

*Title page***Overcoming deterrent metabolites by gaining essential nutrients: a lichen/snail case study**

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Abstract

Lichen secondary metabolites are generally considered as repellent compounds for lichen feeders. Nevertheless, if the only food available consists in lichens rich in secondary metabolites, lichenophages such as *Notodiscus hookeri*, a gastropod native from the Possession Island, seem able to implement strategies to overcome the toxicity of these metabolites. Thus, the balance between phagostimulant nutrients and deterrent metabolites appears to play a key role in their feeding preferences. To further understand lichen-gastropod interactions, we studied the feeding behavior of *Notodiscus hookeri* fed exclusively with *Usnea taylori*, a lichen rich in usnic acid and arabitrol. Snail feeding choice experiments with intact lichens vs acetone-rinsed lichens were carried out to study the influence of secondary metabolites. Simultaneously, usnic acid and arabitrol were quantified and localized within the lichen thallus using HPLC-DAD-MS and *in situ* imaging by mass spectrometry to assess whether their spatial distribution induce preferential snail grazing. Then, no-choice feeding experiments were devised using usnic acid and arabitrol embedded in artificial diet, separately or together. This case study demonstrated that the nutritional activity of *N. hookerii* was governed by the chemical quality of the food and primarily by nutrient availability (arabitrol), despite the presence of deterrent metabolites (usnic acid).

Keywords: Mass spectrometry imaging, feeding choice, lichen, snail, usnic acid, D-arabitrol

1. Introduction

Optimal foraging in generalist species consists in finding the right balance between need to overcome potential toxic compounds and access to useful nutrients. Invertebrates, including gastropods, are considered as the most important lichen feeders (Asplund and Wardle, 2016; Fröberg et al., 2011; Gerson, 1973; Vatne et al., 2010) and because lichens are sessile and slow-growing symbiotic organisms, they must defend strongly against these predators (Solhaug and Gauslaa, 2012). Lichen symbiosis is usually structured with fungus, the mycobiont, and with photosynthetic partners, the photobiont (chlorophytes and/or cyanobacteria). The mycobiont produces hydrosoluble secondary metabolites such as mycosporines (Roullier et al., 2011) and lectines (Huneck and Yoshimura, 1996), but also hydrophobic ones like depsides, depsidones and dibenzofuranes (Stocker-Wörgötter, 2008). These latter can be accumulated as tiny crystals on the external part of the fungus hyphae, in the cortex or the medulla of the lichen thallus (Honegger, 1991). This extracellular localization makes the metabolites easy to remove using the so-called acetone-rinsing methodology, without degrading the lichen viability and palatability (Pöykkö et al., 2005; Solhaug and Gauslaa, 2001). Such experiments have highlighted deterrent and/or toxic activity for some secondary metabolites towards invertebrates, including snails (Emmerich et al., 1993; Gauslaa, 2005; Solhaug and Gauslaa, 2012). Generalist lichenophages species generally preferred lichens with few secondary 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, the nature of the latter turns out to be the main discriminant factor in food preference by lichenophages. In the accidental degradation of the lichen herbarium of the Trieste University, the coleopteran *Lasioderma serricorne* avoided lichen species containing the dibenzofurane usnic acid, the depside atranorin or the depsidone fumarprotocetraric acid (Nimis and Skert, 2006). In a similar way, when snails faced two chemotypes of the lichen *Lobaria pulmonaria*, identified by the presence or absence of stictic acid (depsidone) in the thallus, a preferential grazing of the depsidone-free lichen was observed (Asplund, 2011a).

However, if the only available resources are lichens rich in secondary metabolites, lichenophages have to overcome toxicity of some secondary metabolites or to be able to metabolize them (Gadea et al., 2017; Hesbacher et al., 1995).

Lichen tissues are also a complex matrix containing an important part of carbohydrates, including polysaccharides and reserve carbohydrates such as sugars and polyols. The phagostimulant properties of sugars were previously highlighted for improving slug baits, particularly disaccharides which appeared more attractive than monosaccharides (Clark et al., 1997; Henderson et al., 1992). Indeed, gastropods have the full enzymatic arsenal to degrade and to assimilate sugars and polyols, however the role of polyols in their feeding choices was not confirmed (Charrier et al., 2006; Charrier and Rouland, 2001; Flari et al., 1995). As the subantarctic snail *Notodiscus hookeri* is an exclusive lichen feeder, it appears to be a relevant model to advance in the understanding of the chemical ecology of the lichens/snails interactions. In our previous works, a compromise between appetent polyols and potentially repulsive secondary 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 secondary metabolites, which can be visualized 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 are distributed the primary metabolites, such as sugars and polyols, within the lichen thallus. In this study, we proposed the first spatial mapping of both appetent and repellent metabolites in order to account for the nutritional choices made by the snail. To address this question, the lichen *Usnea taylorii* was selected because of its simple chemistry. First, we followed the snail behavior 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. Second, we intended to explore the spatial localization of the main primary (D-arabitol) and secondary (usnic acid) metabolites of *U. taylorii* using *in situ* mass spectrometry imaging. Patterns of distribution of the metabolites were expected to influence the snail feeding strategy. Third, snails were subjected to no-choice feeding experiments

