Overcoming deterrent metabolites by gaining essential nutrients: A lichen/snail case study

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ARTICLE INFO

Keywords:
Usnea taylorii
Parmeliaceae
Notodiscus hookeri
Mass spectrometry imaging
Feeding choice
Lichen
Snail
Usnic acid
D-arabitol

ABSTRACT

Specialised metabolites in lichens are generally considered repellent compounds by consumers. Nevertheless, if the only food available is lichens rich in specialised metabolites, lichenophages must implement strategies to overcome the toxicity of these metabolites. Thus, the balance between phagostimulant nutrients and deterrent metabolites could play a key role in feeding preferences. To further understand lichen-gastropod interactions, we studied the feeding behaviour and consumption in Notodiscus hookeri, the land snail native to sub-Antarctic islands. The lichen Usnea taylorii was used because of its simple chemistry, its richness in usnic acid (specialised metabolite) and arabitol (primary metabolite) and its presence in snail habitats. Choice tests in arenas with intact lichens versus acetone-rinsed lichens were carried out to study the influence of specialised metabolites on snail behaviour and feeding preference. Simultaneously, usnic acid and arabitol were quantified and located within the lichen thallus using HPLC-DAD-MS and in situ imaging by mass spectrometry to assess whether their spatial distribution explained preferential snail grazing. No-choice feeding experiments, with the pure metabolites embedded in an artificial diet, defined a gradual gustatory response, from strong repellence (usnic acid) to high appetence (D-arabitol). This case study demonstrates that the nutritional activity of N. hookeri is governed by the chemical quality of the food and primarily by nutrient availability (arabitol), despite the presence of deterrent metabolite (usnic acid).

1. Introduction

Lichen symbiosis comprises a fungus, the mycobiont, and photosynthetic partners, the photobiont (chlorophytes and/or cyanobacteria). The mycobiont produces water-soluble specialised metabolites such as mycosporines (Roullier et al., 2011) and lectines (Huneck and Yoshimura, 1996) but also hydrophobic metabolites such as depsides, depsidones and dibenzofurans (Stocker-Wörgötter, 2008). Hydrophobic metabolites accumulate as tiny crystals outside the fungal hyphae, in the cortex or the medulla of the lichen thallus (Honegger, 1991). This extracellular localisation makes the metabolites easy to remove using the acetone-rinsing methodology, without degrading the viability and palatability of the lichen (Pöykkö et al., 2005; Solhaug and Gauslaa, 2001).

Because lichens are sessile and slow-growing symbiotic organisms, they must defend themselves strongly against lichenivores such as gastropods, which are important lichen feeders (Asplund and Wardle, 2016; Fröberg et al., 2011; Solhaug and Gauslaa, 2012; Vatne et al., 2010). Many experiments have highlighted deterrent and/or toxic activity for some specialised metabolites towards invertebrates, including snails (Emmerich et al., 1993; Gauslaa, 2005; Solhaug and Gauslaa, 2012). Generalist lichenophage species prefer lichens with few specialised metabolites (Benesperi and Tretiach, 2004; Boch et al., 2015; Černajová and Svoboda, 2014; Gauslaa, 2005; Goga et al., 2015), but more than the total amount of metabolites, their nature turns out to be the main discriminant factor in food preference. In the accidental
degradation of the lichen herbarium of the Trieste University, the coleopteran Lasioderma serricorne avoided lichen species containing the dibenzofuran usnic acid, the depside atranorin or the depsidone fu-marprotoctenaric acid (Nimis and Skert, 2006). In a similar way, when snails faced a mixture of Lobaria pulmonaria chemotypes with and without stictic acid (depsidone), a preferential grazing on the depsidone-free lichen was observed (Asplund, 2011a).

