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

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Title page

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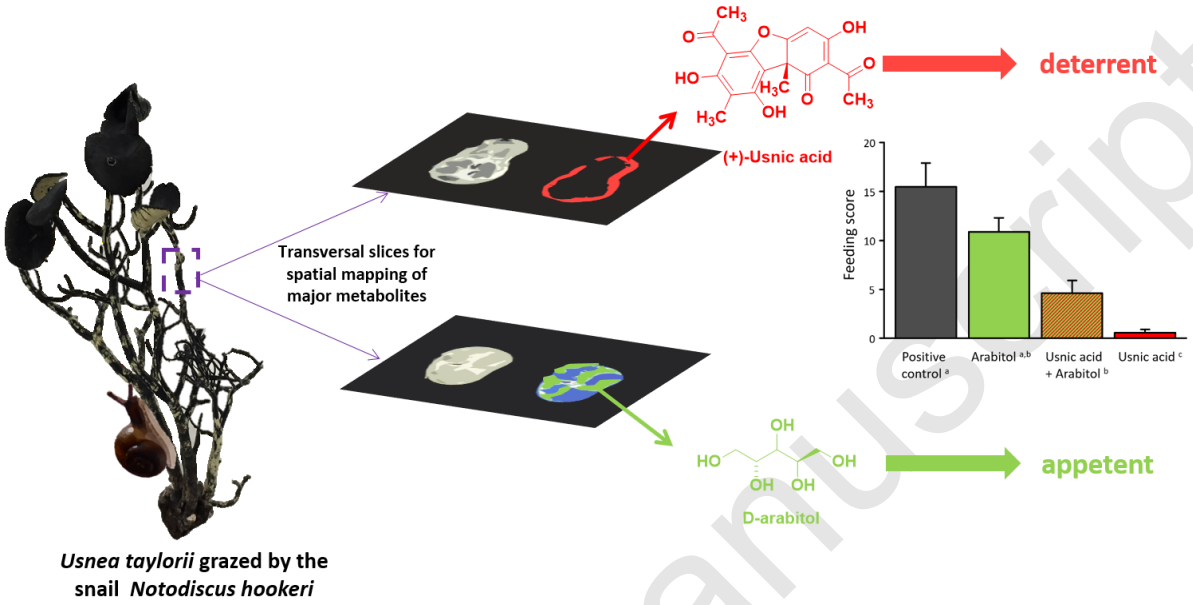
Graphical abstract

The main primary and specialised metabolites in *Usnea taylorii* are *in situ* located by mass spectrometry imaging. Arabitol, which is highly palatable to the snail *Notodiscus hookeri*, counterbalances the deterrent effect of usnic acid and plays a key role in lichen-snail trophic interactions.



Usnea taylorii* grazed by the snail *Notodiscus hookeri

Highlights

- The lichen *Usnea taylorii* contains mainly usnic acid and arabitol.
- *In situ* mass spectrometry imaging reveals the spatial mapping of the metabolites.
- The land snail *Notodiscus hookeri* consumes lichen parts containing both metabolites.
- The nutritional activity of snails is governed by the chemical quality of the lichen.
- Primary metabolites overcome the deterrent effect of specialised metabolites.

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).

Keywords: Usnea taylorii, Parmeliaceae*, Notodiscus hookeri*, Mass spectrometry imaging, feeding choice, lichen, snail, usnic acid, D-arabitol

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 fumarprotocetraric 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 polyols in their feeding choices has not yet been confirmed (Charrier et al., 2006; Charrier and Rouland, 2001; Flari et al., 1995).

