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Intrathalline Metabolite Profiles in the Lichen *Argopsis friesiana* Shape Gastropod Grazing Patterns

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4 1 INTRATHALLINE METABOLITE PROFILES IN THE LICHEN *Argopsis friesiana* SHAPE
5 2 GASTROPOD GRAZING PATTERNS.
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Abstract-Lichen-gastropod interactions generally focus on the potential deterrent or toxic role of secondary metabolites. To better understand lichen-gastropod interactions, a controlled feeding experiment was designed to identify the parts of the lichen *Argopsis friesiana* consumed by the Subantarctic land snail *Notodiscus hookeri*. Besides profiling secondary metabolites in various lichen parts (apothecia, cephalodia, phyllocladia and fungal axis of the pseudopodetium), we investigated potentially beneficial resources that snails can utilize from the lichen (carbohydrates, amino acids, fatty acids, polysaccharides and total nitrogen). *Notodiscus hookeri* preferred cephalodia and algal layers, which had high contents of carbohydrates, nitrogen, or both. Apothecia were avoided, perhaps due to their low contents of sugars and polyols. Although pseudopodetia were characterized by high content of arabitol, they were also rich in medullary secondary compounds, which may explain why they were not consumed. Thus, the balance between nutrients (particularly nitrogen and polyols) and secondary metabolites appears to play a key role in the feeding preferences of this snail.

Key Words-Herbivory, chemical ecology, lichen-gastropod interactions, Subantarctic islands, Stereocaulaceae, *Notodiscus hookeri*.

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INTRODUCTION

Lichens are above all a lifestyle generally presented as a stable organism corresponding to the symbiotic association of fungi with photobionts (Gargas et al. 1995). Fungal symbioses with algal or cyanobacterial photobionts are called chloro- and cyanolichens, respectively. Sometimes the mycobiont coexists with both green algae and cyanobacteria and forms tripartite lichens, referred to as cephalolichens, because cyanobacteria are located inside specialized fungal compartments (cephalodia).

In a lichen thallus, green algae produce polyols while cyanobacteria synthesize sugars. These photosynthetically produced carbohydrates may be metabolized by the fungus partner into mannitol and/or arabitol (Richardson et al., 1968). The mycobiont also produces secondary metabolites (or specialized metabolites) that accumulate in the cortex or in the medullar layer (Molnár and Farkas 2010). In most chlorolichens, the acetyl-polymalonyl pathway leads to the synthesis of secondary metabolites, mainly phenolic compounds such as depsides and depsidones (Stocker-Wörgötter 2008). In most cyanolichens and in cephalodia containing cyanobacteria, terpenoids and mycosporines predominate (Rundel 1978; Roullier et al. 2011). The co-occurrence of two photobionts in cephalolichens is reflected by the diversity and allocation of metabolites produced, cephalodia being devoid of phenolic compounds, which are found in the other parts of the thallus, but rich in N-containing compounds (Roullier et al. 2011). As a general trend, a higher content of nitrogen occurs in cephalo- and cyanolichens as the atmospheric nitrogen fixed by the cyanobacteria is converted to amino acids by the mycobiont (Rai and Bergman 2002).

Phenolic compounds in lichens have various roles. Some may deter lichen feeders such as molluscs (Coker 1967; Asplund et al. 2010b), mammals (Cook et al. 2007; Nybakken et al. 2010) and arthropods (Gerson 1973; Wieners et al. 2018). Various physical and chemical factors may influence the attractiveness of lichens for lichenivorous organisms, such as roughness and

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4 68 growth form, occurrence of defence compounds and N-fixing ability of the lichen (Asplund and
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6 69 Wardle 2016). In studies focussing on interactions between plants and phytophagous animals,
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8 70 different hypotheses have been proposed postulating a trade-off between the cost of operating
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10 71 chemical defence and the benefit of enhancing survival. In his work on plant-herbivore
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12 72 coevolution, McKey (1974) developed the “optimal defence theory” (ODT) which was later
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14 73 applied by Asplund et al. (2010b) to lichen-gastropod interactions. This theory predicts that
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16 74 secondary metabolites in plants are allocated proportionally to the risk of attack for a specific
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18 75 tissue and the value of this tissue in terms of the reduction in fitness that its loss would entail. In
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20 76 many lichens, secondary metabolites occur in higher concentrations in reproductive parts,
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22 77 whether sexual (apothecia) or asexual (isidia, soralia), than in the rest of the thallus, or occur
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24 78 only in these reproductive parts. (Hyvärinen et al. 2000). Rundel (1978) conceptualized another
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26 79 hypothesis, suggesting that the higher the nitrogen content, the higher is the lichen’s palatability.
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31 80 Hence, lichens with low nitrogen content and high concentration of secondary metabolites
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33 81 (particularly depsides and depsidones) should suffer low grazing. Rundel’s hypothesis was
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35 82 confirmed for most chlorolichens, which were indeed more grazed after non-destructive removal
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37 83 of the secondary metabolites by the acetone-rinsing method (Solhaug and Gauslaa 2001;
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39 84 Asplund and Wardle 2013). Conversely, Rundel’s theory was not validated for some
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41 85 cyanolichens which were not consumed although characterised by high nitrogen levels and low
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43 86 content of secondary chemicals. The presence of intracellular repulsive compounds or of
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45 87 metabolites not extractable by acetone (*i.e.* mycosporines) was hypothesized, due to the
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47 88 persistence of a deterrent effect after acetone rinsing (Solhaug and Gauslaa 2012).

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51 89 Lichens are widespread from cold to tropical habitats, from wet to xeric conditions and in
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53 90 nutrient-poor habitats, such as alpine and polar areas, where they are often dominant (Sanders
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55 91 2001). In Subantarctic islands, where lichens are abundant, it could be important for
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57 92 phytophages to exploit such an abundant resource and thus allow an expansion into new
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59 93 environments. The Subantarctic land snail *Notodiscus hookeri*, an exclusive lichen feeder, is

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4 94 particularly widespread on Crozet (Possession Island) and Kerguelen Archipelagos (Charrier et
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6 95 al. 2013). This species provides an appropriate model organism for studying lichen-gastropod
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8 96 interactions. Recently, Gadea et al. (2017) demonstrated the generalist status of *N. hookeri* by a
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10 97 comparative study of snail metabolism after feeding on *Usnea taylorii* (chlorolichen) and
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12 98 *Pseudocyphellaria crocata* (cyanolichen). These authors observed several snail strategies to
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14 99 overcome potentially toxic metabolites present in lichens.
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17 100 Optimal foraging in generalist species, such as most terrestrial gastropods, requires a balance
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19 101 between the access to useful nutrients and the need to overcome toxic compounds. Therefore,
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21 102 the snail should be able to select lichen parts according to their nutritional quality and to avoid
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23 103 toxic compounds. In this study, we focussed on the cephalolichen *Argopsis friesiana* Müll. Arg.
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25 104 (Stereocaulaceae), a tufty ≤ 4 cm tall species, widespread in low-nutrient, windy fell-fields of
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27 105 Subantarctic islands (Lamb 1974; Galloway 1980). *Argopsis friesiana* is characterized by a rigid
28
29 106 pseudopodetium, cylindric-coralloid phyllocladia, concave-scutelliform apothecia and botryose
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31 107 cephalodia. Its main photobiont is a green alga (*Trebouxia* s. lat.), its secondary autotrophic
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33 108 partner is the cyanobacterium *Stigonema* sp., located in the cephalodia. To understand how this
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35 109 snail could benefit from eating this lichen, we examined the influence of lichen chemistry on snail
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37 110 nutrition. For this purpose, we explored how the metabolites were distributed in the different
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39 111 parts of the lichen *A. friesiana* and how this distribution shaped the snail grazing patterns.
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41 112 Intrathalline sectorization of the symbiotic partners facilitated the separation of *A. friesiana* into
42
43 113 four different parts to be analysed separately. Three hypotheses were tested: The snail (1)
44
45 114 avoids lichen parts with high concentrations of secondary metabolites, (2) prefers lichen parts
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47 115 that are rich in essential nutrients (sugars, amino acids, fatty acids, polysaccharides) and (3)
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49 116 prefers thallus parts rich in nitrogen.
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57 117 58 118 MATERIALS AND METHODS

