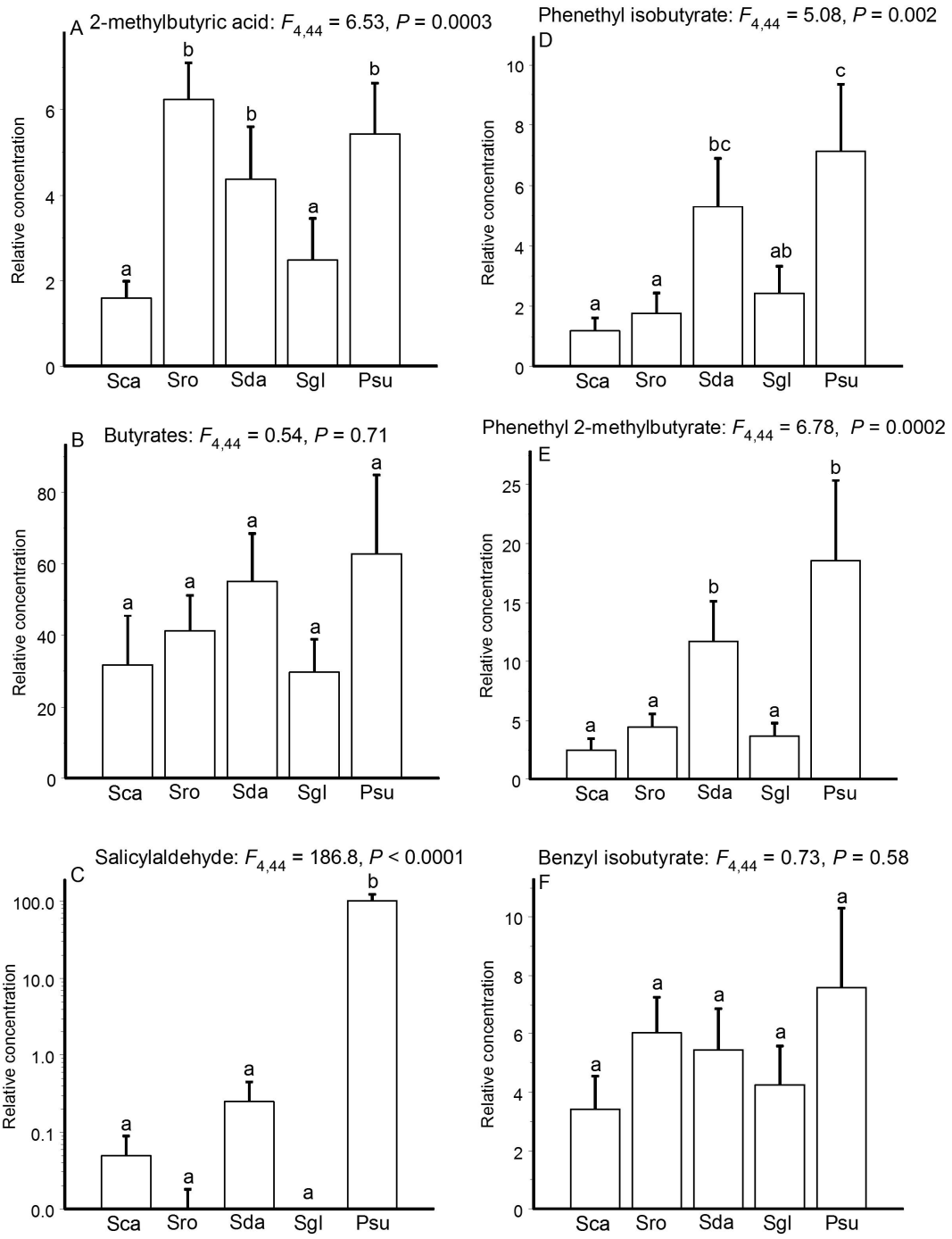
A 2-methylbutyric acid: $F_{4,44}$ = 6.53, P = 0.0003
Relative concentration
0
2
4
6
a
b
b
a
b
Sca
Sro
Sda
Sgl
Psu
D
Phenethyl isobutyrate: $F_{4,44}$ = 5.08, P = 0.002
Relative concentration
0
2
4
6
8
10
a
a
bc
ab
c
Sca
Sro
Sda
Sgl
Psu
B
Butyrates: $F_{4,44}$ = 0.54, P = 0.71
Relative concentration
0
20
40
60
80
a
a
a
a
a
Sca
Sro
Sda
Sgl
Psu
E
Phenethyl 2-methylbutyrate: $F_{4,44}$ = 6.78, P = 0.0002
Relative concentration
0
5
10
15
20
25
a
a
b
a
b
Sca
Sro
Sda
Sgl
Psu
C
Salicylaldehyde: $F_{4,44}$ = 186.8, P < 0.0001
Relative concentration
0.0
0.1
1.0
10.0
100.0
a
a
a
a
b
Sca
Sro
Sda
Sgl
Psu
F
Benzyl isobutyrate: $F_{4,44}$ = 0.73, P = 0.58
Relative concentration
0
2
4
6
8
10
a
a
a
a
a
Sca
Sro
Sda
Sgl
Psu