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 work, a compromise between appetent polyols and

potentially repulsive specialised metabolites was suggested. *N. 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. and Taylor (Parmeliaceae) 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 repellence (usnic acid) to high appetence (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-dry thalli in acetone. Thalli had only one or two main specialised metabolites. The concentration of usnic acid ranged from 2.5 to 5.4 $mg \cdot g^{-1}$ dry mass (DM) of lichen, with a mean value ± s.d. of 4.1 ± 1.1 $mg \cdot g^{-1}$ DM (n = 6). Sugar and polyol profiling was also performed. A low diversity was observed, with only seven metabolites quantified. Arabitol was the most important polyol, reaching 138.4 ± 25.8 $mg \cdot g^{-1}$ 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 ($[M\text{-}Me]^-$) (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 3d) 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 ($Chi^2 = 0.397$, 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 ($Chi^2 = 0.874$, df = 1, P = 0.350) and collecting sites ($Chi^2 = 1.553$, 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 hours. Gel-mixed metabolites had a significant impact on their consumption by snails (Chi^2 = 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 (2010), who observed that usnic acid did not affect snail grazing on the lichen *Lobaria scrobiculata.* In contrast, Asplund (2011b) described snail grazing (*Cochlodina laminata*) 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* appeared closer to the lichens belonging to Physiaceae 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 appetent 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 thalli. 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 *Argopsis friesiana* (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 phytophages. 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 arabitol, 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 arabitol 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 arabitol in *U. taylorii*, thirty times as much as usnic acid, might explain why the feeding stimulus “arabitol” 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 arabitol, 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°50'10.68"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 fruticose 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 Ertz 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 Prominence Shimadzu HPLC system (Marne La Vallée, France) equipped with a Kinetex C_{18} HPLC column (100 x 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 to 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 (λmax) 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 to 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 (Solhaug and Gauslaa, 2001). Acetone extracts (N=6) were used for the chemical analysis while the rinsed lichen thalli were kept for the feeding choice experiments. 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 bi-distilled tetrahydrofuran at the concentration of 0.5 $g{\cdot}L^{-1}$. Then, the extracts were filtered through a Nylon syringe F2504-1 (Thermo Scientific, Rockwood, USA, 0.45 µm x 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 tetrahydrofurane to reach a concentration of 0.5 $mg.mL^{-1}$. Then, six

working standard solutions 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 $mg{\cdot}g^{-1}$ DM of the lichen part.

5.4. *Sugar* and *polyol profiling*

Sugars and polyols were extracted from *U. taylorii* entire fertile thalli (N=6) and profiled by gas chromatography-flame ionisation detector (GC-FID) (Thermo-Fisher Scientific, Waltham, CA, USA), as previously described by Gadea et al. (2018). Adonitol (20 mM) was used as internal standard. The metabolites were identified from their retention time by comparison with external standards. Concentrations were obtained on the basis of internal standards and were expressed in $mg.g^{-1}$ DM of the lichen part.

5.5. Mass Spectrometry Imaging (MSI)

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 microns. 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).

5.6. 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 hours 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 (Plast'it 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 follows: 10 °C day (12 hours) and 6 °C night (12 hours). 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 hours 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 μm^2/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 $mg \cdot g^{-1}$ for usnic acid and 100 $mg \cdot g^{-1}$ 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 hours. 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 (x1 for consumption of the surface layer and x2 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 μm^2) 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: $V = \mu$).

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 ($\chi^2 = 0.69$, 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.