using the pure metabolites embedded in artificial diet, separately or together. We hypothesized a gradual gustatory response, from strong repellence (usnic acid) to high appetite (D-arabitol).

2. Results and discussion

2.1. Morphological and chemical characterisation of *Usnea taylorii*

2.1.1. Description of *Usnea taylorii* morphology

Microscopic analysis of the fruticulose lichen *Usnea taylorii* reveals a specific morphology that deserves a detailed description to improve understanding of the metabolites localisation (Fig 1a; a complete morphological description of the lichen is available in Text S1). Apothecia (reproductive parts of the lichen) are localized at the apex of the *Usnea* branches and are characterized by their high diameter (2-17 mm) and their jet-black pigmented disc. In cross section, under the black epithecium, asci containing spores are present in the hymenium, algal layer is then observed (Fig 1b). The thallus in cross section shows a very thin cortex, protecting a discontinuous algal layer. Unlike other *Usnea* species (Seymour et al., 2007), the central axis of *U. taylorii* is divided into several smaller axial strands by the protruding medulla, which are surrounded by some algae. Between these axial strands, lax medulla is observed (Fig. 1c) as previously described by Walker (1985). This subdivided axis confers to the lichen a strong rigidity, which enables it to resist to windy conditions despite its large size (approximately 10 cm tall).

2.1.2. Quantification of the main metabolites of the lichen *U. taylorii*

Organic extracts derived from the acetone rinsing method led to the identification and quantification of the main metabolites. The extraction yields ranged between 0.5 and 1.0 % for the six replicates of dried lichen materials with a mean value of 0.8 ± 0.2 %. The chemical profile of the secondary metabolites for whole fertile thalli was established by HPLC-DAD-ESI-MS analysis of acetone extracts. Lichens contain a few metabolites diversity with generally one or two main secondary metabolites hiding minor ones. In *U. taylorii*, as in other *Usnea* species belonging to the subgenus *Neuropogon*

(Walker, 1985), usnic acid is the main secondary metabolite detected by HPLC-DAD-ESI-MS (Gadea et al., 2017). Fumarprotocetraric acid, found in trace amounts in some samples of *U. taylorii* thalli, was not detected in our experiments. To validate the chromatographic method used in the study, the precision and linearity of the method were determined (Table 1). Amount of usnic acid ranged from 2.5 to 5.4 mg.g⁻¹ Dry Mass (DM) of lichen, with a mean value \pm s.d. of 4.1 ± 1.1 mg.g⁻¹ DM (n = 6). The concentrations in *U. taylorii* were consistent with those found in other *Usnea* species (Cansaran et al., 2006).

Sugars and polyols profiling was also performed. A low diversity was observed, with only seven metabolites quantified. Arabitol was the most important polyol of the lichen, reaching 138.4 ± 25.8 mg.g⁻¹ DM (mean value \pm s.d., Table S1). The high amount of arabitol can be explained by its role as short-term carbohydrate reserve (Armstrong and Smith, 1994; Dahlman et al., 2003). It is noteworthy that polar or sub-polar lichens, such as the Antarctic lichen *Usnea antarctica*, can contain high levels of polyols (arabitol: 112,9 mg.g⁻¹)(Tearle, 1987).

2.2. Snail behaviour in feeding choice arenas

In order to study the influence of acetone soluble secondary metabolites on snail feeding choice, *U. taylorii* thalli were rinsed by acetone before being tested face to face with intact *U. taylorii* thalli in feeding choice arenas (Fig 2). During the nutritional phase, more than 50% of the snails preferred to stay near the rock shelter, while the rest of them were equally distributed in arms with intact and rinsed lichens (Fig 3). So, in an unknown environment, snails seem first to look for a shelter before looking for food. A recent study on dispersal in this species confirmed that big rock size reduced snail dispersal and that lichen availability had no effect on the decision of snail movements (Dahirel et al., 2018).

