

Response of the lichen-eating moth *Cleorodes lichenaria* larvae to varying amounts of usnic acid in the lichens

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Abstract Lichens are characterized by a great variety of secondary metabolites. The function of these substances remains partly unknown. In this study, we propose that some of these metabolites may expel insect herbivores. To test this hypothesis, we reared larvae of the lichenivorous moth *Cleorodes lichenaria* on three selected lichens, *Cladonia arbuscula* subsp. *mitis*, *Usnea hirta*, and *Usnea dasypoga*. In experimental setup, the secondary metabolite usnic acid was removed from the lichens with acetone prior to feeding, whereas a control was left untreated. On all three lichens, removal of usnic acid from the lichens using acetone significantly prolonged survival of larvae and increased their viability. Larvae reared on control lichens contained significantly more usnic acid than those reared on treated lichens, both in their biomass and their faeces. These results support the hypothesis that usnic acid serves as a repellent against insect feeding, besides its well established functions of UV protection and antimicrobial properties.

Keywords Herbivory · Insect feeding · Moths · Repellent · Usnic acid

Introduction

Lichens are a symbiotic association between fungi (mycobionts) and algae or cyanobacteria (photobionts, phycobionts), representing nearly one fifth of all known fungal species (Hawksworth et al. 1995). In spite of their poorly developed morphology, lichens contribute crucially to the vegetation worldwide and even dominate the vegetation of approximately 8 % of terrestrial ecosystems (Larson 1987).

Lichens produce around thousand different extracellular secondary metabolites known as “lichen substances” (Hauck and Huneck 2007). Their functions include—but are not restricted to—antimicrobial, antiherbivoral, and allelopathic activities (Lawrey 1986; Fahselt 1994), the protection of the photobiont cells against excessive light, the control of photobiont cell division, or chelating of potentially toxic metals (Hauck et al. 2009; Solhaug et al. 2009; Bačkor et al. 2010). Lichen secondary metabolites are usually located extracellularly on the cell surface. Thus, many secondary metabolites can be removed from dry thalli by rinsing with acetone (Solhaug and Gauslaa 1996) without the loss of lichen viability. It is, therefore, possible to manipulate the content of secondary metabolites in lichen thalli, enabling the test of hypotheses on their physiological and ecological functions.

In spite of the production of secondary metabolites, lichenivory, i.e. feeding on lichens, is relatively common. Lichenivorous organisms range from invertebrates such as springtails, mites, gastropods, and larvae of Lepidoptera, to large mammals such as reindeer or even monkeys (Gerson and Seaward 1977). Although Stahl (1904) found increased palatability in herbivores as response to removal of secondary metabolites from lichens, hypotheses on a possible defence function of the secondary metabolites remained unanswered for more than a hundred years (Zukal 1895).

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The selection of a host by insect herbivores is primarily determined by host properties such as nutritional quality and chemical defence against herbivores. For many lichen metabolites, including usnic acid, toxicity and insecticidal activity have been described (e.g. Emmerich et al. 1993; Ingólfssdóttir 2002; Pöykkö et al. 2005; Pöykkö and Tammaru 2010). In feeding experiments with lichenivorous Lepidoptera, these authors demonstrated that usnic acid caused high mortality, growth retardation, and prolonged larval periods. Some lichenivores, however, are specialized in feeding on lichens that are rich in usnic acid, and the response of such specialists to different levels of usnic acid is rarely reported. *Cleorodes lichenaria* (Hufnagel) is a lichen-eating geometrid moth feeding on several *Ramalina* species containing usnic acid (Pöykkö 2006). *C. lichenaria* larvae seem to have adapted to the small amounts of metabolites present in *Ramalina*; neonate larvae are slightly stunted, but later stages remain completely unaffected (Pöykkö et al. 2010).

The aim of this study is to quantify the effects of usnic acid, one of the most frequent cortical secondary metabolites from lichens, on larval survival and growth of *C. lichenaria*, and to evaluate the role of lichen (plant) chemical defense against herbivore feeding.