Fig. 3

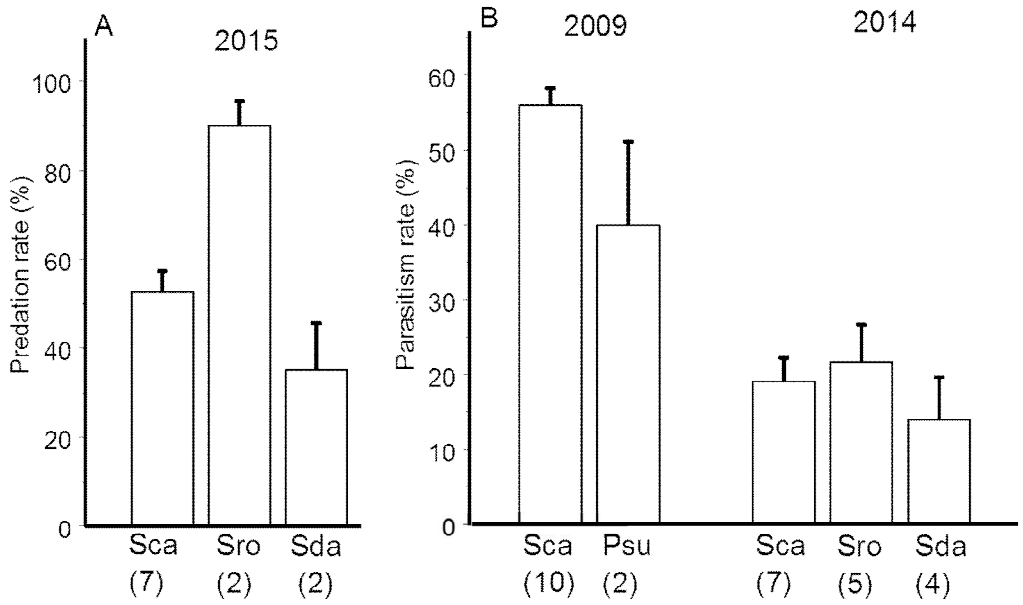
A
2015
Predation rate (%)
100
80
60
40
20
0
Sca
(7)
Sro
(2)
Sda
(2)
B
2009
2014
Parasitism rate (%)
60
50
40
30
20
10
0
Sca
(10)
Psu
(2)
Sca
(7)
Sro
(5)
Sda
(4)

Fig. 4

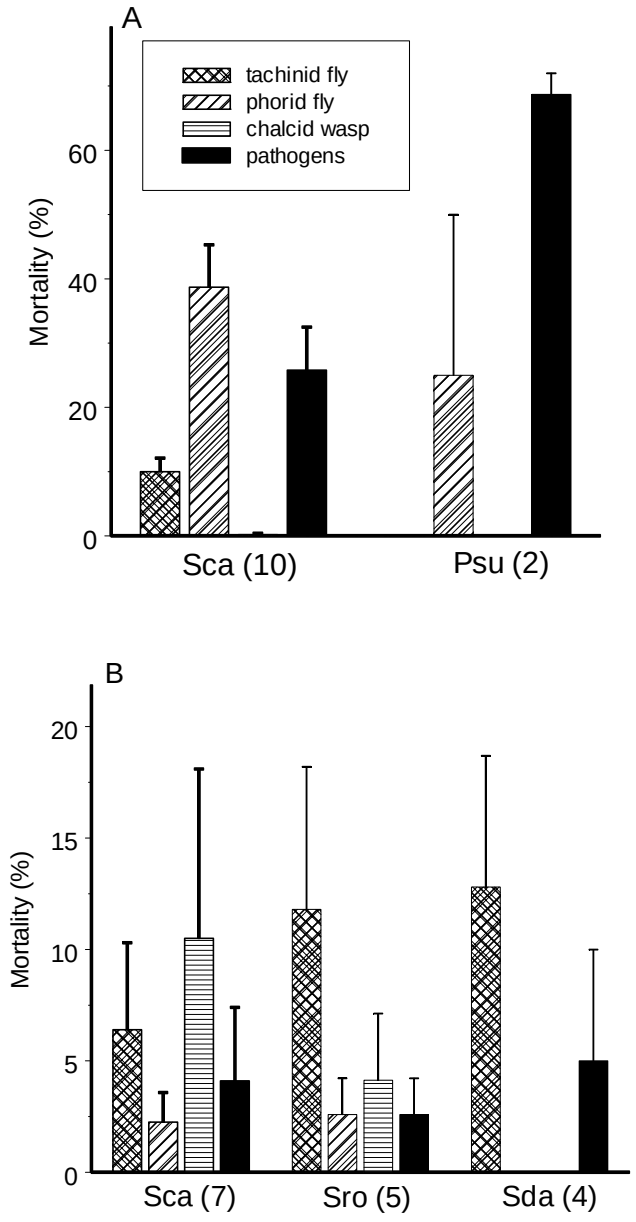
A
tachinid fly
phorid fly
chalcid wasp
pathogens
Mortality (%)
0
20
40
60
Sca (10)
Psu (2)
B
Mortality (%)
0
5
10
15
20
Sca (7)
Sro (5)
Sda (4)

Fig. 5