After 48 hours in feeding choice arenas, *N. hookeri* grazed intact *U. taylorii* thalli as well as rinsed ones (GLM, F = 1.81, P = 0.204). Removal of acetone soluble metabolites did not promote lichen consumption. Consequently, we hypothesised that (i) usnic acid had no repellent effect on *N. hookeri*

and /or (ii) arabitol was a phagostimulant compound. 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 axial strands of branches always remained intact (Fig. 4). Snails ate the external layers of the apothecia, epithecium and hymenium on the upper face and the underside, containing both fungal mycelium and algae. This preferential grazing of some lichen parts could be explained by a sectorial localization of the secondary metabolites in lichens (Gadea et al., 2018; Le Pogam et al., 2016). As an example of this statement, Asplund (2011b) described a perpendicular grazing of the lichens thalli after acetone rinsing in two *Lobaria* species, while snails avoided medulla before removal of acetone soluble metabolites, suggesting the presence of deterrent compounds in the medulla. To assess whether usnic acid and arabitol spatial distributions in the thallus parts influenced the snail feeding strategy, we applied mass spectrometry imaging on sections of *U. taylorii*, before and after snail grazing.

2.3. Localization of the major metabolites of *U. taylorii* by mass spectrometry imaging (LDI-MSI and MALDI-MSI)

Mass spectrometry imaging (MSI) is a powerful tool for studying the chemical role of secondary metabolites in lichen thalli, that couples both spatial and chemical information at the tissue level (Le Pogam et al., 2016). Preliminary LDI-MS analysis of an acetone extract of *Usnea taylorii* confirmed that the (+)-usnic acid was the only secondary metabolite detected through its deprotonated molecule (m/z 343) along with a fragment ion at m/z 329 ([M-Me]⁻). 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. Yet, 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 performed by 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 localized in the peripheral layer of the thallus, *i.e* in the cortical and external algal layers and medulla (Fig. 5a). The same distribution pattern was observed within the apothecia slices, showing

usnic acid allocated to the external layers of the apothecium (epithecium, hymenium and the underside of the apothecium) (Fig. 5c). D-arabitol was present mainly in the lax medulla, the cortex and the algal layer (Fig. 6a). It was detected in lower intensities in the axial strands of the lichen branches. In apothecia, the signal corresponding to the D-arabitol was stronger in the layer containing the lax medulla and algae than in the epithecium and hymenium, containing both the asci and algal cells (Fig. 6b).

The cortical localization of usnic acid by LDI-MSI, surrounding the algal cells, confirmed previous reports (Le Pogam et al., 2016; Liao et al., 2010). Usnic acid might provide photoprotection (Nybakken and Gauslaa, 2007) and/or regulate algal development (Bačkor et al., 2010; Le Pogam et al., 2016). Nevertheless, the presence of usnic acid was not always correlated to the occurrence of algal cells given that no usnic acid was detected around axial strands of *Usnea taylorii*, where some invaginated algal cells were observed. We hypothesize that internal algal cells could be protected by the axial strands and external fungal layers. Hence, they should not need more photoprotection. In apothecia, one can assume that usnic acid acts as UV filter to protect spores during their maturation within asci, avoiding a potential DNA degradation of the spores (Liao et al., 2010).

To perform a comparison, some branches and apothecia eaten by snails 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. 5b and 5d) and D-arabitol (Fig. 6). In a previous study Gadea et al. (2017) showed that usnic acid was retrieved from faeces of *N. hookeri* after *U. taylorii* intake. However, consumption and excretion of the metabolite do not mean that usnic acid was appetent and/or not toxic for the snail. Arabitol and usnic acid were found partly co-localized in the cortex and in the apothecia, and these two parts being eaten by *N. hookeri*, the question then arises whether both metabolites are attractive to the snail. In order to compare the influence of these metabolites on the snail feeding choice, isolated compounds were tested separately and in mixture, in no-choice experiments.

2.4. 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 consumption showed that the molecules incorporated into the gel had a significant impact on the snails feeding choices ($\chi^2 = 57.47$, $df = 3$, $P < 0.001$; Fig 7). Snails confronted to gels with usnic acid alone, mainly avoided it (Tukey, $P < 0.001$). Gels supplemented with arabitol were eaten as well as the positive control starch gel alone (Tukey, $P = 0.795$; Fig. 7). Snails ate gel mixing up arabitol and usnic acid more than gel holding usnic acid alone (Tukey, $P < 0.001$), but less than gel with arabitol alone (Tukey, $P = 0.009$).