Methods and materials

Preparation of lichen material

Three lichen species, *Cladonia arbuscula* (Sandst.) Ruoss., *Usnea dasyposa* (Ach.) Röhl., and *Usnea hirta* (L.) Wigg. were used in our experiments. All of them contain usnic acid as the main cortical secondary metabolite (Culberson et al. 1977). *C. arbuscula* was collected in Slovakia (terricolous lichen, locality Gelnica-Slovenské Cechy), the epiphytic lichens *U. dasyposa* and *U. hirta* were collected from spruce (*Picea abies*) and pine (*Pinus silvestris*), respectively, in parks in Oulu, Finland. Fifteen thalli of each species were collected, one part of each thallus served as a control, and the other was treated. Both treated and control thalli were dried in a desiccator for 2 days.

The treatment thalli were rinsed with dry acetone, five times for 5 min each, in order to remove most of the secondary metabolites (Solhaug and Gauslaa 2004; Asplund et al. 2015). Subsequently, they were placed in a fume hood for 2 days to allow all acetone to evaporate.

Treatment of larvae

As a test system, moth larvae of *C. lichenaria* (Fig. 1) were used. Larvae were fed with the lichen species described above. “No-choice experiments” were performed, meaning



Fig. 1 Larva of *Cleorodes lichenaria* (I instar)

that larvae had no opportunity to choose between treated and non-treated food. The respective contents of usnic acid in the lichens, the larvae and their excrements were determined and correlated using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) techniques (see below).

Larvae for the experiment originated from five females collected on the Åland Island in autumn 2011. One hundred and fifty-eight individuals were used for the experiments. After hatching, the offspring of each female was randomly divided into three groups. The first group was exposed to the experimental diet from hatching to the second instar. The other two groups were reared on *Ramalina fraxinea* from which secondary metabolites were removed in order to exclude any experience of usnic acid before the experiment. Upon reaching the second and the fourth instar, these larvae were exposed to the experimental diet until arriving at the third and fifth instar, respectively.

For the feeding experiment, the larvae were placed individually in small Petri dishes (diameter 35 mm), divided equally on each lichen/treatment combination. The Petri dish contained a small piece of lichen and a piece of filter paper, which was wetted every second to third day with 250–350 μ L deionized water. This treatment provided the humid condition necessary for larval survival, but also ensured fluctuations in humidity required by the lichens. Light regime, temperature and humidity in the cultivation chambers simulated the conditions at the natural habitat during the growth period, as described by Pöykkö et al. (2010)

Survival and growth rate of the larvae were monitored. After reaching each instar, larval mass and the date of measurement were recorded. After each measurement, the larvae were transferred into a new Petri dish. After the feeding experiment, the larvae were killed by freezing and dried for 5 days at 35 °C. The faeces produced by larvae were collected, dried, and analysed as well.

Table 1 Composition of the solvent system A, B, C for TLC

A	Toluene/dioxan/acetic acid	180:45:5
B	Hexane/methyl <i>tert</i> -butyl ether/formic acid	140:72:8
C	Toluene/acetic acid	170:30

Analyses of secondary metabolites by TLC and HPLC

The lichen secondary metabolites were analysed from lichens, larvae, and excrements of larvae by TLC and HPLC. Small samples of lichens (15–20 mg dry mass) were placed into Eppendorf tubes and secondary metabolites from surface of thalli were extracted in 1 mL cold acetone for 60 min (Feige et al. 1993). Extraction was repeated three times. Acetone extracts were evaporated and the remains were dissolved in 1.5 mL of acetone. The same method was used to extract secondary metabolites from larvae and from their excrements. Extraction times for larvae were prolonged to 24 h. Samples of larvae and lichens were not grounded before extraction of secondary compounds because we focused on cortical secondary metabolites. Grinding would lead to complete extraction including medullary compounds.