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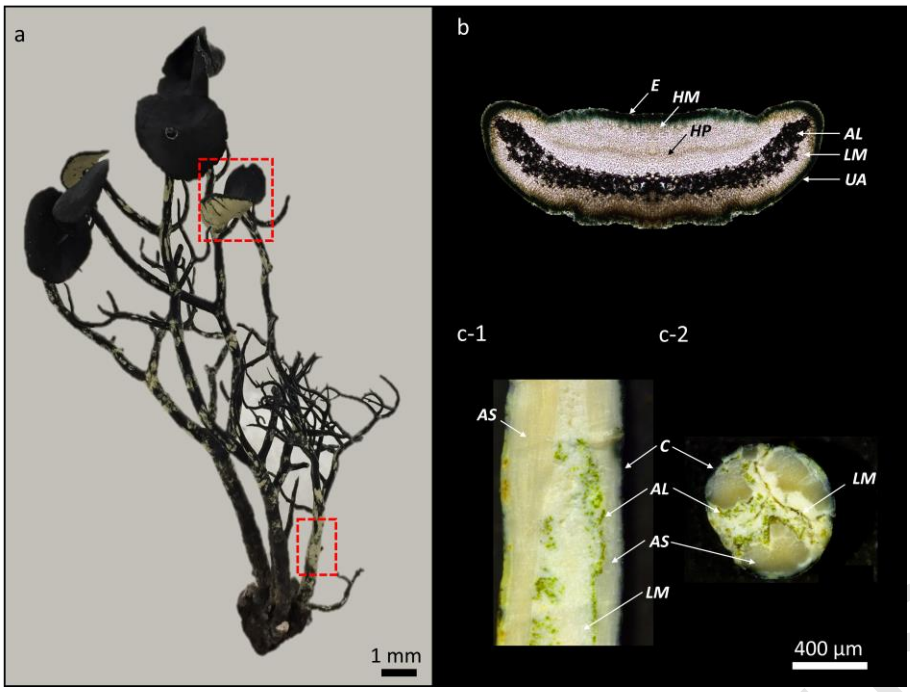
373
374 *Ethical Statement*
375 The French Polar Institute (IPEV) is the authority that supported this research based on the advice of
376 its scientific council. The sites visited during this study did not require any access authorization. All
377 research and data reported here were obtained in compliance with all current French laws. Collecting
378 and transport of specimens of the species *Notodiscus hookeri* were authorized by the Prefect of Ille-
379 et-Vilaine, France, licence N°35–120 delivered in October 2015.
380
381 *Figures and Legends*
382
383 Fig. 1. Morphological description of *U. taylorii.* (a) *U. taylorii* entire thalli. Insets correspond to the
384 magnification used in (b) for the apothecia and in (c) for the branch. (b) Apothecium in cross-section;
385 (c) branches in cross and longitudinal sections. AL = algal layer; AS = axial strands; C = cortical layer and
386 E = epithecium, HM = hymenium containing asci and spores; HP = hypothecium; LM = lax medulla;
387 UA=underside of the apothecium. In colour online.
388
389 Fig. 2. Chemical structures of the two main metabolites of *Usnea taylorii*: the specialised metabolite
390 (+)-usnic acid and the primary metabolite D-arabitol.
391
392 Fig. 3. Distribution of usnic acid ($[M-H]^-$ ion; *m/z* 343) in samples of *U. taylorii*. a. Intact branch; b.
393 Grazed branch (cross-sections); c. Intact apothecium, d. Grazed apothecium (longitudinal sections).
394 Each panel features side by side the optical image of the lichen section (left side) and the mass
395 spectrometry imaging results (right side). The grazing marks were highlighted by blue arrows. Intensity
396 scale was adjusted to maximise the visualisation of usnic acid. In colour online.
397
398 Fig. 4. Distribution of arabitol ($[M+Na]^+$ ion; *m/z* 175) in samples of *U. taylorii*. a. Grazed branches; b.
399 Intact apothecium. On each panel are side by side the optical image of the lichen section (left side) and
400 the mass spectrometry imaging results (right side). The grazing marks on the branches were

401 highlighted by a blue arrow. Intensity scale was adjusted to maximise differences in segregation of
402 arabitol. In colour online.
403
404 Fig. 5. Snail distribution (means + s.e., N= 21 snails x 8 arenas = replicates x 2 sites) in the arms of
405 feeding choice arenas (three-test choice with two identical opposite arms), during the nutritional
406 phase. The letters *a* and *b* indicate significant differences between arena arms containing lichens and
407 arms containing the little rocks.
408
409 Fig. 6. Snails ate the apothecium (a) and the external layers of branches (b). Arrows designate snail
410 grazing marks. In colour online.
411
412 Fig. 7. Gel consumption (Mean ± s.e.) by the snails (N=240), according to the metabolite tested on
413 starch gel. Gel consumption was estimated by a feeding score. The positive control was the starch gel
414 without metabolites. The lowercase superscript letters a, b and c indicate significant differences
415 between positive control and usnic acid containing gels (alone or in mixture with arabitol). Gels with
416 arabitol were not significantly different from the positive control.
417
418 Fig. 8. Choice arena device. Lichen samples, intact or acetone rinsed, and rocks were arranged in
419 opposite arms. Snails (N=21 x 8 arenas x 2 sites) were placed in the central area at the beginning of the
420 experiment and 48 hours later, snail distribution and lichen consumption were analysed.
421