Usnic acid contained in the lichen was consumed while it turned out to be deterrent for *N. hookeri* when extracted and incorporated in a waxy starch gel. The results are partly in line with those obtained with the lichen *Lobaria scrobiculata*, eaten by the snail *Cochlodina laminata* before and after usnic acid removal (Asplund et al., 2010). However, its deterrent effect on snails when given as pure metabolite was counteracted by D-arabitol in mixture with usnic acid. The phagostimulant effect of D-arabitol for the snail also occurred when 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 of high interest for snails as nutrient metabolites to provide energy after oxidative conversion, but also for production of mucus which enables body hydration, locomotion, and food foraging among others (Ng et al., 2013). In lichen, arabitol is supposed to be localised in the cytosol of the fungus cells (Eisenreich et al., 2011) conversely to usnic acid that is localised extracellularly (Honegger, 1986). In wet conditions, after a rain event for example, lichens exudate until 10% of the polyols pool (Dudley and Lechowicz, 1987). Consequently, snails might be first attracted by the exudate arabitol and then start grazing the lichen including the cortex containing usnic acid.

This case study revealed that as in plant-phytophage interactions (Jamieson et al., 2017), primary metabolites should be considered as well as secondary metabolites in lichen-lichenophage interactions.

3. Conclusion or concluding remarks

High amount of arabitol, useful for its 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, mass spectrometry imaging helped to improve 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 enlarged to other biological models, for instance terrestrial gastropods well known as crop pests in agriculture, horticulture and orchards.

4. Experimental

4.1. Snail Collection and Lichen Material

Adult individuals of *Notodiscus hookeri* Reeve were collected on Possession Island during 2015 Austral summer. Snails came from two fell-fields, Mont Branca (BRA, GPS, 300 m) and Mascarin Summit (MAS, 46°26'10.09" S; 51°45'20.58" E, 600 m) (a map of the sampling sites is available in Gadea et al. (2018)). The fruticose chlorolichen *Usnea taylorii* D. Hook., encountered at MAS in the snail habitat, was harvested on Possession Island during 2015 Austral summer. 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.

4.2. 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 well-developed thicker branches. The ratio “% of axis thickness/% of medulla thickness” or 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 in longitudinal sections of the branches.

4.3. HPLC-DAD-MS analysis of secondary metabolites

4.3.1. 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 C₁₈ HPLC column (100 x 4.6 mm, 2.6 µm, 6A, Phenomenex) and consisting of 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, U.S.A.). 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 (λ_{max}) of main metabolites. Peaks were assigned according the retention time, UV-spectra as well as based on mass spectra for both the standards and the samples under the same chromatographic method.

4.3.2. Samples and standard preparation

Six specimens of the whole fertile thallus of *Usnea taylorii* (642 to 1000 mg) were extracted by acetone rinsing method three times (5 mL, 20 min) at room temperature and were allowed to dry in air 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 lichen powder, was calculated for each extraction. All extracts obtained from *Usnea taylorii* were dissolved in bidistilled tetrahydrofuran at the concentration of 0.5 g.L⁻¹. Then, they 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.

4.3.3. 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 interday and intraday. Four stock standard solutions of (+)-usnic acid (Sigma-Aldrich ; 329967) were prepared by dissolving about 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 g.L⁻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. Limit 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 g.L⁻¹) was injected six times the same day. These assays were repeated on four different days for inter-day repeatability. Coefficient of variation and standard deviation were then calculated. Variation coefficients of less than 10 % for intra-day and for inter-day

were accepted. Concentrations were obtained on the basis of calibration curves and were expressed in mg.g⁻¹ DM of the lichen part.

4.4. Sugars and polyols profiling

Sugars and polyols were extracted from *U. taylorii* entire fertile thalli (N=6) and profiled by Gas Chromatography - Flame Ionization 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.

4.5. Mass Spectrometry Imaging (MSI)

Usnea taylorii samples were hand-cut using a razor blade to afford slices of about 100 µm thick. Branches and apothecia slices were fixed on a carbon-conductive adhesive tape which was in turn fixed on an indium tin oxide (ITO) slide (Bruker Daltonics, Bremen, Germany, cat no 237001). For Laser Desorption Ionization (LDI) MSI measurement, no further preparation step was required. For Matrix Assisted Laser Desorption Ionization (MALDI) MSI measurements, DHB matrix solution (50 mg/mL in 50% methanol) was homogeneously applied with a home-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 optimized 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).

