To eat or not to eat—relationship of lichen herbivory by snails with secondary compounds and field frequency of lichens

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Abstract

Aims
The biochemical defense of lichens against herbivores and its relationship to lichen frequency are poorly understood. Therefore, we tested whether chemical compounds in lichens act as feeding defense or rather as stimulus for snail herbivory among lichens and whether experimental feeding by snails is related to lichen frequency in the field.

Methods
In a no-choice feeding experiment, we fed 24 lichen species to snails of two taxa from the Clausilidae and Enidae families and compared untreated lichens and lichens with compounds removed by acetone rinsing. Then, we related experimental lichen consumption with the frequency of lichen species among 158 forest plots in the field (Schwäbische Alb, Germany), where we had also sampled snail and lichen species.

Important findings
In five lichen species, snails preferred treated samples over untreated controls, indicating chemical feeding defense, and vice versa in two species, indicating chemical feeding stimulus. Interestingly, compared with less frequent lichen species, snails consumed more of untreated and less of treated samples of more frequent lichen species. Removing one outlier species resulted in the loss of a significant positive relationship when untreated samples were analyzed separately. However, the interaction between treatment and lichen frequency remained significant when excluding single species or including snail genus instead of taxa, indicating that our results were robust and that lumping the species to two taxa was justified. Our results imply lichen-feeding snails to prefer frequent lichens and avoid less frequent ones because of secondary compound recognition. This supports the idea that consumers adapt to the most abundant food source.

Keywords: acetone rinsing, biodiversity exploratories, defense strategies, gastropoda, herbivore resistance, lichenivory, mollusk grazing, plant–animal interaction

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INTRODUCTION
Biochemical plant defense against natural enemies, especially herbivores, is among the most prominent examples of evolutionary adaptation. Plants evolved many defense strategies against herbivores, including chemical defense by secondary compounds, expressed either constitutively or after induction by herbivore attack (Karban and Baldwin 1997; Kempel et al. 2013; Walling 2000). Herbivores, in turn, may adapt to such chemical defenses (Futuyma 2009). Thus, plant–herbivore systems represent some of the best examples of coevolution (Ehrlich and Raven 1964; Garrido et al. 2012; Parachnowitsch and Lajeunesse 2012; Thompson 2009). Interestingly, despite the large interest in plant defenses, their relation to plant biogeography is poorly understood. A plausible assumption is that better defended plants occur more widely. On the other hand, more common plant species may also be more affected by enemies because commonness may facilitate the adaptation of herbivores (Speiser 2001; apparency hypothesis: Feeny 1976; but see Endara and Coley 2011). However, while some studies assessed the links between abundance and defense in single plant species (e.g. Rand 2002), testing this relationships...
across a larger set of species has hardly ever been done, and this is especially true for nonvascular plants.

Lichens represent one of the most successful forms of symbiosis occurring in essentially all terrestrial and some aquatic habitats (Lutzoni and Miadlikowska 2009). They share many attributes with plants, including sessile life-form, richness of secondary compounds and being consumed by ‘herbivores’. As lichens are consumed by various animals, including gastropods, they represent an important food resource, embedded in complex food webs (Gerson and Seaward 1977; Richardson and Young 1977; Seaward 2008). Gastropods are expected to feed selectively and feeding rates differ strongly among lichen species (Gauslaa 2005; Lawrey 1983; Rundel 1978). Selective feeding of gastropods may well be related to differences in the nutrient content, presence of toxic compounds and also availability of the food resources (Lawrey 1983; Speiser 2001). In fact, lichens are known to produce a wide range of secondary compounds (Huneck and Yoshimura 1996; Rambold et al. 2014) deposited as crystals in the cortex or on the outer surface of hyphae in the medulla. These compounds can play different ecological roles, including allelopathic inhibition of competitors, UV protection and defense against pathogens and lichen-feeding animals (Lawrey 1995; Rundel 1978). Furthermore, they can affect lichen palatability and decomposition (Asplund and Wardle 2013). With acetone, carbon-based compounds can be almost completely extracted from desiccated lichen thalli without harming symbionts (Reutimann and Scheidegger 1987; Solhaug and Gauslaa 2001). Thus, lichens are perfect study organisms to manipulate antitherbivore effects of secondary compounds in feeding experiments.