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119 *Biological Material. Argopsis friesiana* Müll. Arg., endemic to the Subantarctic islands in the
120 Indian Ocean, was collected on Possession Island (Crozet archipelago) at « Le Donjon »
121 (46°25'01"S, 51°50'15"E, 200 m, DON) during the austral summer 2015. The identification of the
122 species was confirmed by one of us (DE) by comparison with type material and the species was
123 sequenced for the first time. A sequence of nuclear ribosomal internal transcribed spacer (ITS)
124 of the material used in this study was deposited in GenBank (MG947382) as a reference for our
125 material because ITS is used as a universal DNA barcode marker for fungi (Schoch et al. 2012).
126 The species resembles the sympatric *Stereocaulon cymosum* Cromb., which differs by its
127 transversely septate ascospores versus muriform ascospores in *A. friesiana*. Voucher
128 specimens were deposited at the herbarium of the Faculty of Pharmacy of Rennes 1,
129 Department of Pharmacognosy and Mycology, under the reference REN000140.

130 To investigate the palatability of *A. friesiana*, snails were collected from sites for which
131 we had an official collection permit. Seventy-five adults (shell size ≥ 5 mm) were collected during
132 2015 austral summer at Mascarin (46°26'10.09"S; 51°45'20.58"E, 600 m - 930 m) (Online
133 Resource 1, Fig. S1). The specimens, found in fell-fields including the lichen habitats, belong to
134 the soft-shell ecophenotype described by Charrier et al. (2013).

135
136 *Lichen Preparation.* *Argopsis friesiana* was cut into four parts as explained in Fig. 1a. The
137 pseudopodetium consists of (i) a central axis, the medulla, made up of compact fungus hyphae
138 (Pf) and (ii) an outer layer with fungi and green algae (Pa). Phyllocladia are the terminal
139 branchlets of pseudopodetia containing algae (Ph). Cyanobacteria live inside numerous black
140 cephalodia (Ce), external gall-like structures emerging laterally from the pseudopodetium.
141 Apothecia, the sexual reproductive parts of the lichen, are scattered on the upper surface of the
142 thallus. The underside of the apothecium is similar to the algal layer of the pseudopodetium. For
143 chemical analyses, algal layer and phyllocladia were combined because of their structural
144 similarity.

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146 *Experimental Design.* Snails were reared in boxes (7.5 x 6 x 4.5 cm) under a thermal cycle, 6°C
147 during night (10h) and 10°C during day (14h). A moist synthetic foam in the bottom (5 mm thick)
148 ensured snail activity. After four days of starvation, the snails (n=75) were divided into 15 groups
149 of five individuals. They were allowed to feed upon *A. friesiana* for two nights (48 h experiment).
150 Each group received a fertile thallus, on which apothecia and cephalodia were counted. Snail
151 consumption was controlled after one night and damage was measured after two nights under a
152 stereomicroscope (Stemi 2000-C, Zeiss, France).

153 Level of grazing (LD) was adapted from Nimis and Skert (2006) and measured as
154 follows: LD0 intact, LD1= low grazing (<25%), LD2=medium damage (>25-50% consumed) and
155 LD3= high damage (>50%). For pseudopodetia, grazing on the algae-containing cortical layer
156 (Pa) was assessed separately from that on the fungus axis (Pf) and the levels of grazing were
157 defined as: LD0 = algal layer and fungal axis of the pseudopodetia remained intact, LD1 =
158 minimum damage (algal layer consumed <25%, fungal axis intact), LD2 = medium damage
159 (algal layer consumed <50%, fungal axis intact), LD3 = high damage (algal layer Pa and fungal
160 axis Pf consumed, > 50%). To assess the damage to cephalodia and apothecia, a grazing ratio
161 was determined as follows: Number of apothecia or cephalodia with grazing marks / Number of
162 apothecia or cephalodia available. To compare grazing on cephalodia and apothecia with
163 damage to algal layer, fungal axis and phyllocladia, the levels described above (LD in %) were
164 utilized.

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4 166 *Sample Preparation for Chemical Analyses.* To determine a sectorial distribution of metabolites,
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6 167 three samples of several *A. friesiana* thalli (about one gram each) were prepared. Each thallus
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8 168 was cut into the four parts apothecia, cephalodia, fungal axis and algal layers. The 12 samples
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10 169 were ground separately with mortar and pestle in liquid nitrogen. To quantify secondary
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12 170 metabolites, freeze-dried samples (40 mg each) were used. Primary metabolites, including
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14 171 amino acids, free sugars and polyols, were analysed with 10 mg of each thallus part, while 50
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16 172 mg of each part was needed for quantification of fatty acid and sugar-forming polysaccharides
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18 173 and for determination of nitrogen content.
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24 175 *Secondary Metabolite Profiling.* Each part of the air-dried lichen thalli was extracted with 100%
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26 176 acetone (3 x 500 μ L). Acetone extracts were solubilized in tetrahydrofuran (THF), filtered and
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28 177 injected (10 μ L at 0.5 mg.mL⁻¹) in a Liquid Chromatography - Diode Array Detector (LC-DAD)
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30 178 (Shimadzu®, Marne La Vallée, France, LC-20AD SP, injector SIL-20A HI, column oven CTO-
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32 179 20A, diode array detector SPD-M20A) and a mass spectrometer (MS) (Advion® expression
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34 180 CMS, Ithaca, USA). Ionization was performed by negative-ion mode electrospray (ESI-). A
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36 181 gradient system was applied: A (0.1% formic acid in water) and B (0.1% formic acid in
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38 182 acetonitrile) for 48 min on a C₁₈ column (Phenomenex®, Kinetex 2.6 μ C₁₈ 100A, temperature
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40 183 40°C) at a flow rate of 0.5 mL.min⁻¹: initial, 20% B; T 0-5 min, 20% B linear; T 30 min 80% B; T
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42 184 35-42 min, 100% B linear; T 45-48 min, 20% B linear. Linear equations based on the calibration
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44 185 curve were used to quantify atranorin and the results were expressed in mg.g⁻¹ Dry Mass (DM).
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46 186 Atranorin (JB/A/142) used for calibration belong to the library of single lichenic compounds of
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48 187 UMR 6226, being isolated during previous phytochemical investigations. For the other
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50 188 metabolites, also detected by UV on HPLC chromatograms, a relative quantification was
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52 189 performed, by multiplying the area under the curve (AUC) and the extraction yield of the lichen
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54 190 extract. Full scan mass spectra were recorded in negative-ion mode in a mass range of 100 to
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56 191 1200 Da, applying the following parameters: detector gain 1200, ESI voltage 3.5 kV, capillary
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4 192 voltage 180 V, source voltage 20 V, source voltage dynamic 20 V, nebulizer gas pressure 60
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6 193 psig, desolvation flow gas rate 4 L.min⁻¹, capillary temperature 250°C and gas temperature
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8 194 20°C. Data processing and evaluation for MS measurement were performed with the Data and
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10 195 Mass Express 2.2.29.2 software (Advion). As a complement to LC-DAD-MS experiments, in situ
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12 196 analyses of each part of the lichen were made using the ambient air source DART-HRMS,
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14 197 enabling the chemical profiling of unprocessed pieces of thallus (Le Pogam et al. 2015, 2016);
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16 198 more detailed protocol in Online Resource 2). The identification of metabolites was done against
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18 199 compounds formerly identified within the genus *Argopsis* and the family Stereocaulaceae (Bodo
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20 200 and Molho 1974; Lamb 1974; Galloway 1980).