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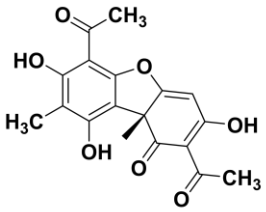
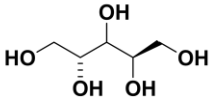


(+)-usnic acid



D-arabitol

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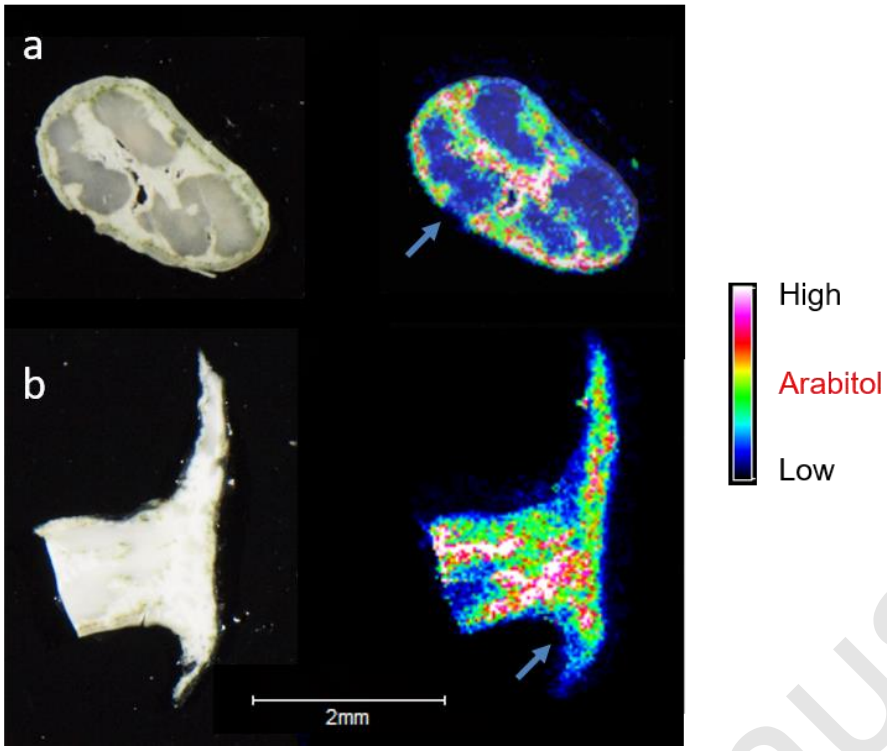

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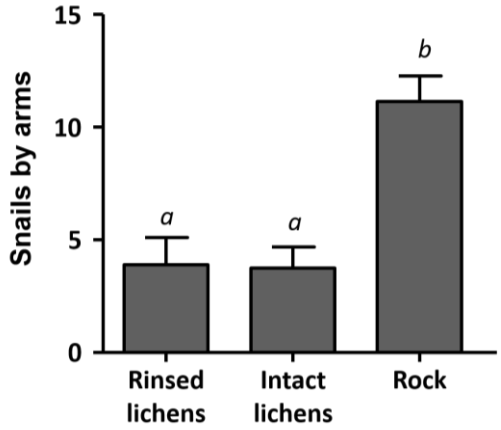