4.6. Snails behaviour experiments

4.6.1. Snails feeding choice arena

Each fertile thallus of *U. taylorii* was divided into two pieces of equal sizes, always containing a branch with the terminal apothecia. Half of these thalli were randomly taken to extract the secondary metabolites and constituted the rinsed lichens ("Rinsed"). The other group contained intact lichens ("Intact"). Secondary metabolites were removed using the acetone rinsing technique described by Solhaug and Gauslaa (2001). Acetone allowed the extraction of extracellular secondary metabolites, without altering the lichen viability and without impacting the nutritional value (Pöykkö et al., 2005). Residual acetone was evaporated for at least 20 hours before the experiment.

One hundred and sixty-eight *N. hookeri* were divided randomly into eight subgroups of 21 snails. No significant differences in shell size were observed among snails subgroups (mean \pm s.d. = 4.80 ± 0.37 ; Kruskal-Wallis, $\chi^2 = 7.04$, $P > 0.05$). Two days before the experiment, snails were starved in order 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 M. Charrier, Fig. 2). Wet synthetic foam was placed at the bottom of each arena to maintain a sufficient humidity necessary for snails activity. Similar lichen samples were placed at both ends in opposite arms, giving the choice between two: two "Intact" and two "Rinsed" samples. The two latter arms contained a small bare rock each, representing secure refuges always used by snails in the field during resting phases.

Eight feeding choice arenas, also called “Notodromes” received 21 snails each and were maintained in a climatic chamber under a light/dark photoperiod set as follows: 10 °C day (12 h) and 6 °C night (12 h). Experiments started at the beginning of the nocturnal phase and ended 48 hours after. The localisation of each snail in the Notodrome was reported, radula marks on lichen thalli were observed.

4.6.2. No-choice experiments on isolated compounds

Two hundred and forty adult snails were separated in four subgroups: one control and three tested groups of 60 snails each (usnic acid, usnic acid + arabitol and arabitol). No significant difference in the shell size was observed among subgroups (mean \pm s.d. = 4.81 \pm 0.33 mm ; Kruskal-Wallis, χ^2 = 5.26, df=3, $P > 0.05$). No-choice experiments were performed in two-compartments Petri dishes (Greiner Bio One, Austria). Wet nonwoven sterile gauze was placed in each compartment to maintain humidity. Waxy starch gels were prepared at the concentration of 15% and small discs (diameter: 6 mm, thickness 1 mm) were manufactured using homemade device (Plast’it supplier, Rennes, France, under the concept proposed by M. 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 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 (1 for consumption of the surface layer and 2 for a total consumption; Fig S1).

4.6.3. Statistical analyses

Snail distribution in the feeding choices arenas’ arms was analysed by a Generalized Linear Model (GLM, distribution: poisson, link function: logit) in which the fixed factor “sampling site” was added.

In the no-choice experiments, to look whether metabolites were differently consumed by comparison with the control, a likelihood ratio test was used on a Generalized Linear Model (GLM, distribution: negative binomial, link function: log ; function “glm.nb”, package MASS (Venables and Ripley, 2002), in which the sampling site was introduced as covariable. Post-hoc Tukey pairwise comparisons were realised with the function “glht” (package “multcomp”, (Hothorn et al., 2008)). As no differences between sampling sites being observed for the feeding score ($\chi^2 = 0.69$, $df = 1$, $P = 0.408$), results of both sampling sites were grouped. All statistical analyses were made using R software V. 3.4.3 (R Core Team, 2017).

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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.