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24 202 *Primary Metabolite Profiling.* Soluble carbohydrates, polyols and amino acids were extracted
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26 203 from each part of the lichen, following the methodology of Gravot et al. (2010). Twenty mM of
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28 204 adonitol (soluble carbohydrates and polyols) and 10mM of 3-aminobutyric acid (BABA) (amino
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30 205 acids) were used as internal standards. Soluble carbohydrates and polyols were profiled by a
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32 206 Gas Chromatography - Flame Ionization Detector (GC-FID) (Thermo-Fisher Scientific, Waltham,
33
34 207 CA, USA) according to Adams et al. (1999) and Lugan et al. (2009). Amino acids were detected
35
36 208 with an Ultra High Performance Liquid Chromatography – Diode Array Detector (UPLC-DAD,
37
38 209 Waters), by using an AccQ-Tag Ultra derivatization kit (Waters Corporation, Milford, MA, USA)
39
40 210 following the protocol described in Gravot et al. (2010). The metabolites were identified from
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42 211 their retention time by comparison with external standards. Concentrations (mg.g⁻¹ DM) were
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44 212 obtained on the basis of internal standards.

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48 214 *Fatty Acids Profiling.* Samples were extracted three times in a parallel synthesis apparatus
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50 215 (Heidolph Parallel Synthesis Apparatus®-Instruction Synthesis 1, France), for 1 h each, with
51
52 216 chloroform/methanol (2:1, v/v, 6 mL) at 80°C, under constant shaking at 700 rpm (Vu et al.
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54 217 2016). To quantify fatty acids (FAs), 100 µg of heneicosanoic acid (C21:0) were added as an

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4 218 internal standard. The organic phase containing the total lipids was recovered and evaporated to
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6 219 dryness under a nitrogen flow. Sample derivatization and GC-FID/GC-MS (Agilent 6890N,
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9 220 Toulouse, France) analyses were conducted using the protocol of Vu et al. (2016). Identification
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11 221 of fatty acid methyl esters (FAMES) was based on the retention times. The quantity of total FAs
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13 222 (in mg.g⁻¹ DM) was calculated using the internal standard.
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17 224 *Extraction of Sugar-Forming Polysaccharides.* Approximately 50 mg of each dried lichen tissue
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19 225 were reduced to a fine powder with a pestle and transferred in 22 mL cells of an accelerated
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22 226 solvent extraction unit ASE® 350 (ASE™ 350, Thermo Scientific™ Dionex™), dried at 40°C
23
24 227 overnight under vacuum over P₂O₅, prior to weighing. Alcohol-insoluble residues (AIRs) were
25
26 228 obtained by using 80% ethanol at 2 mL.min⁻¹ flow. The conditions for the ASE extraction were
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29 229 set at 100°C for 20 min, followed by rinse volume of 150%, and a purge time (N₂) of 30 s. Cells
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31 230 of the ASE® 350 containing AIRs were dried at 40°C overnight under vacuum over P₂O₅, prior to
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33 231 weighing, in order to determine the extraction yield.
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35 232 Identification and quantification of polysaccharide neutral sugars were performed by GC
36
37 233 after sulfuric acid degradation (Hoebler et al. 1989). Five mg of AIR were dispersed in 13 M
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40 234 sulfuric acid for 30 min at 30°C, then hydrolysed in 1 M sulfuric acid (2 h, 100°C). Sugars were
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42 235 converted to alditol acetates according to Blakeney et al. (1983) and chromatographed on a TG-
43
44 236 225 GC Column (30 x 0.32 mm ID) using TRACE™ Ultra Gas Chromatograph (Thermo
45
46 237 Scientific™; Waltham, CA, USA; temperature 205°C, carrier gas H₂). Inositol was used as
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48
49 238 internal standard. The quantity of each sugar (in mg.g⁻¹ DM) was determined, using the
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51 239 calibration curves drawn from standard solutions.
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55 241 *Total Nitrogen (TN) Measurement.* TN (in % of DM) was determined with an automated dry
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57 242 combustion method (Dumas method ; Rhee 2001) using an elemental analyser (vario MICRO
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60 243 cube, Elementar Analysensysteme, Hanau, Germany).
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245 *Statistical Analyses.* A Fisher's exact test was applied to LD values obtained for damage to algal
246 layer and fungal axis of the pseudopodetia, phyllocladia, cephalodia and apothecia, in order to
247 compare the observed frequencies among lichen parts. In the text, mean \pm standard deviation is
248 given. A Spearman correlation was used to compare LD values with the number of cephalodia or
249 apothecia carried out by the thallus.

250 To analyse the metabolic data, we first proceeded to fourth-root transformation of the
251 values, centring and autoscaling, because null values and great differences in concentrations
252 according to metabolite profiles were observed (van den Berg et al. 2006). Powered Partial
253 Least Squares – Discriminant Analysis (PPLS-DA) was performed on the normalized values to
254 discriminate the four parts of *A. friesiana* on the basis of their metabolic profiles (function `cppls()`,
255 package "pls" (Indahl et al. 2009)). The significance of the discrimination was tested using a
256 cross-model validation (functions `MVA.cmv()` and `MVA.test()`), developed in the package
257 "RVaidememoire" (Hervé 2016).

258 All statistical analyses were made using R software V. 3.4.0 (R Core Team 2017).

260 RESULTS

261 *Snail Feeding Preferences.* Snails from Mascarin site had shell sizes of 4.9 ± 0.5 mm (mean
262 \pm s.d.). Shell size did not differ among groups ($n = 15 \times 5$ individuals per group; ANOVA, $F_{14,60} =$
263 0.415 , $P = 0.964$).

264 Apothecia were completely avoided by *N. hookeri* (Fig. 1a-2). Similarly, the fungal axis
265 was not consumed, while the cortical algal layer and phyllocladia were grazed (Fig. 1a-2). The
266 levels of damage (LD) on cortical algal layer and phyllocladia ranged from $< 25\%$ to $< 50\%$ and
267 were similar (Fisher's exact test, $P=0.710$) (Fig. 2). Cephalodia were the preferred part, more
268 than 40% of them being highly damaged (= LD3) and significant grazing differences being
269 observed between cyanobacterial (cephalodia) and green algal parts (cortical algal layer and

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270 phyllocladia) of the lichen (Fisher's exact tests, $P \leq 0.001$) (Fig. 1b-2, Fig. 2). The mean (\pm s.e.)
271 number of cephalodia grazed reached 4.9 ± 0.5 in 48 hours and was dependent on the total
272 number of cephalodia per thallus (Spearman's rank correlation, $\rho = 0.59$, $P = 0.021$).