For some of these secondary lichen compounds, the constitutive defensive nature against gastropods has already been documented (Asplund et al. 2009, 2010b; Gauslaa 2005; Lawrey 1983). In contrast, some snails are feeding selectively on lichens containing deterrent secondary compounds, suggesting adaptation of some snails to these compounds (Hesbacher et al. 1995). Furthermore, some secondary lichen compounds were found to attract and even stimulate lichen-feeding oribatid mites (Reutimann and Scheidegger 1987). So far such an effect has not been shown for any other group of lichen feeders, including the ecologically very important gastropods.

A further limitation of the few previous experiments in which untreated lichens and lichens with experimentally removed secondary compounds had been fed to herbivores is their focus on foliose or fruticose lichens, i.e. those forming leaf-like or shrub-like growth forms. In contrast, crustose lichen species, despite their high species diversity and importance as early colonizers, have never been examined in feeding experiments using the acetone-rinsing technique. Thus, it remains open whether lichen growth form, in addition to secondary lichen compounds, has an impact on feeding preferences.

The distribution, abundance and diversity of lichens depend on various factors including their substrate and habitat specificity, tolerance to land use and environmental pollution (Boch et al. 2013c; Purvis et al. 2010) and on their colonization, dispersal and establishment ability (Shriver et al. 2012; Werth et al. 2006). Lichens may reproduce by vegetative propagules, thallus fragments or fungal spores. In addition to wind and water dispersal and exozoochory (Seaward 2008), our recent study showed that all these structures also can be dispersed by snail endozoochory (Boch et al. 2011). Endozoochorous dispersal by gastropods is also known from bryophytes, ferns and seed plants (Boch et al. 2013a; Türke et al. 2012), which might play an important role for their diversity and abundance (Boch et al. 2015; Türke et al. 2012). However, whether lichen–gastropod interactions including endozoochorous lichen dispersal have an impact on lichen frequency and composition in natural habitats is still unclear.

We present a no-choice experiment feeding thalli of 24 lichen species of different growth form (crustose/noncrustose) to individuals of two common snail taxa and compared feeding rates of untreated lichens with lichens whose secondary compounds had been removed by acetone rinsing. We tested whether (i) chemical compounds in the 24 lichen species acted as feeding defense or rather as feeding stimulus for the two snail taxa, (ii) lichen growth form has an impact on feeding preferences, (iii) effects are consistent across the two snail taxa and (iv) the field frequency of lichen species in the region, where we had sampled experimental snails and lichens, is related to the feeding behavior of the two snail taxa.

**MATERIALS AND METHODS**

**Study system**

This study forms part of the Biodiversity-Exploratories project (Fischer et al. 2010), with field parts in the biosphere area Schwäbische Alb (southwest Germany; for details, see Boch et al. 2013d) and laboratory experiments in Bern (Switzerland; 46°57′12″N, 7°26′42″E).

**Vegetation data**

During 2007 and 2008, we recorded the presence of lichen species for each type of substrate (bark, dead wood, soil and rocks) occurring up to a height of 2 m in 158 forest plots of 20 m × 20 m. We assessed the frequency of each lichen species as the total number of plots where the particular lichen species occurred. The 158 plots are distributed over an area of ~422 km². Together, the plots comprised different management types and habitat characteristics relevant for lichens and snails: the seven unmanaged forest plots harbored mature, deciduous forests dominated mainly by European beech (*Fagus sylvatica* L.). The 131 age-class forest plots were dominated by European beech (94 plots) and Norway spruce (*Picea abies* (L.) H. Karst.; 37 plots) and had different developmental stages of even-aged structure due to
harvests at 80- to 120-year intervals. The 20 selection forest plots harbored uneven-aged deciduous stands dominated by European beech, in which single or small groups of trees were harvested selectively. Based on a large forest inventory (Boch et al. 2013c), the number of our sampled plots is proportional to the frequency of these forest types in this region, thus our sampling resulted in unbiased estimates of regional lichen abundance.