If the only available resources are lichens rich in specialised metabolites, lichenophages must overcome or be able to metabolise potentially toxic specialised metabolites (Gadea et al., 2017; Hesbacher et al., 1995). Thus, optimal foraging in generalist species implies a balance between defending against toxics and accessing useful nutrients. Indeed, lichen tissues also contain carbohydrates, including polysaccharides and reserve carbohydrates such as sugars and polyols. The phagostimulant properties of sugars have previously been highlighted to improve slug baits, particularly disaccharides that appeared more attractive than monosaccharides (Clark et al., 1997; Henderson et al., 1992). Gastropods have the full enzymatic arsenal to degrade and to assimilate sugars and polyols. However, the role of polysaccharides in feeding choices has not yet been confirmed (Charrier et al., 2006; Charrier and Rouland, 2001; Flari et al., 1992).

As the sub-Antarctic snail Notodiscus hookeri Reeve (Charopidae) is an exclusive lichen feeder, it appears to be a relevant model to advance the understanding of the chemical ecology of the lichen/snail interactions. In our previous works, a compromise between appettent polysaccharides and potentially repulsive specialised metabolites was suggested. Notodiscus hookeri is able to deal with potential toxic metabolites (Gadea et al., 2017) and to select lichen parts according to their gustatory quality (Gadea et al., 2018). Lichens are known to have a sectorial distribution of their specialised metabolites, which can be visualised by spectroscopic experiments as LDI-MSI or vibrational spectroscopy (Le Pogam et al., 2016; Liao et al., 2010). However, it is still not known how the primary metabolites such as sugars and polyols are distributed within the lichen thallus.

In this study, we present the first spatial mapping of both attractive and repellent metabolites to account for the nutritional choices made by the snail. To address this question, the lichen Usnea taylorii Hook. f. & Taylor (Parmeliaceae) was selected because of its simple chemistry. The sub-Antarctic snail Notodiscus hookeri Reeve (Charopidae) was selected because of its simple chemistry. First, we followed the snail behaviour in feeding choice arenas to assess the defensive-compound hypothesis described by Gauslaa (2005). We expected that acetone-rinsed thalli would promote food consumption compared to control thalli. At the same time, snails were subjected to no-choice feeding experiments using the pure metabolites embedded in an artificial diet, separately or together. We hypothesised a gradual gustatory response, from strong repulsion (usnic acid) to high appettence (D-arabitol). Second, we intended to explore the spatial localisation of the main primary (D-arabitol) and specialised (usnic acid) metabolites of U. taylorii using in-situ mass spectrometry imaging. The patterns of distribution of the metabolites were expected to influence the snail feeding strategy.

2. Results

2.1. Morphological and chemical characterisation of Usnea taylorii

2.1.1. Description of Usnea taylorii morphology

Microscopic analysis of the fruticose lichen Usnea taylorii reveals a specific morphology that deserves a detailed description to better understand the location of metabolites (Fig. 1a; a complete morphological description of the lichen is available in Text S1). Apothecia (the reproductive parts of the lichen) are located at the apex of the Usnea branches and are characterised by their large diameter (2–17 mm) and their jet-black pigmented disc. In cross-section, underneath the black epithecium, asci containing spores are present in the hymenium, covering the algal layer (Fig. 1b). The thallus in cross-section shows a very thin cortex, protecting a discontinuous algal layer. The central axis of U. taylorii is divided into several smaller axial strands by the protruding medullae, which are surrounded by some algae. Between these axial strands, lax medulla is observed (Fig. 1c).

2.1.2. Chemical characterisation of Usnea taylorii

The extraction yields with acetone ranged between 0.5 and 1.0% of specialised metabolites for the six replicates of dried lichen materials with a mean value of 0.8 ± 0.2%. Preliminary experiments showed that more than 95% of the specialised compounds were extracted from the lichen U. taylorii by rinsing intact air-dried thalli in acetone. Thalli had only one or two main specialised metabolites. The concentration of usnic acid ranged from 2.5 to 5.4 mg g⁻¹ dry mass (DM) of lichen, with a mean value ± s.d. of 4.1 ± 1.1 mg g⁻¹ DM (n = 6). Sugar and polyol profiling was also performed. A low diversity was observed, with only four metabolites quantified. Arabitol was the most important polyol, reaching 138.4 ± 25.8 mg g⁻¹ DM (mean value ± s.d., Table S1, Fig. 2).