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274 *Metabolite Profiling of A. friesiana.* The chemical profiles in secondary metabolites of each part
275 of the lichen *A. friesiana* were established by LC-DAD-ESI-MS from their acetone extracts.
276 Three main compounds were identified: the depside atranorin (m/z 373) and two chlorinated
277 compounds (Table 1, Fig. S2). These two chlorodepsidones were identified as the dichlorinated
278 argopsin (m/z 395) and as the trichlorinated caloploicin (m/z 401) through isolation and
279 spectroscopic data analysis (Purification protocol and NMR data are given in Online Resource 2,
280 Fig. S3-S4, Table S1-S2). *In situ* DART-HRMS analysis confirmed identification of chlorinated
281 compounds detected by LC-DAD-MS from unprocessed pieces of lichens through exact mass
282 measurements (Online Resource 2, Table S3). Algal layers had the highest concentration of
283 secondary metabolites, particularly for atranorin, reaching 10.6 ± 2.3 mg.g⁻¹ DM. In comparison,
284 atranorin varied from 0.5 ± 0.2 mg.g⁻¹ DM in cephalodia to 1.1 ± 0.6 mg.g⁻¹ DM in apothecia. In
285 pseudopodetia, the medullar caloploicin with argopsin accounted for about two-thirds of the
286 secondary metabolites, while atranorin alone approximately reached this proportion in apothecia
287 and in green algae-containing tissues (Table S4).

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289 Among all detectable metabolites, 36 were found in apothecia, 33 in the medullar axis of
290 pseudopodetia, 41 in phyllocladia and algal layer of pseudopodetia and 42 in cephalodia (Table
291 1). The multivariate analysis (PPLS-DA) showed partitioning in the metabolite profiles among
292 parts of the lichen *A. friesiana* (Fig. 3a, Fig. 3b, contributions to PC1 axis, Online Resource 3
293 Fig. S5). The cross model validation used for the discrimination of the lichen parts (8
294 components, 999 permutations, NMC = 0.042; $P = 0.001$) indicated statistically different
295 chemical profiles. The PC2 axis distinguished the presence or the absence of photobionts while

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the biont identity was discriminated on the first axis (PC1). Green algal layers were characterized by proline and GABA, and by the secondary metabolites atranorin, argopsin and caloploicin. On the opposite side of the first axis, discrimination of the cephalodia was mainly due to trehalose, FA_{unsat} (n-7) family, galactose, mannose and rhamnose from polysaccharides, amino acids such as lysine and arginine, and by high nitrogen concentration (TN). The position of cephalodia was negatively correlated with the abundance in glucose issued from the degradation of polysaccharides. On the second axis (contribution to PC2 axis, Online Resource Fig. S6), the position of apothecia was negatively associated with the occurrence of amino acids and polyols (especially arabitol) and a positive but limited association with some FA (FA_{sat} and FA_{unsat} (n-9) families) was observed. The position of the fungal axis of pseudopodetia on the third axis (PC3) was positively correlated with the presence of fatty acids (FA_{sat}, C18:2 n-6, C16:1 n-9), mannitol) and α -alanine (Fig. 3c, Fig. 3d, contributions to PC3 axis, Online Resource 3 Fig. S7).

DISCUSSION

Among the three major secondary metabolites detected in *Argopsis friesiana*, the depside atranorin and the chlorodepsidone argopsin have already been reported in this species (Huneck and Lamb 1975). Yosioka et al. (1973) revealed the chlorodepsidone caloploicin in the genus *Caloplaca*, but here, for the first time, caloploicin has been isolated from the genus *Argopsis* with the complete NMR assignment for this compound (Online resource 2).

When consuming *A. friesiana*, *N. hookeri* at first meets external layers high in secondary metabolites. Despite the abundance of atranorin in the cortex, it had no deterrent effect on snail grazing. In phytophagous arthropods, atranorin was reported to elicit a significant avoidance (Nimis and Skert 2006) or to reduce growth of insect larvae (Slansky 1979; Pöykkö et al. 2005) but no protective effect of atranorin was noticed in snails (Hesbacher et al. 1995; Gauslaa 2005).

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322 In the soft bodies of various snail species, Hesbacher et al. (1995) detected a compound
323 presumably originating from hydrolysis of atranorin and suggested its sequestration in snail
324 tissues. While the retention of lichen compounds in bodies of snails was not analysed as part of
325 the current study, this hypothesis is consistent with the previously reported ability of *N. hookeri*
326 to hydrolyse ester bonds of the tridepside tenuiorin, due to the alkalinity of its gut lumen (Gadea
327 et al. 2017). According to Lawrey (2009), cortical compounds (i.e. atranorin in *A. friesiana*) are
328 assigned a protective role against high irradiance, whereas a number of medullary secondary
329 metabolites deter lichenivores. As caloploicin and argopsin are depsidones, they were expected
330 to occur predominantly in the medulla. In *A. friesiana*, much higher concentrations of these
331 compounds were found in the external green algal layers, but expressed in percentages they
332 represented less than 25% of the detected secondary metabolites in this part. By contrast,
333 caloploicin and argopsin reached 70% of the secondary metabolites found in the central axis of
334 the pseudopodetia. The medulla of *A. friesiana* remained intact after snail feeding, consistent
335 with a deterrent role of these compounds. This is in agreement with Asplund (2011), who
336 demonstrated that snails avoid the medulla of *Lobaria* species due to the presence of
337 depsidones.

338 According to the Optimal Defence Theory (McKey 1974; Asplund et al. 2010b),
339 reproductive tissues should be well defended. However, our results did not support the theory. In
340 *A. friesiana*, apothecia and cephalodia had similar concentrations of medullary secondary
341 metabolites. Unlike cephalodia, apothecia were avoided by *N. hookeri*, suggesting that (i) other
342 undetected toxic compounds deter snail grazing, or (ii) their avoidance may be due to low
343 nutrient content, or (iii) the roughness of apothecia may present a physical barrier to radula
344 motion. Therefore, the food choices made by *N. hookeri* cannot be explained simply by the
345 avoidance of secondary metabolites, supporting the conclusion of Lawrey (1983). Other factors
346 or compounds such as essential nutrients for snail growth and reproduction might be involved.

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4 347 Cephalodia and algal layers were selected by *N. hookeri* when given a choice among the
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6 348 four parts of *A. friesiana*. These lichen parts were characterized by their richness in some
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8 349 nutrients: (i) polyols and free sugars, among which trehalose was encountered in cephalodia
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10 350 only, (ii) amino acids, among which proline was exclusive of algal layers. Avoidance of apothecia
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12 351 by the snail might be linked to their low content of phagostimulant compounds. However, we
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14 352 cannot explain why the snails did not consume the fungal axis of pseudopodetia. Although rich in
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16 353 arabitol, the fungal axis was characterized by a lack of diversity in nutrients compared to that
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18 354 found in apothecia. Moreover, the central axis made of compact hyphae, as well as apothecia,
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20 355 may be resistant to grazing. Indeed, the radular teeth of *N. hookeri* are smooth and more or less
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22 356 bulging or flat, features of non-specialized radulae potentially sensitive to rough (apothecia) and
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24 357 fibrous (fungal axis) textures (Fig. S8). By contrast, the soft and gelatinous structure of the
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26 358 cephalodia could promote grazing. This observation is in line with the study of Baur et al. (2000),
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28 359 who compared the ultrastructure of snail radulae and concluded that hook-shaped teeth, such as
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30 360 those of *Chondrina clienta*, are necessary to scrape efficiently on crustose lichens.