Lichen species

We collected thalli of 24 lichen species from bark of recently felled European beech, large-leaved lime (Tilia platyphyllos Scop.) and living blackthorn (Prunus spinosa L.) in the Schwäbische Alb near Münsingen (48°23′N, 9°27′E; Table 1). All 24 lichen species are common across large parts of Central Europe (Smith et al. 2009) but their abundances vary strongly among regions and habitats (Boch et al. 2013b; Stofer 2006) because of their different ecological requirements (Smith et al. 2009). Lichen nomenclature follows Smith et al. (2009). We obtained the number and identity of secondary compounds of lichen species from Tanahashi et al. (1997, 2003) and Smith et al. (2009). Prior to the start of the experiment, lichen material was air dried and then stored at −18°C. We removed foliose and fruticose species from bark and cut out crustose species, only leaving a small piece of bark underneath the lichen shape. Half of the lichen material of each species was leached four times in acetone (100%) for 20 min in each run to extract carbon-based compounds (Solhaug and Gauslaa 2001). This method does not affect the palatability of

<table>
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<tr>
<th>Lichen species</th>
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<th>Frequency among 158 plots</th>
<th>Secondary compounds</th>
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<td>53</td>
<td>Atranorin, norstictic acid and other substances of the stictic acid complex&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fagus sylvatica</td>
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<td>Dimerella pineti</td>
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<td>98</td>
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<td>Evernia prunastri</td>
<td>Fruticose</td>
<td>22</td>
<td>Atranorin, evernic acid and usnic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F. sylvatica</td>
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<tr>
<td>Graphis scripta</td>
<td>Crustose</td>
<td>93</td>
<td>No substances detected by TLC&lt;sup&gt;b&lt;/sup&gt;, graphislactones and 6H-dibenzo[b,d] pyran-6-one&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Hypocenomyce scalaris</td>
<td>Crustose</td>
<td>6</td>
<td>Lecanoric acid and unidentified substances&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tilia platyphyllos</td>
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<td>Hypogymnia physodes</td>
<td>Foliose</td>
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<td>Atranorin, chloratranorin, physodic acid, physodalic acid, 3-hydroxyphysodic acid and protocetraric acid&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Crustose</td>
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<td>Arthothelin, granulosin and 4,5 dichlorolichexanthone&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Physcia adscendens</td>
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<td>Xanthoria parietina</td>
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<td>50</td>
<td>Physcion (=parietin)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Prunus spinosa</td>
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<sup>a</sup>Secondary compounds identified by TLC according to Smith et al. (2009).
<sup>b</sup>Secondary compounds identified by TLC according to Tanahashi et al. (1997, 2003).
lichens (Černajová and Svoboda 2014). As we collected all crustose species from F. sylvatica (except Hypocenomyce scalaris that was collected from T. platyphyllos and showed a positive effect of acetone rinsing on the consumed lichen biomass; Table 1 and Fig. 1), we can exclude that compounds accidentally extracted from the tree bark can explain differences in consumed lichen biomass. Before and after feeding, we weighed lichen thalli after storing them for 2 days in a drying oven at 35°C and another day in silica gel. Thus, we measured the total lichen mass consumed but did not further distinguish whether snails preferred or avoided particular parts of the lichen thallus (e.g. vegetative vs. reproductive parts).

Snail taxa

We collected adult individuals of Ena montana (Enidae; N = 20) and species from the Clausilidae family (Balea biplicata, N = 8; Bulgarica cana, N = 1; Clausilia dubia, N = 1; Cochlodina laminata, N = 13; Cochlodina orthostoma, N = 2; Macrogastra attenuata, N = 1; Macrogastra plicatula, N = 14; Macrogastra ventricosa, N = 1), which, due to their similarity and their close relatedness in terms of habitat type, were considered as representatives of one single taxon for analysis. All snail species are common in temperate Europe, where they occur in the litter layer and on the trunk mainly of deciduous trees in humid forest sites and mossy and rocky limestone habitats (Boschi 2011; Turner et al. 1998). They are known to supplement their diet with fungi and lichens or even to feed exclusively on lichens and algae (Boschi 2011; Speiser 2001). We sampled snails from trunks of living European beech trees in forests in the vicinity of our plots near Münsingen (48°23′N, 9°27′E) at the same sites where we collected lichens and released them at the sampling site after the experiment. Snail nomenclature follows Turner et al. (1998).