Figures and Legends

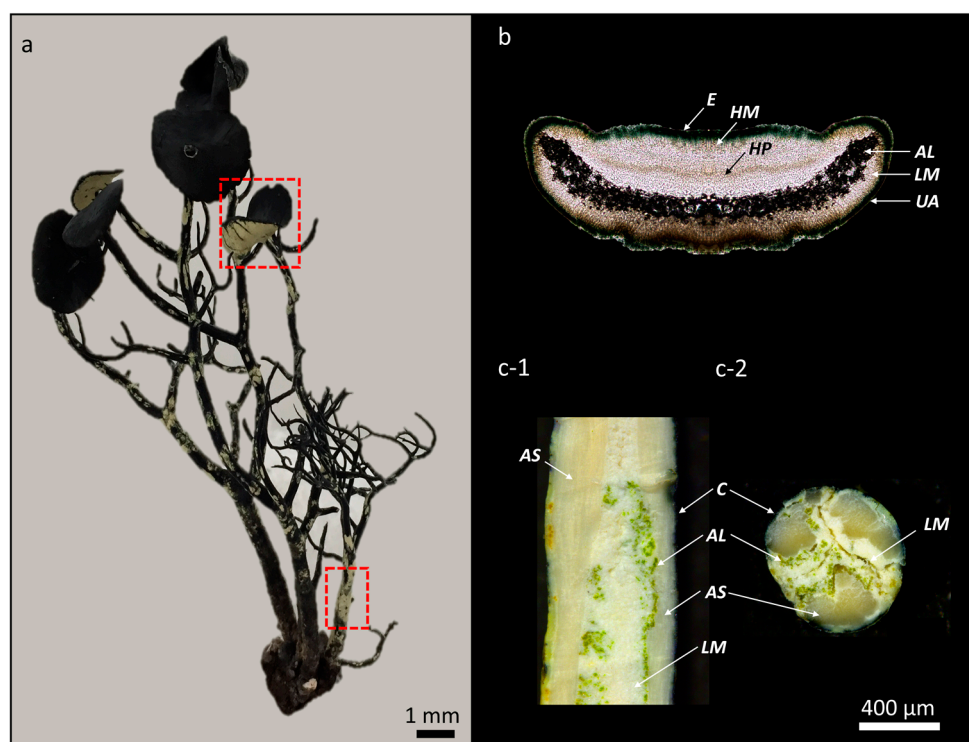


Fig. 1 Morphological description of *U. taylorii*. (a) *U. taylorii* entire thalli. Insets correspond to the magnification used in (b) for the apothecia and in (c) for the branches. (b) Apothecium in cross section; (c) branches in cross and longitudinal sections. AL = algal layer; AS = axial strands; C = cortical layer and E = epithecium, HM = Hymenium containing asci and spores; HP = hypothecium; LM = lax medulla; UA=underside of the apothecium. In colour online.

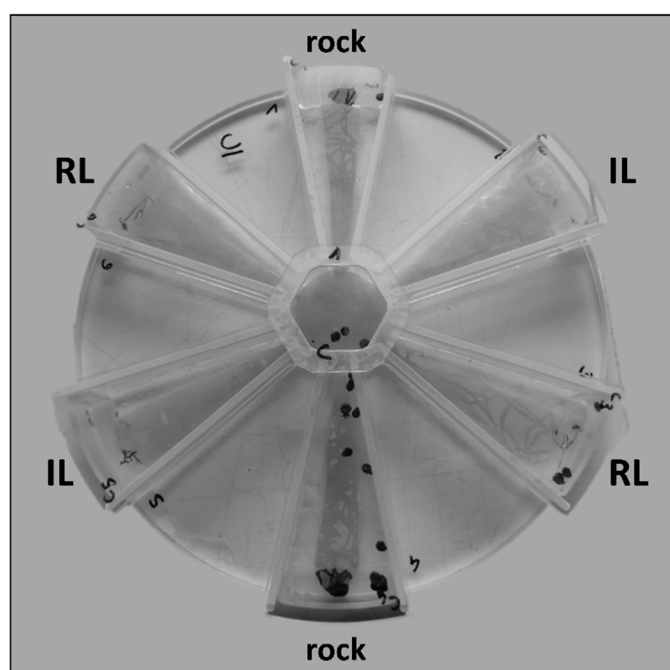


Fig. 2 Choice arena dispositive or “Notodrome”. Lichen samples, intact (IL) or acetone rinsed (RL), and rocks were arranged in opposite arms. Snails (N=21 x 8 notodromes) were placed in the central area at the beginning of the experiment and 48 hours later, snail distribution and lichen consumption were analyzed.

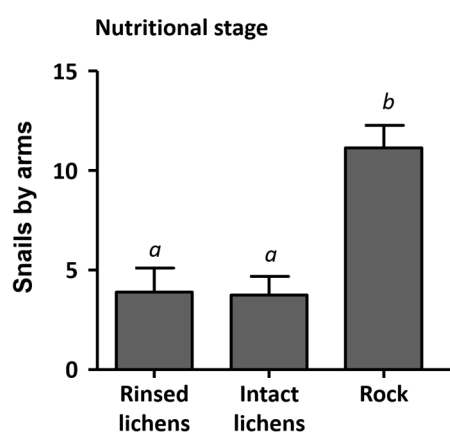


Fig. 3 Snail by arms (means + s.e., N= 21 x 8 notodromes = replicates) in the arms of feeding choices arenas (N= 8 notodromes x 2 identical arms), during the nutritional phase. The letters *a* and *b* indicate significant differences between arena arms containing lichens and arms containing the little rocks.