Preliminary LDI-MS analysis of an acetone extract of Usnea taylorii confirmed that the (+)-usnic acid was the only specialised metabolite detected through its deprotonated molecule (m/z 343) along with a fragment ion at m/z 329 ([M-Me]⁻) (Fig. S1). Usnic acid was consequently imaged through the m/z 343 ion. D-arabitol, the major polyol quantified by GC, was also imaged by mass spectrometry. However, because D-arabitol does not absorb at the wavelength of the laser used for LDI-MSI, a MALDI matrix solution was sprayed on U. taylorii slices.
and images were obtained using MALDI-MSI (through its sodium adduct observed at m/z 175).

In situ LDI-MSI experiments applied to a slice of Usnea taylorii's branch revealed that usnic acid was located in the peripheral layer of the thallus, i.e., in the cortex and in the uppermost parts of the medulla including the photobiont layer (Fig. 3a). Likewise, usnic acid was allocated to the external layers of the apothecium (epithecium, hymenium and the underside of the apothecium) (Fig. 3c). D-arabitol was present mainly in the lax medulla, the cortex and the algal layer (Fig. 4a), but occurred in lower intensities in the axial strands of the lichen branches. In apothecia, the signal corresponding to D-arabitol was stronger in the layer containing the lax medulla and algae than in the external layers of apothecium (epithecium and hymenium) (Fig. 4b). To perform a comparison between lichen parts eaten by snails or not, some branches and apothecia were sliced and analysed by LDI-MSI. Snails consumed parts of the cortex, the algal layer and the lax medulla of the branches. In apothecia, epithecium, hymenium as well as the underside of the apothecium were grazed. All these tissues contain usnic acid (Fig. 3b and d) and D-arabitol (Fig. 4).
2.2. Snail behaviour and lichen consumption experiments

2.2.1. Feeding choice arenas

There was no site effect on the distribution of snails in the feeding choice arenas (Chi² = 0.397, df = 2, P = 0.820). During the nutritional phase, 67.9% (± se = 4.11%) of the snails preferred to stay near the rock shelter (rock versus intact lichen P < 0.001, rock versus rinsed lichen P < 0.001), while the rest of the snails were equally distributed (P = 0.70) in arms with intact (16.7 ± 2.5%) and rinsed lichens (15.5 ± 3.2%) (Fig. 5).

The removal of acetone-soluble metabolites did not promote lichen consumption (mean ± s. e. = 0.38 ± 0.12 μm²/snail; BRA = 0.25 ± 0.11 μm²/snail; MAS = 0.50 ± 0.21 μm²/snail). No differences were detected among treatments (Chi² = 0.874, df = 1, P = 0.350) and collecting sites (Chi² = 1.553, df = 1, P = 0.213). Morphological analyses of the consumed parts showed distinct marks of radula on the cortex, the algal layer and the lax medulla of the branches, while the axial strands of branches always remained intact (Fig. 6).

2.2.2. No-choice experiments

Tested metabolites (usnic acid and/or arabitol) were added to a starch gel at the mean concentrations found in the thalli. The feeding experiment lasted 48 h. Gel-mixed metabolites had a significant impact on their consumption by snails (Chi² = 57.47, df = 3, P < 0.001; Fig. 7). Snails offered gels with usnic acid alone mainly avoided it (Tukey, P < 0.001). Gels supplemented with arabitol were eaten as much as the starch control alone (Tukey, P = 0.795; Fig. 7). Gels containing a mixture of arabitol and usnic acid were consumed more than usnic acid gels alone (Tukey, P < 0.001), but less than arabitol gels alone (Tukey, P = 0.009).