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33 361 We demonstrated the high palatability of cephalodia and algal layers for *N. hookeri* and
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35 362 that the more numerous the cephalodia, the more consumed they were. Various factors could
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37 363 explain these feeding choices. Due to their low levels of defences, cephalodia may be preferred
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39 364 by snails (Renner 1982; Asplund and Gauslaa 2010). Second, the snails could acquire a
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41 365 balanced intake of essential nutrients by consuming several parts of the lichen with intrathalline
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43 366 chemical specificities. If so, the snail would have to perceive nutrient quality even in the case of
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45 367 sub-lethal toxicity particularly due to the occurrence of argopsin and caloploicin in *A. friesiana*.
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47 368 An ability to select foods that contain essential organic or mineral nutrients despite ingestion of
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49 369 secondary metabolites was evidenced in slugs by Cook et al. (2000) and in snails by Chevalier
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51 370 et al. (2003).

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53 371 The glucose residues in polysaccharides, although they were half as abundant in
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55 372 cephalodia as in other parts, they suggested the presence of glucans, probably isolichenan (α -
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373 glucan), as found for *Stereocaulon ramulosum* (Baron et al. 1991). The abundance of galactose
374 and mannose units in the cephalodia could reflect the synthesis of galactomannan, a hypothesis
375 supported by the description of these heteropolymers in *Stereocaulon paschale* (Baron et al.
376 1989). Because lichenan, mannan and galactomannan degrading-enzymes occur in land snails
377 (Flari et al. 1995; Charrier and Rouland 2001), *N. hookeri* can probably exploit this source of
378 glycosidic residues and thereby meet its energy needs. Indeed, gastropod metabolism is
379 considered to be polysaccharide-orientated as energy is mainly provided by glycogen storage
380 (Nicolai et al. 2012). Sugars and amino acids are also needed to synthesize mucus
381 proteoglycans for snail locomotion, adhesion, body hydration and chemical communication (Ng
382 et al. 2013). Because mucus production requires around 20% of ingested energy (Denny 1980),
383 the consumption of food rich in potential mucin-like compounds would be beneficial to snail
384 survival.

385 Two fatty acids (C16:1 n-7 and C18:1 n-7) discriminated cephalodia. FA_{unsat} (n-7) family
386 is specific to cyanobacteria (Rezanka and Dembitsky 1999; Vu et al. 2016), raising the question
387 of whether the snail feeding preference for cyanobacteria is governed by phagostimulating
388 properties of this FAs family. Lipids are mainly in cellular membranes, phospholipids and sterols
389 representing over 80% of the total lipids (Van der Horst and Zandee, 1973). In *A. friesiana*,
390 linoleic acid (C18:2 n-6) is by far the most available FA_{unsat} for *N. hookeri*. Yet, Arakelova et al.
391 (2009) reported more than 10% of linoleic acid in triglycerides in the digestive gland of
392 phytophagous pond snails. These authors showed that linoleic acid and linolenic acid (C18:3 n-
393 3), were provided by the algal food and acted as precursors of long-chain C22 FAs that play a
394 role in the activity of the pedal muscle.

395 Cephalodia were also characterized by their richness in N, suggesting that the grazing
396 preferences of *N. hookeri* were not reduced by nitrogen abundance. Asplund and Gauslaa
397 (2010) showed that gastropods also preferred cephalodia when feeding on the foliose lichen
398 *Nephroma arcticum*. By contrast, Asplund et al. (2010a) demonstrated species-specific reduction

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399 in lichen palatability following artificial N deposition, and Asplund and Wardle (2013) found that
400 N-enriched lichens were less palatable. However, these authors showed that the effect on
401 consumption rate depended on an interaction between nitrogen-fixing ability and growth form.
402 The cephalodiate and fruticose *A. friesiana* could be included in the group of fruticose N-fixing
403 species described by Asplund and Wardle (2013), which are consumed more than non-N-fixing
404 fruticose species. Hence, our results do not contradict those of Asplund and Wardle (2013).
405 Because the snail tissues contain 10% nitrogen (Speiser 2001), we hypothesize that *N. hookeri*
406 should benefit from the higher N concentrations (1.6%) in cephalodia than in other lichen
407 tissues.

408 Studies on lichen-gastropod interactions have often examined only the repellent role of
409 secondary metabolites, but we showed here that the contents in nitrogen and primary
410 metabolites related compounds (such as amino acids, sugars and polyols) should not be
411 underestimated. Moreover, physical criteria such as the hardness of lichens thalli could be able
412 to modulate the chemical defence strategy of the snail. Additional study should focus on fatty
413 acids of the snail's tissues to investigate the role of the lichen food in provisioning lipid
414 components.

415
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417
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47 599 FIGURES

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51 601 **Fig. 1** *Argopsis friesiana* (Stereocaulaceae) entire thallus (a) and cephalodia (b) before and after
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53 602 snail consumption. Phyllocladia (Ph), algal layer of pseudopodetia (Pa) and cephalodia (Ce)
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55 603 were consumed while apothecia (Ap) and fungal axis of pseudopodetia (Pf) remained
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57 604 untouched. The specimens were dry before consumption (a-1 and b-1) and wet after snail
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4 605 feeding (a-2 and b-2). The black line shows the transversal section of a pseudopodetium to
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6 606 discriminate between the cortical layer (Pa) and the fungal axis (Pf).

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10 608 **Fig. 2** Differential grazing and level of damage (LD) to *Argopsis friesiana* by the snail *Notodiscus*
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13 609 *hookeri*. N = 15 lichen thalli given to snails. Pa = algae-containing layer of pseudopodetia, Ph =
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15 610 phyllocladia, Ce = cephalodia; apothecia and medullar part of pseudopodetia that remained
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17 611 untouched are not shown. The level damage classes are LD1 = minimum damage (>0% and
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19 612 <25%), LD2 = medium damage (25%-50%) and LD3 = high damage (>50%).