Lichen metabolites were identified by a standard TLC (Orange et al. 2001). Acetone extracts were transferred to plates coated with Merck silica gel 60 F-254. The composition of the solvents is shown in Table 1. Components were visualised by sprinkling the plate with concentrated sulfuric acid at 110 °C for 30 min.

Acetone extracts were filtrated and analyzed by gradient HPLC (Feige et al. 1993) under the following conditions: column Tessek SGX C₁₈ (4 × 250 mm; Tessek, Prague, Czech Republic), flow rate 0.7 mL min⁻¹. The composition of the mobile phase is shown in Table 2. Gradient program: 0 min 25 % B, 5 min 50 % B, 20 min 100 % B, 25 min 25 % B. Detection was performed at 245 nm (detector Ecom LCD 2084; Ecom, Prague, Czech Republic). Pure usnic acid (Aldrich) was used as a standard.

Localization of photobionts and secondary metabolites

For localization of secondary metabolites (usnic acid) and photobionts within the lichen, a Leica DM6000 CS with a TCS SP5 X scan head confocal microscope was used. Emission spectra of secondary metabolites and photobionts were acquired from crystalline usnic acid and photobiont cultures. Because of the overlapping spectra of the metabolites and the photobionts, spectral dye separation was used for visualisation. Metabolites were quantified at a detection wavelength of 500–550 nm with fixed settings for gain and offset. Fluorescence intensities were measured in the surface layer of both treatment and control thalli.

Table 2 Composition of the mobile phase for HPLC

Mobile phase		
A	H ₂ O/C ₂ H ₅ N/H ₃ PO ₄	80:19:1
B	95 % Acetonitrile	

Statistical analyses

One-way analysis of variance (Tukey's pairwise comparisons) was used to test for differences in all measured parameters, and Pearson's correlation coefficient was used for analyses of correlations; $P < 0.05$ was regarded as significant. To compare the weight of excrements, ANOVA Tukey's HSD post hoc was applied; $P < 0.01$ was regarded as significant. For microscopic analyses, software SPSS 21 with the non-parametric Mann–Whitney test for non-normal distributed samples was used.

Results

Survival of larvae *Cleorodes lichenaria*

Rearing of larvae on untreated thalli of *C. arbuscula* ($n = 10$) and *U. hirta* ($n = 9$) led to complete mortality of tested larvae (100 % mortality). Rearing of larvae on untreated thalli of lichen *U. dasypoga* ($n = 10$) resulted in 60 % mortality. Larvae reared on thalli without usnic acid showed increased survival compared to larvae reared on untreated lichen thalli. Larvae reared on *C. arbuscula* without usnic acid showed a mortality of 40 %, similar to larvae reared on the lichen *U. dasypoga*. Larvae reared on *U. hirta* without usnic acid showed approximately 33 % mortality. Significant differences between treatments were observed in *C. arbuscula* and *U. hirta*.

Average survival rate of larvae *C. lichenaria* was strongly affected by experimental removal of usnic acid from tested lichens (Fig. 2). On average, larvae reared on control *C. arbuscula* survived for 9.1 days, while removal of usnic acid tripled the average survival of up to 31.5 days ($P = 0.021$). Larvae reared on thalli of *U. hirta* had an increased average survival time due to removal of usnic acid from 7.66 to 36.3 days (approximately 4.7 times, $P < 0.001$). Removal of usnic acid from lichen *U. dasypoga* increased the survival of larvae from 22.8 to 32.5 days, but this effect was not significant ($P = 0.323$). For the evaluation of survival times, a value of days was assigned to the larvae that were still alive at the end of the experiment (Fig. 2).