3. Discussion

The fruticose lichen U. taylorii (Parmeliaceae), found in nutrient-poor environments, was expected to be increasingly grazed by the snail N. hookeri, after acetone rinsing. Indeed, Gauslaa (2005) evidenced that acetone-rinsed lichens belonging to Parmeliaceae, rich in specialised metabolites, were more palatable to generalist land snails. Contrary to our expectation, snails were equally distributed between rinsed and intact thalli in the four arms of the feeding choice arenas. Most of the snails were counted on the rocks in the two other arms, suggesting that...
U. taylorii could not be a resting place after feeding. Although information about lichen choice was lost because 68% of the snails preferred the rocks, simulations made from the real data set confirmed that absence of difference was not a bias due to a very low power of the test (Table S2). Lichen consumption was not significantly different between treatments. Thus, removal of acetone-soluble metabolites of U. taylorii did not increase lichen consumption by the snail. This result is consistent with the results of Asplund et al. (2010), who observed that usnic acid did not affect snail grazing on the lichen Lobaria scrobiculata. In contrast, Asplund (2011b) described snail grazing (Cochlodina lamina) in depth after acetone rinsing of two Lobaria species, while the snail avoided the medulla before removal of acetone-soluble metabolites. However, Lobaria pulmonaria did not contain usnic acid (Asplund et al., 2018), suggesting that other compounds (stictic acid derivatives) were deterrent. According to the study of Boch et al. (2015) only one of three lichen species containing at least usnic acid showed chemical snail defence. From a relationship with lichen frequency in field, the authors deduced that the more the lichen was frequent, the higher was the consumption by snails. This relationship cannot be the rule in the present study since U. taylorii was absent from Branca site. By a deficit in specialised metabolites and a low concentration in usnic acid (4.1 mg g^-1), U. taylorii appears closer to the lichens belonging to Physciaceae and Teloschistales described by Gauslaa (2005) although common in nutrient-rich environments. In these lichens, the author suggested that the specialised metabolites could protect thalli against excess light but could not deter grazing. In line with this, usnic acid is known to provide photoprotection to the algal cells (Nybakken and Gauslaa, 2007) and regulate their development (Bačkor et al., 2010). Gadea et al. (2017) showed that usnic acid was retrieved from faeces of N. hookeri after U. taylorii intake, meaning that the snail excreted the metabolite, but that did not mean that usnic acid was apparent and/or not toxic for the snail. None of the studies cited above considered primary metabolites as feeding stimulants. For this reason, isolated compounds were tested separately and in mixture, in no-choice experiments. Usnic acid deterred N. hookeri when extracted and incorporated in a waxy starch gel, while it was consumed in lichen thallus. In contrast, Asplund et al. (2010) observed that filter paper added with usnic acid from the lichen Lobaria scrobiculata did not affect Cepaea hortensis grazing, despite the high content used (47.6 mg g^-1). Filter paper, made of cellulose and known to be highly attractive to helicid snails, could be much more palatable than waxy starch was to N. hookeri. In the present study, the repellent effect of pure usnic acid in the starch gel was mitigated by the addition of D-arabitol. The phagostimulant effect of D-arabitol for the snail has already been observed when the snail fed on the lichen Argyrosis frisiana (Gadea et al., 2018). In a similar way, sucrose increased the amount of agar baits ingested by the slug Deroceras reticulatum, more than glucose, lactose and fructose (Henderson et al., 1992). These studies highlight the key role of primary metabolites for phytophagous. Polyols are attractive for snails as nutrient metabolites that provide energy after oxidative conversion and help produce mucus, which enables body hydration, locomotion, and food foraging (Ng et al., 2013). In sub-Arctic lichens, polyol leaching was positively correlated with polyol concentration and in wet conditions (after a rain event, for example, which is so common in the sub-Antarctic), lichens lose up to 10% of their polyols through leaching (Dudley and Lechowicz, 1987). Consequently, snails might be first attracted by the exudate of arbutin, licking it and then would start grazing the cortex containing usnic acid. In contrast, the gustatory receptors of the snails (i.e. buccal lips and anterior tentacles) were in direct contact with usnic acid when the metabolite was incorporated to starch gels, thus revealing its role in chemical snail defence. Mass spectrometry imaging of the two metabolites gave arguments in support of the snail feeding pattern in the field. Spatial mapping revealed that arbutin and usnic acid were found partly collocated in the cortex and in the apothecia. Accordingly, the snails preferred to graze the apothecia, the lax medulla and the cortex that contained both fungal mycelium and algae with usnic acid and arabitol. In addition to arabitol leaching, the high concentration of arbutin in U. taylorii, thirty times as much as usnic acid, might explain why the feeding stimulus “arbutin” was stronger than the repellent stimulus “usnic acid”. This case study reveals that, as in plant-phytophage interactions (Jamieson et al., 2017), primary metabolites are able to overcome deterrent metabolites and should be considered as important as specialised metabolites in lichen-lichenophage interactions.