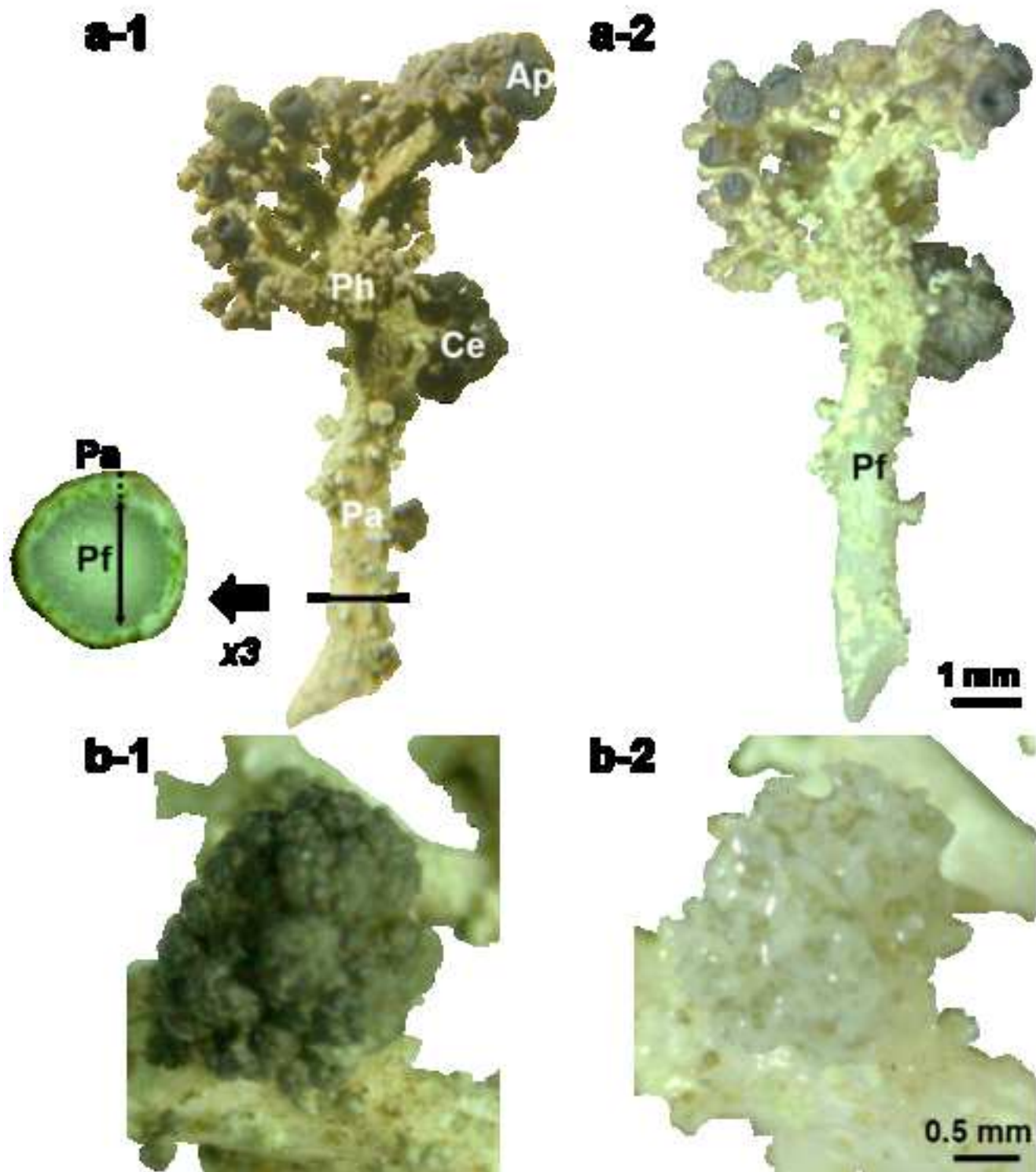
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21 613
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24 614 **Fig. 3** Graphs of the Powered Partial Least Squares - Discriminant Analysis (PPLS-DA)
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26 615 performed on the chemical composition of the four lichen parts of *Argopsis friesiana*. **a.** Score
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28 616 plot 1-2: Ap = apothecia, Ce = cephalodia; Ph + Pa = phyllocladia + cortical algal layer, Pf =
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30 617 fungal layer of pseudopodetia. **b.** Corresponding loading plots of metabolites, including
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32 618 secondary metabolites, amino acids, free sugars and polyols, sugar-forming polysaccharides,
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34 619 saturated and unsaturated fatty acids. Saturated fatty acids (FA_{sat}) included C14:0, C15:0,
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36 620 C16:0, isoC17:0, C18:0, C20:0, C22:0, C23:0, C24:0. Abbreviations used are given in Table 1.
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38 621 For clarity, in the presence of collinearity ($\geq 95\%$), only one of the two correlated variables was
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40 622 considered (Table S3). **c.** Score plot 2-3 and **d.** corresponding loading plots.

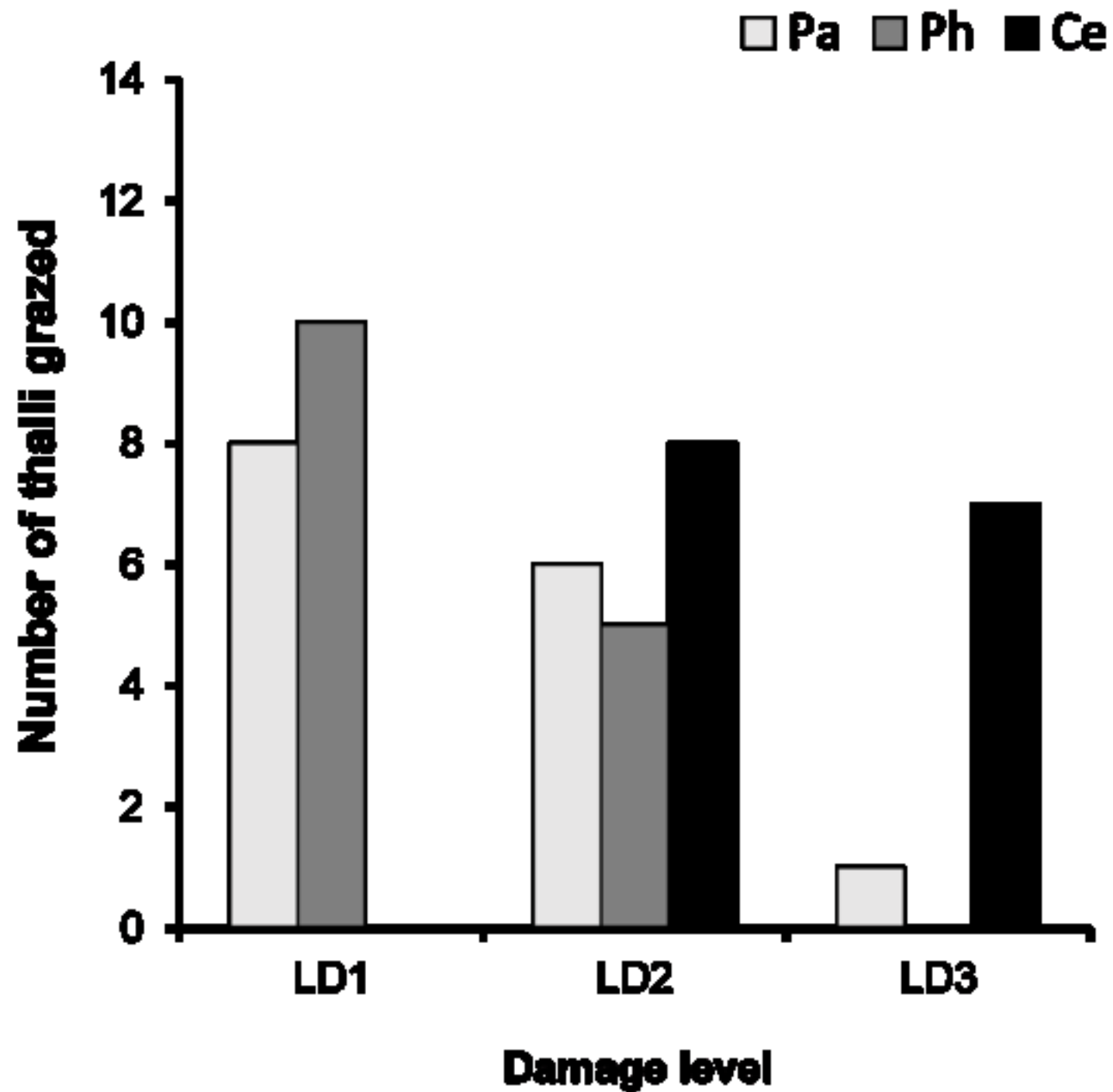
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44 624 SUPPLEMENTARY DATA