The relative growth rate (RGR) values for fourth instar (Fig. 5) did not show any significant differences between control and treatment ($F_{1,13} = 1.5374$; $P = 0.2287$), nor between the three species ($F_{2,4} = 0.38$; $P = 0.6885$). The

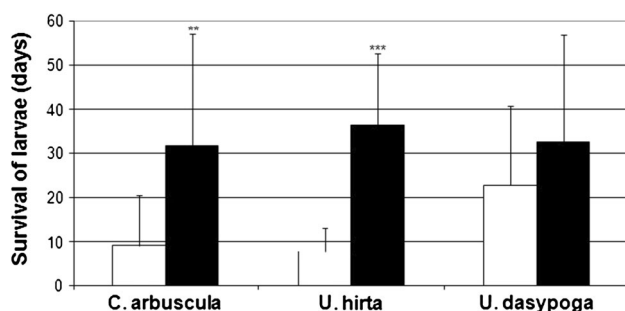


Fig. 2 Average survival (days) of larvae *Cleorodes lichenaria* reared on treated thalli of lichens (*Cladonia arbuscula* subsp. *mitis*, *Usnea hirta*, and *Usnea dasypoga*). White bars represent larvae reared on control thalli, black bars represent larvae reared on treated thalli, $n = 10$ for *Cladonia arbuscula* subsp. *mitis* and *Usnea dasypoga*, $n = 9$ for *Usnea hirta*

RGR values for first instar and second instar were not analyzed due to the death of all control larvae.

Content of usnic acid in lichens, larvae, and their excrements

The average content of usnic acid in the control thalli of *C. arbuscula* was 2.77 % of the dry weight, while removal of usnic acid from the lichen decreased its content to 0.09 % (Fig. 3a, $P < 0.001$). In control thalli of *U. hirta*, the average content of usnic acid was 5.89 % of the dry weight as compared to 0.3 % ($P < 0.001$) acid content after removal of usnic acid. Control thalli of *U. dasypoga* contained an average of 5.63 % usnic acid while removal of usnic acid from this lichen decreased its content to 0.46 % ($P < 0.001$). Other secondary compounds were detected only in trace amounts and therefore were not analyzed qualitatively (less than 1 % of the main secondary metabolite, usnic acid). Usnic acid is localized by its autofluorescence in the cortex of untreated thalli, as demonstrated in cross sections at the confocal microscope (Fig. 4). Further observation of these sections also revealed a significant reduction of the fluorescence by acetone treatment ($P < 0.01$ for all three species).

The average content of usnic acid in the larvae reared on untreated *C. arbuscula* was 0.05 ‰ (Fig. 3b) compared to 0.043 ‰ in larvae reared on treated lichens ($P = 0.842$). The average content of usnic acid in the larvae reared on untreated *U. hirta* was 0.48 ‰, compared to 0.05 ‰ in larvae reared on treated lichens ($P = 0.009$). In larvae reared on *U. dasypoga*, the average content of usnic acid decreased from 0.21 (control thalli) to 0.045 ‰ (treated thalli, $P < 0.001$).

The average content of usnic acid in the excrements of larvae reared on untreated *C. arbuscula* was 0.24 % (Fig. 3c), but only 0.057 % in faeces from treated lichens