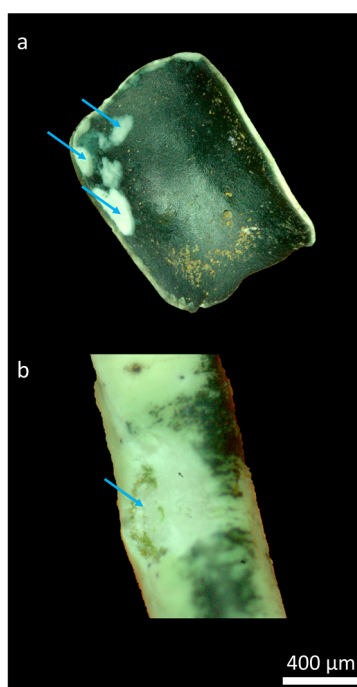


Fig. 4 Snails ate the apothecium (a) and the external layers of branches (b). Arrows design snails grazing marks.

In colour online.

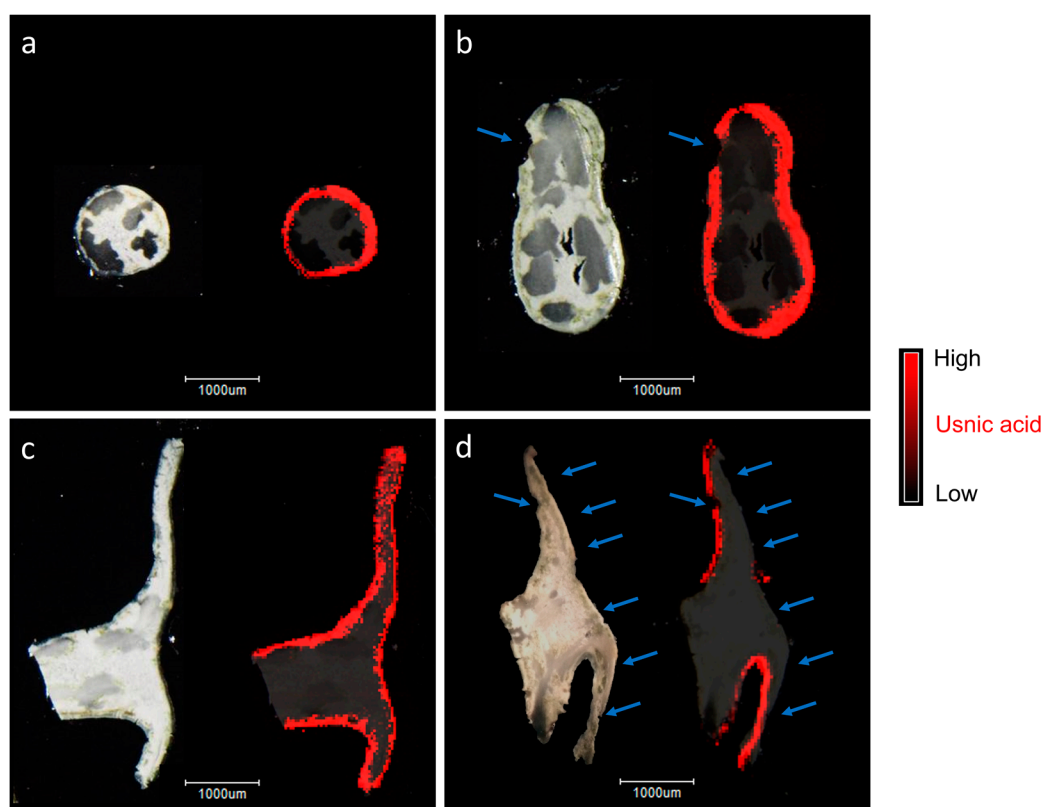


Fig. 5 Distribution of usnic acid ($[M-H]^-$ ion; m/z 343) in samples of *U. taylorii*. a. Intact branch; b. Consumed branch (cross sections); c. Intact apothecium, d. Consumed apothecium (longitudinal sections). Each panel features side by side the optical image of the lichen section (left side) and the mass spectrometry imaging results (right side). The consumed parts (radula marks) were highlighted by blue arrows. Intensity scale was adjusted to maximize the visualization of usnic acid. In colour online.