Feeding experiment

We conducted a no-choice experiment, offering always one of four replicates per treatment (untreated vs. acetone rinsed) of moistened thalli of each lichen species (circa 0.2 g dry mass) randomly assigned to one individual of each snail taxon for 48 h (22°C; 14/10 h light/dark cycle; 60% relative air humidity). We assured that each lichen species was offered only once to the same snail individual to avoid food conditioning (as reported by Speiser 2001) or starvation of individuals that were exposed to toxic or deterrent lichen samples. Before the first run, and between runs, snails were individually kept in Petri dishes for 48 h and fed with tissue paper. Sixty-one snail individuals were used in a total number of 384 runs (24 lichen species × 2 treatments × 2 snail taxa × 4 replicate individuals).

Statistical analysis

We examined differences in consumed lichen mass between snail taxa (E. montana vs. Clausilidae species), among lichen species and the effect of treatment (presence/absence of secondary compounds) and their interactions with analysis of variance. A mixed model was fitted using individual snails as a

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**Figure 1**: consumed lichen mass of untreated minus acetone-treated replicates (mg) of the 24 lichen species (±pooled SD) fed to two snail taxa (Ena montana and Clausilidae species) in a no-choice experiment. Lichen species with chemical snail defense are highlighted by light gray and those with chemical snail stimulus by dark gray color. Significant deviation from zero derived by t-tests. *P < 0.05; **P < 0.01; ***P < 0.001.
random effect for significance testing because individual snails were fed in several feeding runs. In addition, to explain variation among lichen species, we included the field frequency of each lichen species and its interaction with the treatment as a contrast indicated in italics to test whether feeding patterns differed between frequent and less frequent lichens. Alternatively, we also tested potential effects of the growth form of lichens and of the number of secondary compounds per lichen species but excluded them as they did not explain variation in consumed lichen biomass (Table 2). Model assumptions were met and therefore no transformation was needed. We also fitted the model by including only well replicated species (B. biplicata, C. laminata, M. plicatula and E. montana) and by including genus instead of taxon. This analysis gave qualitatively similar results, indicating that grouping the species of the Clausiliidae to one taxon was justified in our case. To test the model's robustness and to assess whether individual lichen species disproportionally affected the results, we refitted the model by omitting a single lichen species at the time. Finally, we calculated the effect size for acetone rinsing for each lichen species as the difference between the means of consumed biomass for acetone-rinsed experimental and control group divided by the pooled standard deviation $S$:

$$S = \sqrt{\frac{(N^C - 1)(S^C)^2 + (N^E - 1)(S^E)^2}{N^E + N^C - 2}},$$

where $S^C$ and $S^E$ represent the standard deviation and $N^C$ and $N^E$ the sample sizes for control and treatment group, respectively. We then tested for significant deviations from zero with $t$-tests. All analyses were done with R, Version 2.8.0 (R Development Core Team 2011).

**RESULTS**

**Consumed lichen mass**

The two snails differed slightly in the amount of consumed lichen mass (Table 2), with Clausiliidae consuming 23% less than E. montana. Acetone-rinsed lichens were consumed slightly, but significantly, more (+15.5%) than untreated lichens were, and this effect was consistent across snail taxa (nonsignificant snail taxon × treatment interaction; Table 2). There was large variation in the amount consumed among lichen species, which further depended on the snail taxon, as indicated by the significant snail taxon × lichen species interaction.