4. Conclusions

High amounts of arbutol, useful for lichen survival in cold places, makes Usnea taylorii vulnerable to grazing by a generalist gastropod, despite the production of a deterrent compound, namely, usnic acid. In addition to classical feeding experiments, the mass spectrometry imaging of deterrent and attractive compounds improved our knowledge of lichen/snail trophic interactions. Although snails are often classified as opportunistic feeders, their nutritional activity appears governed by the chemical quality of the food and primarily by nutrient availability. This lichen/snail case study could be applied to other biological models, for instance, terrestrial gastropods well known as crop pests in agriculture, horticulture and orchards.

5. Experimental

5.1. Snail collection and lichen material

Adult individuals of Notodiscus hookeri Reeve (Charopidae) were collected on Possession Island during Austral summer in November 2015. Information about climate and topography is reported in Charrier et al. (2013). Snails came from two fell-fields, Mont Branca (BRA, 46°26′5.61″ S; 51°50′0.68″ E, 200 m - 383 m) and Mascarin Summit (MAS, 46°26′10.09″ S; 51°45′20.58″ E, 600 m - 930 m) (a map of the sampling sites is available in Gadea et al. (2018)). The fruticosic chlorolichen Usnea taylorii Hook. f. & Taylor (Parmeliaceae), encountered at MAS in the snail habitat, was harvested on Possession Island during Austral summer in November 2015. It was identified by Dr Damien Erz and confirmed by Dr Philippe Clerc. Voucher specimens were deposited at the herbarium of the Faculty of Pharmacy of Rennes 1, Department of Pharmacognosy and Mycology, under the reference REN000141.

5.2. Macroscopic and microscopic analysis of Usnea taylorii

Thallus morphology was examined using a dissecting microscope Leica MZ6 (Leica Microsystems GmbH, Wetzlar, Germany). Anatomical observations were made using a Leica DM2000 LED microscope (Leica Microsystems GmbH, Wetzlar, Germany). Longitudinal sections of apothecia and cross sections of thalli were made using a hand-razor blade, and longitudinal sections of thalli were cut from the well-developed thicker branches. The ratio % of axis thickness/% of medulla thickness = ratio A/M, which is a good discriminator of Usnea species (Truong et al., 2011), was calculated. K (potassium hydroxide) and P (para-phenylenediamine) spot tests according to Hale (1979) were directly applied to the medulla on longitudinal sections of the branches.