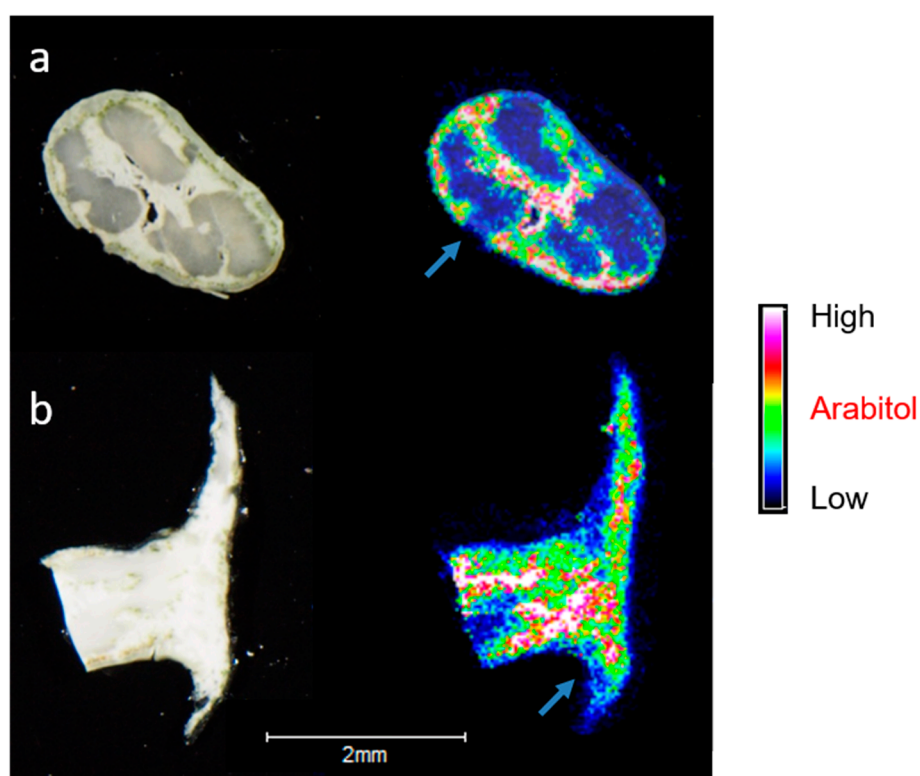


Fig. 6 Distribution of arabitrol ($[M+Na]^+$ ion; m/z 175) in samples of *U. taylorii*. a. Grazed branches; b. Intact apothecium. On each panel are side by side the optical image of the lichen section (left side) and the mass spectrometry imaging results (right side). The consumed part (radula mark) on the branches is highlighted by a blue arrow. Intensity scale was adjusted to maximise differences in segregation of arabitrol. In colour online.

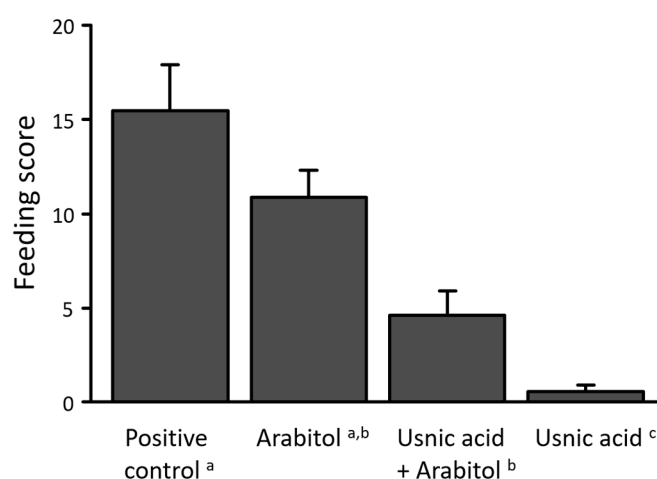


Fig. 7 Gel consumption (Mean \pm s.e., N=240) by the snail, according to the metabolite tested on starch gel. Gel consumption was estimated by a feeding score. Positive control selected was the starch gel without metabolites. The lowercase superscript letters a, b and c indicate significant differences between positive control and usnic acid containing gels (alone or in association with arabitol). Gels with arabitol were not significantly different from positive controls.

Tables

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

Parameters	Usnic acid
Calibration curve equation	$y = 65613076x - 95165$
Correlation coefficient value (R^2)	0.994
LOD ($\mu\text{g.mL}^{-1}$)	0.1
LOQ ($\mu\text{g.mL}^{-1}$)	6.9
Calibration range ($\mu\text{g.mL}^{-1}$)	7.0 to 90.0
Intraday precision (RSD, n=6)	less than 3%
Interday precision (RSD, n=24)	less than 2%

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

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