Importantly, the consumed lichen mass varied considerably across lichen species, also depending on whether they were acetone-rinsed or not. Single-species analyses showed significant acetone-rinsing effects on consumed biomass for 7 of the 24 species, which is five times more frequent than expected by chance. Snails preferred acetone-rinsed samples of Evernia prunastri, Hypocenomyce scalaris, Melanohalea exasperatula, Pseudevernia furfuracea and Xanthoria parietina indicating that their secondary compounds acted as chemical defense against snails. Consumed lichen mass did not differ significantly between treatments in Buellia griseovirens, Dimerella pineti, Hypogymnia physodes, Lecanora expallens, Lecidella elaechroma, Lepraria incana, Opegrapha rufescens, Parmelia sulcata, Pertusaria amara, P. leioplaca, Phlyctis argena, Physcia adscendens, Pleurosticta acetabulum, Porina aenea, Pyremula nitida, Ramalina

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<th>df2</th>
<th>Sum of squares</th>
<th>Mean squares</th>
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<th>P</th>
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<td>510.3</td>
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<td>Snail taxa × lichen species</td>
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Table 2: analysis of variance results for differences in consumed lichen mass between the two snail taxa (E. montana vs. Clausiliidae species) and among the 24 lichen species considering their frequency in forests of the Schwäbische Alb region and the treatment effect (presence/absence of secondary compounds).
farinacea and Ropalospora viridis. In contrast, snails preferred untreated samples of Graphis scripta and Lecanora chlarotera to the acetone-rinsed samples indicating feeding stimulants for snails (Fig. 1).

**Relationship with the frequency of lichen species in the field**

Overall, we found that the amount consumed in treated and untreated lichen interacted with their frequency in the field, as indicated by a significant lichen frequency × treatment interaction, whereas in untreated lichens that still contained their secondary compounds, snails consumed more on lichen species that were more frequent in the field. In contrast, in treated lichens from which secondary compounds had been removed, snails consumed more on lichen species that were less frequent in the field (Table 2 and Fig. 2). The exclusion of L. chlarotera from the analysis resulted in a nonsignificant relationship between consumed lichen mass and frequency in the field among the untreated lichens. However, the interaction between lichen frequency and the acetone-rinsing treatment remained significant when single lichen species were excluded (all P values < 0.0275), indicating that this result was robust and not driven by an exceptional lichen species. Thus, our results show that the frequency of lichens in the field explained a significant part of the variation in consumed mass and differences in consumed mass among treated and untreated lichens.

**DISCUSSION**

**Snail defense of lichens by secondary compounds**

In a group of lichen species, we found no differences in the consumed lichen mass between the control and acetone-rinsing, i.e. secondary compound removal, treatments. As we did not measure the exact level of reduction of thin layer chromatography (TLC) detectable and nondetectable secondary compounds by acetone rinsing, it may well be that some of these species still contained acetone nondissolvable compounds, which acted as defense against snails. However, we demonstrated that feeding on some lichen species was reduced or even avoided because of their secondary compounds, in accordance with previous studies (e.g. Asplund et al. 2010b; Gauslaa 2005; Lawrey 1983). In contrast to Černajová and Svoboda (2014) who fed individuals of six foliose Parmeliaceae species (untreated lichens vs. lichens whose secondary compounds had been removed) to two gastropod taxa, we found indications for chemical defense in M. exasperatula. In addition, we found untreated samples of G. scripta to be preferred to acetone-rinsed ones. This is interesting because these two species contain no secondary compounds detectable by TLC (Table 1; but see Tanahashi et al. 1997, 2003 for G. scripta, which contradicts general information on secondary compounds of this species, e.g. given by Smith et al. 2009). However, it might be that compounds such as fatty acids (potentially preferred by gastropods, see Lawrey 1983) or pigments (as it was the case for M. exasperatula) were removed by acetone rinsing, which acted as defense or feeding stimulus, respectively, for the snails used in our experiment. Lawrey (1984) further mentioned that vegetative parts of the lichen thallus might be less protected against grazing than reproductive structures (e.g. apothecia and perithecia) and that the hymenium (lower part of the apothecium) can contain substances that can prevent grazing. It might thus well be that some lichen species contain acetone dissolvable substances that stimulate snail feeding. In addition, Fröberg et al. (1993) observed on the island of Öland (Sweden) that gastropods consumed more apothecia than perithecia in
saxicolous lichens. However, as we only measured the total consumed lichen mass, it remains open whether snails preferred or avoided particular parts of the lichen thallus (e.g. vegetative vs. reproductive parts or apothecia vs. perithecia) in our study. This needs further investigation in future studies. Contrary to the findings of Lawrey (1983), the number of secondary compounds in the lichen thallus had no influence on lichen consumption by snails in our experiment.