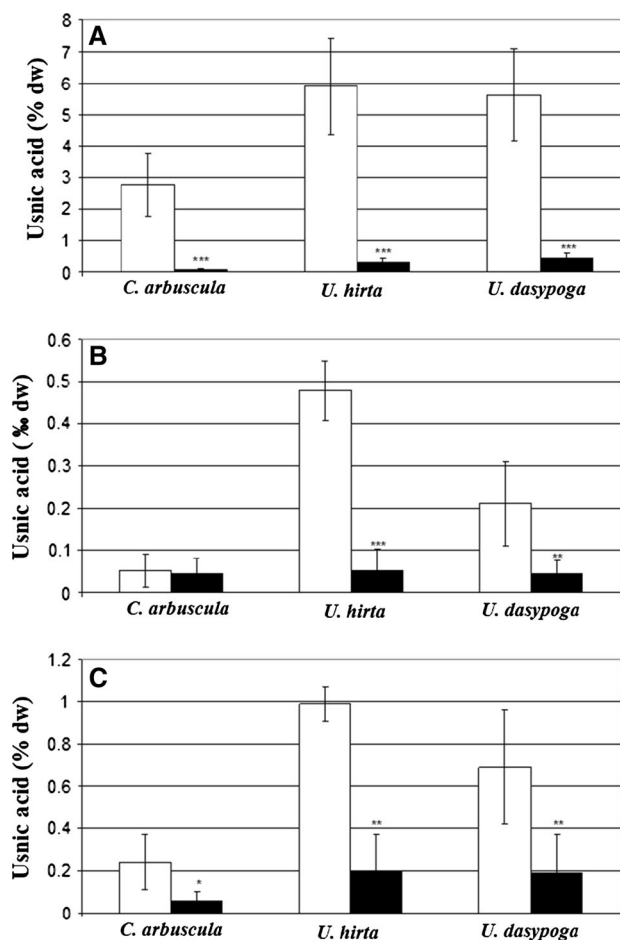


Fig. 3 a Content of usnic acid in lichens thallus (% of dry weight). b Content of usnic acid in larvae (‰ dry weight). c Content of usnic acid in larval excrements (% dry weight). White bars represent control thalli, black bars represent treated thalli, $n = 10$ for each lichen

($P = 0.02$). In *U. hirta*, the average content of usnic acid in the excrements decreased from 0.69 (control lichens) to 0.19 % (treated lichens, $P = 0.003$). In faeces of larvae from *U. dasypoga*, the content of usnic acids decreased from 0.99 to 0.2 % due to the treatment ($P = 0.006$). Usnic acid concentrations in the faeces are considerably higher than in the larvae. Thus, excretion via the faeces may be an important strategy for the detoxification of usnic acid. Weight of excrements of larvae which were fed on studied lichens showed significant differences between *C. arbuscula* and *Usnea* species ($***P < 0.001$) (Fig. 6).

Relationship between usnic acid and larval survival

Survival of larvae on *C. arbuscula* ($R^2 = 0.1391$; $P = 0.015369$) and *U. dasypoga* ($R^2 = 0.03137$; $P = 0.455025$) was not correlated to usnic acid. The survival of larvae on *U. hirta* ($R^2 = 0.4969$; $**P = 0.00109$)

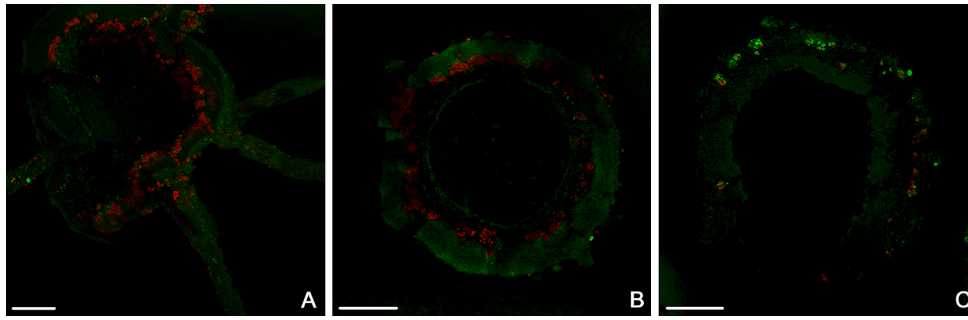


Fig. 4 Cross sections of **a** *Usnea hirta*, **b** *Usnea dasypoga*, **c** *Cladonia arbuscula* subsp. *mitis* in the confocal microscope. Green color represents secondary metabolites and red color represents green algae (untreated thallus). Localisation of green algae in *Usnea* species is

more protected by secondary metabolites in comparison with *Cladonia*. Secondary metabolites form a physical barrier as well as a chemical barrier and herbivores are first in contact with secondary compounds produced by lichens. Bar 200 μm

was strongly negatively correlated with the concentration of usnic acid in the thalli. Usnic acid concentrations in the faeces correlated significantly with survival rates (Fig. 3).