5.3. HPLC-DAD-MS analysis of specialised metabolites

5.3.1. Instrumental settings

HPLC separation and quantification of usnic acid and Usnea taylorii extracts were performed on a Prominece Shimadzu HPLC system (Marne La Vallée, France) equipped with a Kinetex C18 HPLC column (100 × 4.6 mm, 2.6 μm, 6A, Phenomenex, Torrance, CA, USA). The HPLC system comprises a quaternary pump (LC20ASDP), a surveyor autosampler (SIL-20AHT) and a diode array detector (SPD-M20A). The
separation was achieved using an acidic water/acetonitrile system as previously described (Gadea et al., 2017). The ESI-mass spectra were obtained from an Expression Advion CMS apparatus (Advion, Ithaca, USA). The mass spectra were recorded in the negative-ion mode in a mass range of 100–1200 Da, applying the same parameters previously described by Gadea et al. (2017). The spectral data from the photodiode array detector were collected 48 min over the 200–500 nm range of the absorption spectrum, and the chromatograms were plotted at the maximum wavelength of absorption (λ max) of the main metabolites. Peaks were assigned according to the retention time, UV spectra and mass spectra. The same chromatographic method was applied for the standards and the samples.

5.3.2. Sample and standard preparation

Specialised metabolites of six specimens of the whole fertile thallus of Usnea taylorii (642–1000 mg) were extracted by the acetone rinsing method, three times (5 mL, 20 min), at room temperature and were air-dried until the solvent had evaporated (Solbøga and Gauslaa, 2001). Acetone extracts (N = 6) were used for the chemical analysis while the dry lichens, was calculated for each extraction. The yield, defined as the ratio between the masses of the extracts and the dry lichens, was calculated for each extraction. All extracts obtained from Usnea taylorii were dissolved in di-distilled tetrahydrofuran at the concentration of 0.5 g L⁻¹. Then, the extracts were filtered through a Nylon syringe F2504-1 (Thermo Scientific, Rockwood, USA, 0.45 μm × 4 mm) and transferred to an appropriate vial for automatic injection of 10 μL aliquot into the HPLC system.

5.3.3. Analytical method validation

All validation parameters were determined following the International Conference on Harmonization (ICH) Guidelines (ICH, 2005) using HPLC analyses, and the following characteristics were evaluated: linearity, limits of detection (LOD) and quantification (LOQ), repeatability inter-day and intra-day. Four stock standard solutions of (+)-usnic acid (Sigma-Aldrich; 329967) were prepared by dissolving approximately 2 mg in 4 mL tetrahydrofuran to reach a concentration of 0.5 mg mL⁻¹. Then, the working standards of usnic acid were prepared by appropriate dilution of each stock solution with acetonitrile to generate concentrations ranging from 0.07 to 0.09 mg mL⁻¹ for the external standard calibration curve and the determination of the regression line. Hence, the concentrations of usnic acid in U. taylorii were calculated, based on peak areas. The limits of detection and quantification were determined from the y-intercept standard deviation and the slope of the calibration curve. For the calculation of the intra-day repeatability, a dilution of usnic acid (0.05 mg mL⁻¹) was injected six times the same day. These assays were repeated on four different days for inter-day repeatability. The coefficient of variation and standard deviation were then calculated. Coefficients of variation of less than 10% for intra-day and for inter-day were accepted. The results of the validation are available in Table 1. Concentrations were obtained on the basis of calibration curves and were expressed in mg g⁻¹ DM of the lichen part.

Table 1: Results of various parameters of validation studies for usnic acid quantification.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Usnic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve equation</td>
<td>y = 65613076x – 95165</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
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</tr>
<tr>
<td>LOD (mg mL⁻¹)</td>
<td>0.0001</td>
</tr>
<tr>
<td>LOQ (mg mL⁻¹)</td>
<td>0.0070</td>
</tr>
<tr>
<td>Calibration range (μg mL⁻¹)</td>
<td>0.0070 to 0.0900</td>
</tr>
<tr>
<td>Intraday precision (RSD, n = 6)</td>
<td>less than 3%</td>
</tr>
<tr>
<td>Interday precision (RSD, n = 24)</td>
<td>less than 2%</td>
</tr>
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</table>

5.4. Sugar and polyol profiling

Usnea taylorii samples were hand-cut using a razor blade to afford slices approximately 100 μm thick. Branches and apothecia slices were fixed on a carbon-conductive adhesive tape that was, in turn, fixed on an indium tin oxide (ITO) slide (Bruker Daltonics, Bremen, Germany, cat. no. 237001). For laser desorption ionisation (LDI)-MSI measurement, no further preparation step was required. For matrix-assisted laser desorption Ionisation (MALDI)-MSI measurements, DHB matrix solution (50 mg mL⁻¹ in 50% methanol) was homogeneously applied with a custom-designed spraying robot.