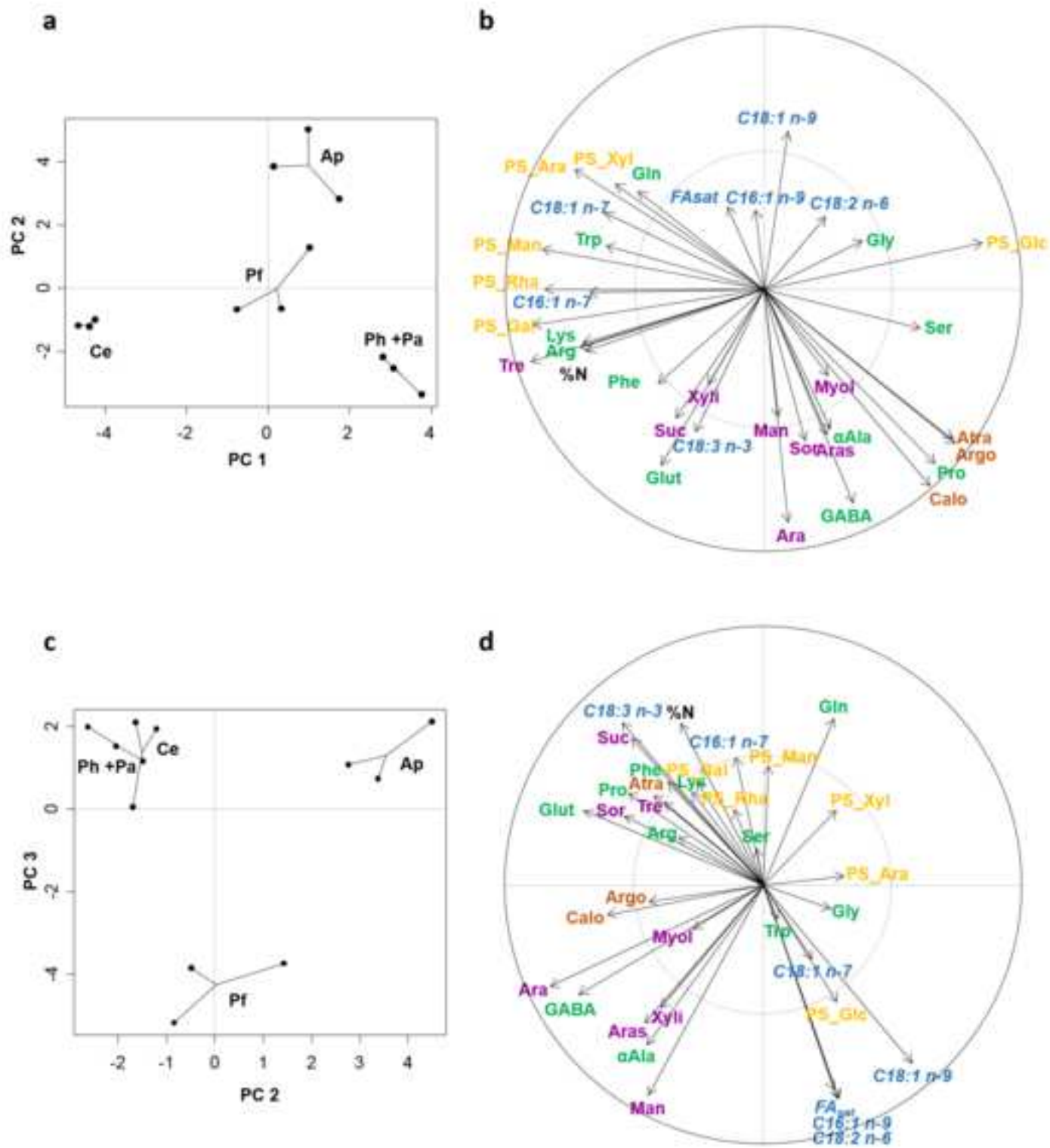
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49 626 The following supplementary data are related to this article: see Supplementary data file
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52 627 Online resource 1. Fig. S1: Study sites on Possession Island, which belongs to Crozet
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54 628 Archipelago (45° 30' – 46° 30' S; 50° 00' – 52° 30' E) in the Subantarctic region.

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56 629 Online Resource 2: Chemical and spectroscopic data. Fig. S2: Three main secondary
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58 630 metabolites of *Argopsis friesiana*: the chlorodepsidones argopsin (1) and caloploicin (2) and the
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4 631 depside atranorine (3). Fig. S3: NMR spectra (^1H , 300 MHz and ^{13}C , 75 MHz) of argopsin in
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6 632 CDCl_3 and argopsin formula, Table S1: ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) data for
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8 633 argopsin. Data recorded in CDCl_3 , δ in ppm; Fig. S4: NMR spectra (^1H , 500 MHz and ^{13}C , 125
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10 634 MHz) of caloploicin in acetone (d_6 -acetone) and caloploicin formula, Table S2: ^1H NMR (500
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12 635 MHz, cryo) and ^{13}C NMR (125 MHz) data for caloploicin. Data recorded in acetone- d_6 , δ in ppm,
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15 636 Table S3: Results of exact mass measurements in negative-ion mode DART-HRMS of the
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17 637 molecules retrieved in *Argopsis friesiana*, Table S4: Each secondary metabolite in the four lichen
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19 638 parts, calculated as a percentage of all the compounds detected in this chemical group.
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22 639 Online Resource 3: Multivariate analysis supplementary data. Fig. S5: Projection to the axis PC1
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24 640 of the metabolites analysed by PPLS-DA. Fig. S6: Projection to the axis PC2 of the metabolites
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26 641 analysed by PPLS-DA. ; Fig. S7: Projection to the axis PC3 of the metabolites analysed by
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28 642 PPLS-DA. Table S5: Correlated compounds (> 95%) in the PPLS-DA analysis from the data set
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30 643 of the compounds identified in the lichen *A. friesiana*.
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33 644 Online Resource 4: Fig. S8: Scanning Electron Microscopy of the radula of *Notodiscus hookeri*,
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35 645 extracted from the buccal mass.
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Figures

INTRATHALLINE METABOLITE PROFILES IN THE LICHEN *Argopsis friesiana* SHAPE GASTROPOD GRAZING PATTERNS.

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Solenn FERRON¹, Daniel CATHELIN⁶, Damien ERTZ⁷, Pierre LE POGAM⁸, Joël
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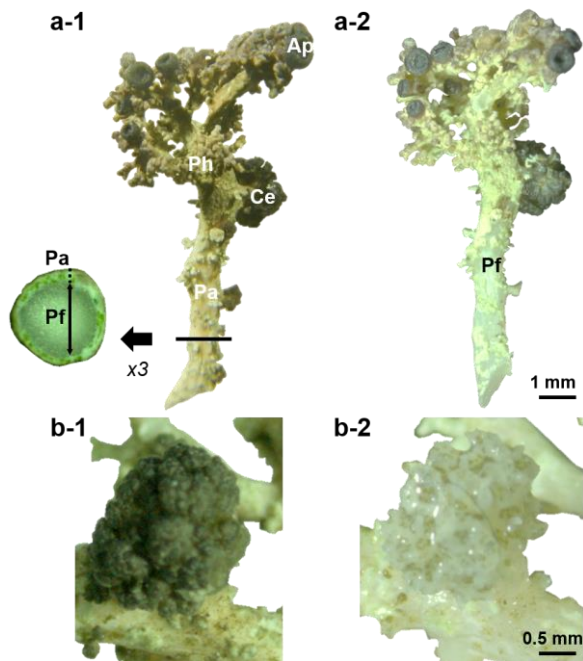


Fig. 1 *Argopsis friesiana* (Stereocaulaceae) entire thallus (a) and cephalodia (b) before and after snail consumption. Phyllocladia (Ph), algal layer of pseudopodetia (Pa) and cephalodia (Ce) were consumed while apothecia (Ap) and fungal axis of pseudopodetia (Pf) remained untouched. The specimens were dry before consumption (a-1 and b-1) and wet after snail feeding (a-2 and b-2). The black line shows the transversal section of a pseudopodetium to discriminate between the cortical algal layer (Pa) and the fungal axis (Pf).