Discussion

Though larvae of *C. lichenaria* are usually exposed to usnic acid, we found detrimental effects of usnic acid on its survival and growth. Many authors reported similar effects of lichen secondary metabolites by using polyphagous or generalist model species (Zukal 1895; Slansky 1979; Reutimann and Scheidegger 1987; Blewitt and Cooper-Driver 1990; Fahselt 1994; Giez et al. 1994; Hyvärinen and Crittenden 2000; Gauslaa 2005; Pöykkö et al. 2005; Lawrey 1983; Blewitt and Cooper-Driver 1990; Emmerich et al. 1993; Giez et al. 1994). Though in good accordance with these studies, our experiments are among the first to show that even specialized lichen feeders are negatively affected by usnic acid concentrations, which are typically found in widely distributed lichens (see also Pöykkö et al. 2010; Pöykkö 2006, 2011a, b). *Ramalina*, the natural host of *C. lichenaria*, contains relatively low concentrations of usnic acid, and these concentrations are easily handled by the larvae (Pöykkö et al. 2010). The selection pressure to cope with higher concentrations of usnic acid, as present in the lichen species tested in this experiment, has been low for larvae of the studied population. Larvae of *C. lichenaria* could theoretically feed successfully on *U. dasypoga*, whereas *C. arbuscula* and *U. hirta* are toxic.

According to RGR results (Fig. 5), it appears that the grown larvae are less affected by usnic acid. Thus, it may be the strategy of the lichen to repel or kill their herbivores in their earliest developmental stages when they are most sensitive. This way, the produced amount of usnic acid can be kept at a minimum, whereas a greater amount would be

required to kill adult larvae. However, the chemical defense is still sufficient since the larvae do not get a chance to grow up. Our results show that larvae are able to exclude excrements from *C. arbuscula* better than from *Usnea* species (Fig. 6). Furthermore, the concentration of usnic acid is much lower in *C. arbuscula*. Lower weight of excrements of larvae from *Usnea* species were probably caused by reduced consumption of lichen species with higher concentrations of usnic acid, or larvae could not digest lichen species with elevated amounts of usnic acid.

To date, the most studied secondary metabolites of lichens with respect to antiherbivore effects are usnic acid, vulpinic acid, and atranorin (Slansky 1979; Stephenson and Rundel 1979; Emmerich et al. 1993). Studies in *V. pinastri*, *H. physodes*, and *P. sulcata* on host selection behaviour of neonate larvae showed that toxicity of secondary metabolites has a strong effect on the survival of larvae (Pöykkö et al. 2005; Pöykkö et al. 2010). All larvae of *E. depressum*, which were reared on untreated thalli of *V. pinastri* and *H. physodes*, died over a short period of a few days. Either usnic acid alone or in combination with pinastric acid in *V. pinastri*, had fatal effects on the survival of *E. depressum* larvae.

Those thalli from which secondary metabolites were removed increased larvae survival. According to Asplund and Wardle (2013), the acetone treatment significantly increased overall palatability. On the other hand, reduction of secondary metabolites such as atranorin from *Parmelia sulcata* or parietin from *X. parietina* had no effect on the survival of larvae (Pöykkö 2005). Thus, lichen secondary metabolites exhibit different toxicity to herbivores (Pöykkö et al. 2005) which may reflect the diversity of their ecological functions.