All MSI measurements were performed using an Autoflex-Speed MALDI-TOF/TOF spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser (355 nm, 1000 Hz) and controlled using the Flex Control 3.4 software package. The mass spectrometer was used in the reflectron mode with a negative polarity for LDI-MSI and a positive polarity for MALDI-MSI. Spectra were acquired in the mass range of m/z 100–600 for all (x, y) coordinates corresponding to the imaged tissue.

The laser raster size was set at 35 μm. The signal was initially optimised by manually adjusting the laser power and the number of laser shots fired. Accordingly, full-scan MS experiments were run by accumulating 400 laser shots per raster position and by using the laser power leading to the best signal-to-noise ratio. Image acquisition was performed using the Flex Imaging 4.0 (Bruker Daltonics) software package. The correlation of the target plate with the optical image was obtained from three distinct teaching points following the procedure of the Flex Imaging software (Bruker Daltonics).

5.5. Mass spectrometry imaging (MSI)

5.6. Snail behaviour experiments

5.6.1. Snail feeding choice arena

Each fertile thallus of U. taylorii was divided into two pieces of equal size, always containing a branch with the terminal apothecia. Half of these thalli were randomly taken to extract the specialised metabolites and constituted the rinsed lichens (“Rinsed”). The other group contained intact lichens (“Intact”). Residual acetone was evaporated for at least 20 h before the experiment.

One hundred and sixty-eight N. hookeri per site were divided randomly into eight subgroups of 21 snails. No significant differences in shell size were observed among snail subgroups (mean ± s.d. = 4.80 ± 0.37; ANOVA, F = 1.590, df = 7, P = 0.137). Two days before the experiment, snails were starved to enhance their feeding motivation. The snails were placed inside a feeding choice arena with six arms, made of plexiglass (Plastit supplier, Rennes, France, under the concept proposed by Dr. Maryvonne Charrier, Fig. 8). Wet synthetic foam was placed at the bottom of each arena to maintain strong humidity necessary for snail activity. Similar lichen samples were placed at both ends in opposite arms, giving the choice between two: two “Intact” and two “Rinsed” samples. To avoid a stressful condition for snails used to aggregate under a shelter (Dahirel et al., 2018), the two latter arms contained a small bare rock each, representing secure refuges during resting phases. The experiment was replicated eight times per site. Each feeding choice arena received 21 snails and was maintained in a climatic chamber under a light/dark photoperiod set as
Fig. 8. Choice arena device. Lichen samples, intact or acetone rinsed, and rocks were arranged in opposite arms. Snails (N = 21 × 8 arenas × 2 sites) were placed in the central area at the beginning of the experiment and 48 h later, snail distribution and lichen consumption were analysed.

follows: 10 °C day (12 h) and 6 °C night (12 h). A rotation of the arenas was done four times a day to avoid a position effect in the climatic chamber. Experiments started at the beginning of the nocturnal phase and ended 48 h after.

To analyse snail distribution, the position of each snail in the arena was reported. To evaluate lichen consumption, radula marks on lichen thalli were observed under stereomicroscope (Zeiss, France). The area of radula impacts was measured (in μm²/snail) with Labscope software (version 2.0, Zeiss, France).