Differences in lichen herbivory among snail taxa
The differences in lichen herbivory between the two snail taxa of our experiment may well reflect general variation in lichen herbivory among gastropod species, most likely due to adaptation to secondary lichen compounds. This is supported by Gauslaa (2005) who used two snail species of the Helicidae, *Cepaea hortensis* and *Arianta arbustorum*, and Černajová and Svoboda (2014) who used a Clausiliidae snail species, *Cochlodina cerata*, and a Limacidae slug species, *Lehmannia marginata*. In contrast to the snail taxa in our experiment, the Helicidae did not differentiate between acetone-rinsed and control samples of *X. parietina* and preferred acetone-rinsed *H. physodes* and *P. sulcata*, indicating chemical defense in these species (Gauslaa 2005). Moreover, *C. cerata* and *L. marginata* did not differentiate between acetone rinsed and control for *M. exasperatula* and showed ambiguous for *P. sulcata* (Černajová and Svoboda 2014). Combining these results with ours extends our finding that lichen herbivory and adaptation to secondary lichen compounds differs among snail taxa, which is in line with the conclusions of previous studies (Baur et al. 1994; Fröberg et al. 1993; Hesbacher et al. 1995).

Food recognition and secondary compounds
Interestingly, in our study, the snail taxa even preferred untreated samples of two lichen species compared with acetone-rinsed samples. Therefore, at least some secondary compounds in lichens may stimulate snail feeding. This implies food recognition by secondary compounds, as it had been shown for plant-feeding insects (van der Meijden 1996) and for lichen-feeding oribatid mites (Reutimann and Scheidegger 1987). Speiser (2001) mentioned that the slow movement of terrestrial gastropods is costly because of mucus secretion, which may have favored the evolution of a generalized feeding behavior to minimize foraging efforts. The feeding behavior of terrestrial gastropods involves food sampling and learning (e.g. unknown food is first consumed in only small quantities before either rejecting or accepting it), and the final mass consumed depends not only on the nutritional quality such as nutrient content, toxic compounds and palatability but also on the quantity and availability of lichens (Speiser 2001). As a consequence, this may result in completely different diets of the same gastropod species in different habitats and regions. Thus, such a feeding behavior may serve as a plausible mechanism for our finding of an interaction between consumed lichen mass and lichen frequency in the field (Fig. 2). Because the positive relationship between lichen frequency and lichen consumption in the control treatment occurred only when the most consumed and most frequent lichen (i.e. *L. chlorotera*) was included in the analysis, further studies, e.g. multiple-choice experiments, are needed to test whether lichen-feeding snails generally prefer the common lichen species in different habitats. Nevertheless, we hypothesize that snails might recognize the most frequent lichen species by their secondary compounds, and vice versa they avoid feeding less frequent or ‘unknown’ lichen species. Thus, our data support the idea that abundance of a food source may promote adaptation of the consumer, along the lines of the apparency hypothesis (Feeny 1976).

Mutualistic lichen–snail interactions
In addition to antagonistic herbivorous interactions between gastropods and lichens (e.g. Asplund et al. 2010a; Seaward 2008), snails might also interact mutualistically with lichens by promoting fragmentation, proliferation and dispersal of lichen thalli (Boch et al. 2011). Therefore, selective grazing by gastropods of frequent lichen species may benefit population growth, whereas less frequent lichen species may contain chemical compounds that reduce snail feeding and, as such, limit opportunities for dispersal. Thus, higher lichen abundance may result from increased herbivory, which may be promoted by improved herbivore adaptation to more frequent lichens, which even could eventually feedback on lichen abundance. Our study calls for field tests of both directions of such mutualistic lichen–snail interactions.

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