It can be concluded that the cortical secondary metabolite usnic acid plays an important role in the defense of lichens against herbivores. We found that even herbivores adapted to moderate concentrations of usnic acid are

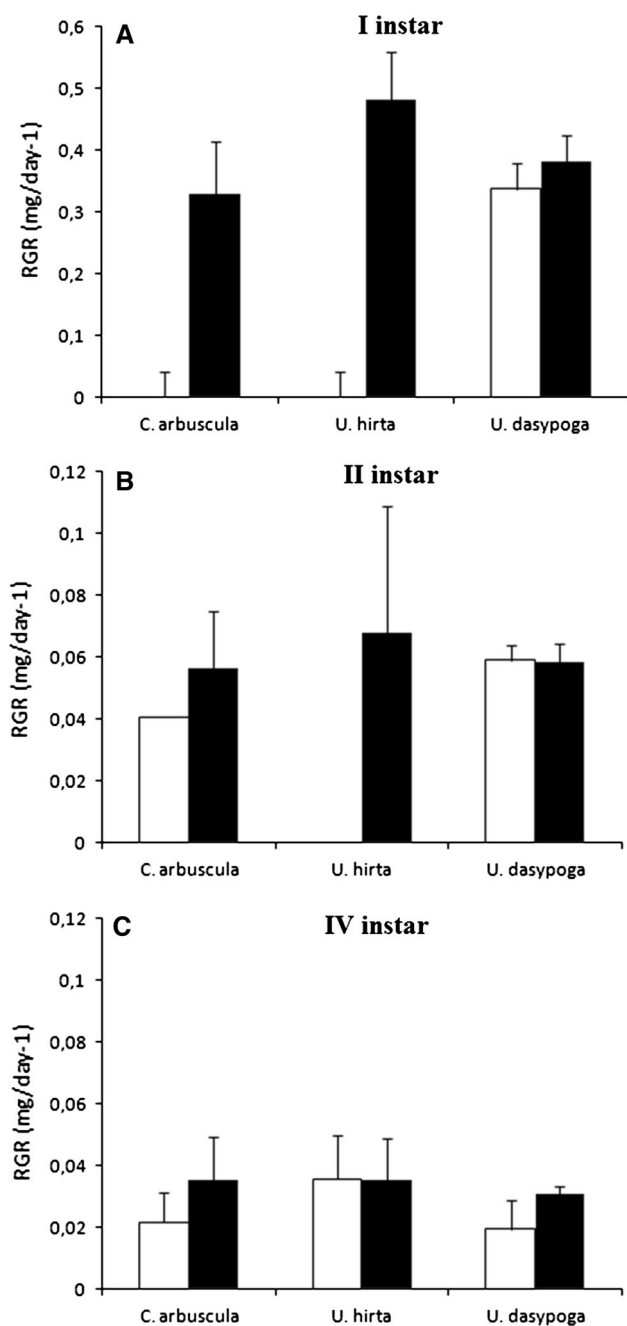


Fig. 5 RGR (relative growth rate) of larvae representing **a** I instar, **b** II instar, and **c** IV instar. *White bars* represent larvae which fed on control thalli and *black bars* represent larvae which fed on treated thalli

seriously affected by its higher concentrations. The effects of usnic acid on the larvae included retarded growth, increased mortality and enhanced concentrations of usnic acid in the animal tissue. The removal of secondary metabolites from living lichens by acetone enables a wide field of studies on their ecological function.

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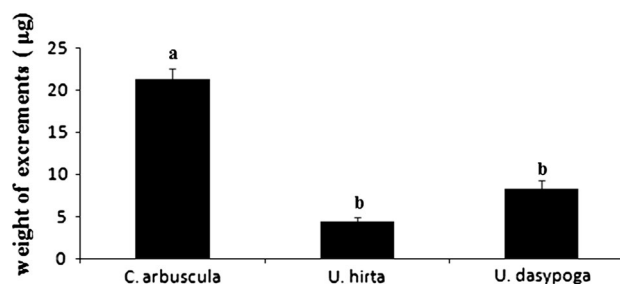


Fig. 6 Weight of excrements of larvae (V instar) which fed on *Cladonia arbuscula* subsp. *mitis*, *Usnea hirta*, *Usnea dasypoga*, respectively ($P < 0.001$, ANOVA Tukey HSD post hoc test).

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