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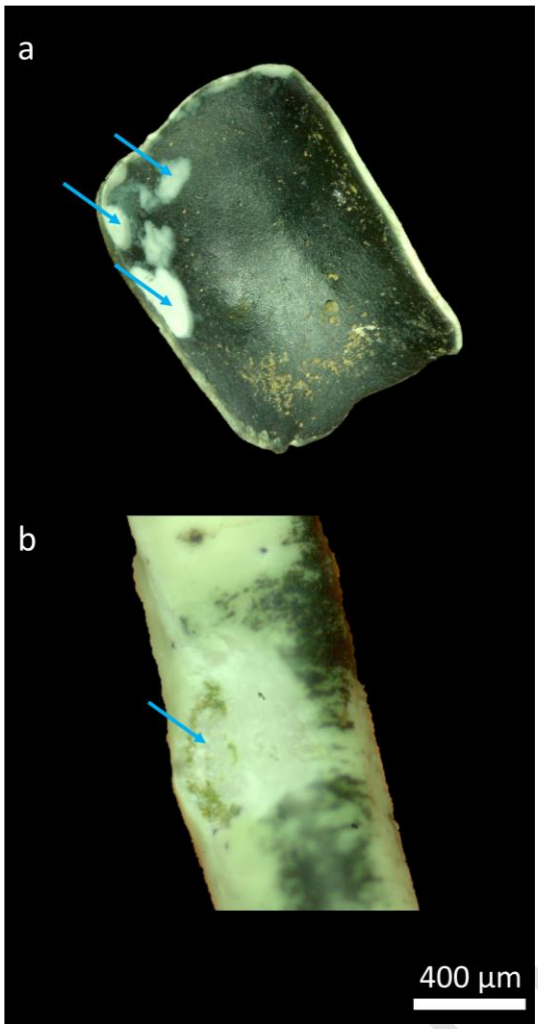

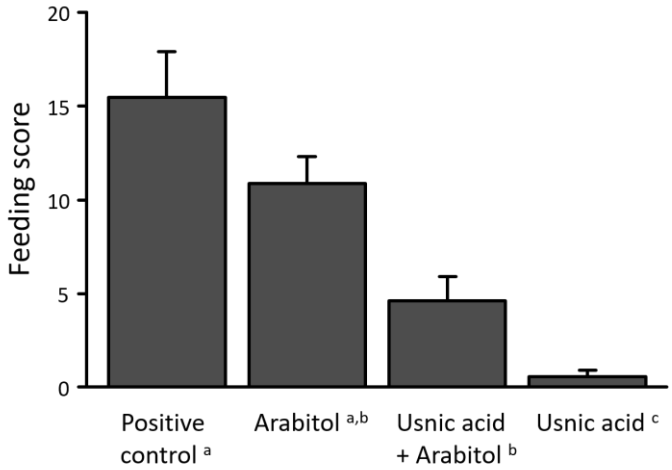

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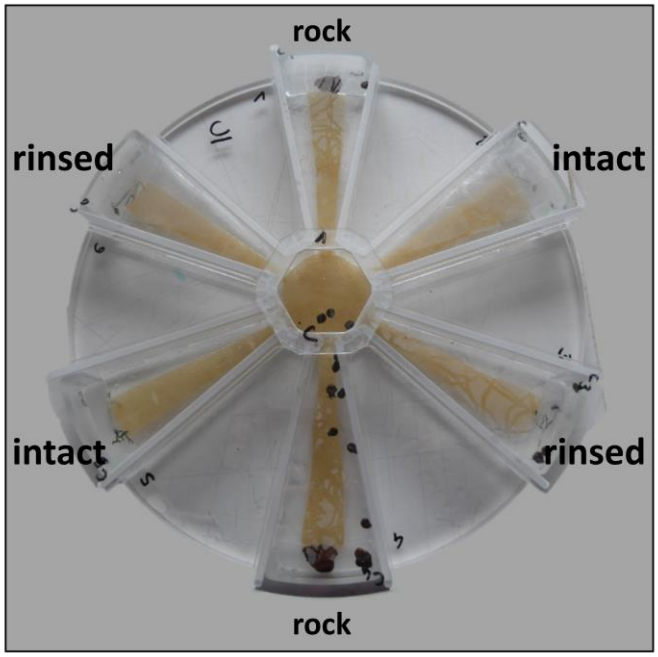

436

437 *Tables*

438

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

440

Parameters	Usnic acid
Calibration curve equation	y = 65613076x – 95165
Correlation coefficient value (R^2)	0.9940
LOD ($mg.mL^{-1}$)	0.0001
LOQ ($mg.mL^{-1}$)	0.0070
Calibration range ($\mu g.mL^{-1}$)	0.0070 to 0.0900
Intraday precision (RSD, n=6)	less than 3%
Interday precision (RSD, n=24)	less than 2%

441 LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation

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