5.6.2. No-choice experiments on isolated compounds

Two hundred and forty adult snails were separated into four subgroups: one control and three tested groups of 60 snails each (usnic acid, usnic acid + arabitol and arabitol). No significant difference in shell size was observed among subgroups (mean ± s.d. = 4.81 ± 0.33 mm; ANOVA, F = 2.472, df = 3, P = 0.062). No-choice experiments were performed in two-compartment Petri dishes (Greiner Bio One, Austria). Wet nonwoven sterile gauze was placed in each compartment to maintain high humidity. Waxy starch gels were prepared at the concentration of 15% and small discs (diameter: 5 mm, thickness 2 mm) were manufactured using a custom-designed device (Plast’it supplier, Rennes, France), under the concept proposed by Dr. Maryvonne Charrier). Metabolites (usnic acid, arabitol or a mixture of usnic acid and arabitol) were added to the gel at the same concentrations as those quantified in the entire fertile thalli (4 mg g⁻¹ for usnic acid and 100 mg g⁻¹ for arabitol). Waxy starch gels alone were considered as positive controls. Snails had no choice, either facing a gel containing metabolite or a control gel, for 48 h. At the end of the experiment, gels were photographed, and a feeding score was estimated. To calculate the feeding score, a calibrated grid placed on the gel provided the area consumed, then a correction factor was applied (×1 for consumption of the surface layer and ×2 when the gel was consumed in its full thickness; Fig. S2).

5.6.3. Statistical analyses

Snail distribution in the arenas was analysed by a multidimensional logistic model for proportion data. The effect of the site (BRA versus MAS) was tested using a likelihood ratio test on a first model. Since the site did not have a significant effect, a second model was built without any independent variable, that allowed the comparison of the proportion of individuals choosing each treatment (intact lichen, rinsed lichen and rock) using a series of Wald tests where p-values were corrected using the false discovery rate method. To analyse lichen consumption (areas in μm²) according to the treatment (intact versus rinsed) and the snail sampling site, a Wald test was applied on a GLMM (family: quasi, link: identity, variance: V = μ).

In the no-choice experiments, to determine if metabolites were differently consumed compared to the control, a likelihood ratio test was used on a generalised linear model (GLM, distribution: negative binomial, link function: log), in which the snail sampling site was introduced as a fixed factor. Tukey’s post hoc pairwise comparisons were realised. As no differences between sampling sites were observed on the feeding score (χ² = 0.69, df = 1, P = 0.408), the results of both sampling sites were combined. All statistical analyses were made using R software V. 3.4.3 (R Core Team, 2017). The R packages used were: car, emmeans, lme4, nnet, MASS, MuMin and RVAideMemoire.

Ethical statement

The French Polar Institute (IPEV) is the authority that supported this research based on the advice of its scientific council. The sites visited during this study did not require any access authorization. All research and data reported here were obtained in compliance with all current French laws. Collecting and transport of specimens of the species Notodiscus hookeri were authorized by the Prefect of Ille-et-Vilaine, France, licence N°35–120 delivered in October 2015.

Acknowledgments

Subantarctic field trip was funded by l’Institut Polaire Paul-Émile Victor, Plouzané, France (IPEV, programme 136). Dr Damien Erzsch is acknowledged for the identification on the field of the lichen Usnea taylorii and for his help with the snails’ collection. Julien Tommasino and Grichka Biver are thanked for their contribution in producing a part of this data set. Aude Boutet, Julien Tommasino and Benjamin Ferlay are warmly thanked for their help during the fieldwork. Sugars and polyols quantifications were conducted at the P2M2 Platform, thanks to Pr Alain Bouchereau, and with the technical assistance of Catherine Jonard and Nathalie Marnet, which are warmly thanked. Authors are also indebted to Dr Maxime Dahirel for his advices in statistics and to Dr Maxime Hervé who wrote the R script that referred to feeding choice experiments (Link to his page: https://www.maximeherve.com/r-et-statistiques, and package ‘RVAideMemoire’). We thank Dr Béatrice Legouin for her guidance on the analytical method validation. We are grateful to the anonymous referees for their useful comments that contributed to improve the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2019.04.019.

References