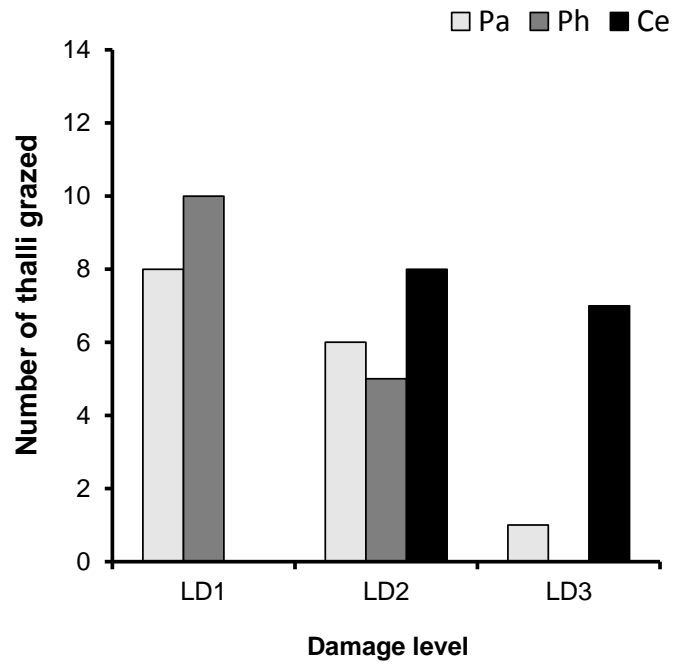


Fig. 2 Differential grazing and level of damage (LD) to *Argopsis friesiana* by the snail *Notodiscus hookeri*. N = 15 lichen thalli given to snails. Pa = algae-containing layer of pseudopodetia, Ph = phyllocladia, Ce = cephalodia; apothecia and medullar part of pseudopodetia that remained untouched are not shown. The level damage classes are LD1 = minimum damage (>0% and <25%), LD2 = medium damage (25%-50%) and LD3 = high damage (>50%).

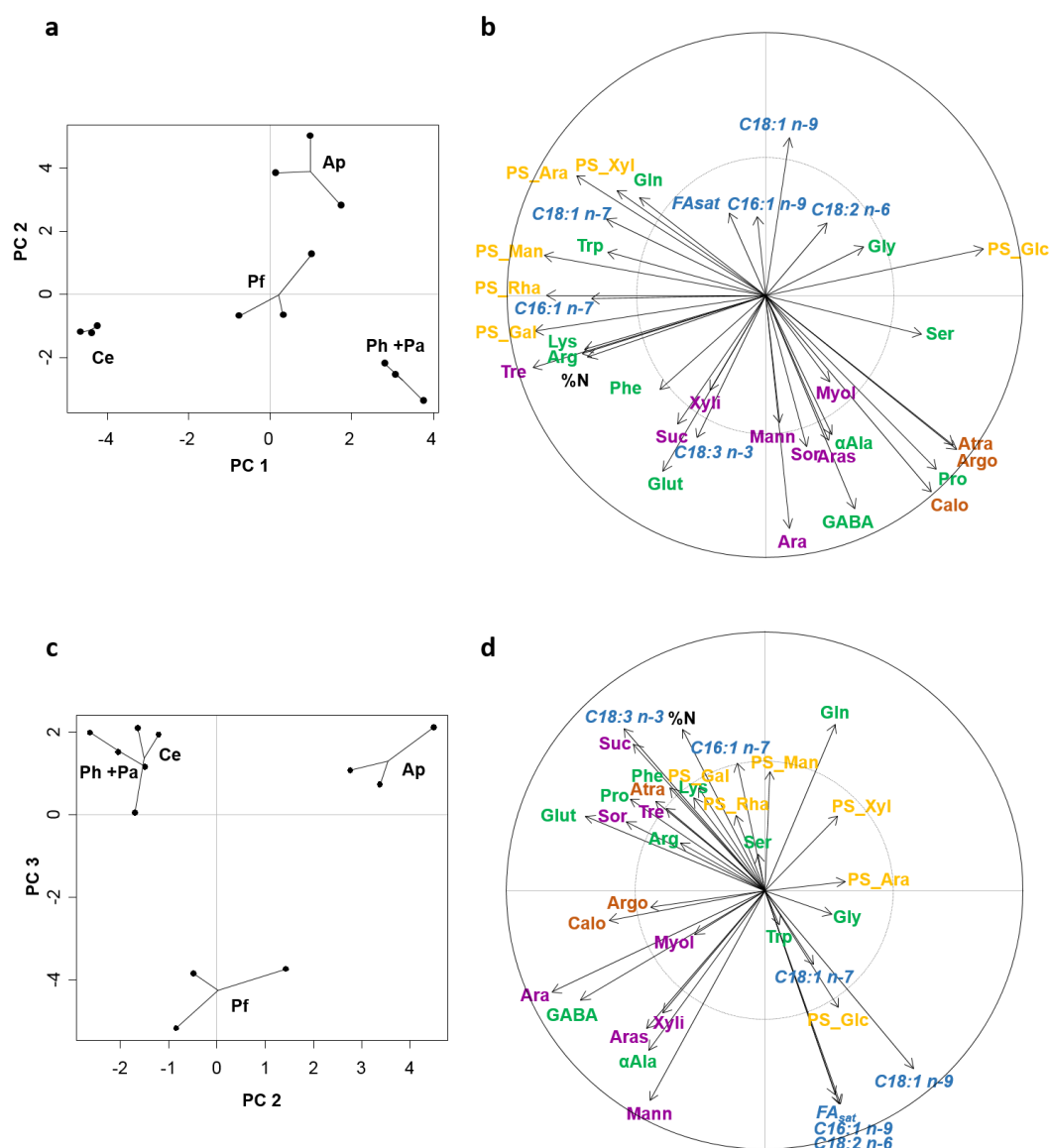


Fig. 3 Graphs of the Powered Partial Least Squares - Discriminant Analysis (PPLS-DA) performed on the chemical composition of the four lichen parts of *Argopsis friesiana*. **a.** Score plot 1-2: Ap = apothecia, Ce = cephalodia; Ph + Pa = phyllocladia + cortical algal layer, Pf = fungal layer of pseudopodetia. **b.** Corresponding loading plots of metabolites, including secondary metabolites, amino acids, free sugars and polyols, sugar-forming polysaccharides, saturated and unsaturated fatty acids. Saturated fatty acids (FA_{sat}) included C14:0, C15:0, C16:0, isoC17:0, C18:0, C20:0, C22:0, C23:0, C24:0. Abbreviations used are given in Table 1. For clarity, in the presence of collinearity ($\geq 95\%$), only one of the two correlated variables was considered (Table S3). **c.** Score plot 2-3 and **d.** corresponding loading plots.

Table 1 Data set of the compounds identified in the four parts of the lichen *Argopsis friesiana* (Ap= apothecia, Ce= cephalodia, Ph= phyllocladia, Pa= algal layer of pseudopodetia and Pf= fungal axis of pseudopodetia): secondary metabolites, free sugars and polyols, amino acids, fatty acids and sugar forming polysaccharides. Values are means (n=3 replicates) in mg.g⁻¹ dry mass with (*minimum - maximum*) in brackets, except the secondary metabolites, which are expressed in Area Under the Curve (samples: 0.5 mg.ml⁻¹) *extraction yield (x10⁵). Total polysaccharides (Total PS) and total nitrogen (TN) are expressed in percentage of DM. The sign ‘-’ means that the metabolite was not detected.

Secondary Metabolites (Area x 10⁵):	Ap	Pf	Ph + Pa	Ce
Atranorin (Atra)	3.20 (1.23 - 6.25)	1.87 (1.16 - 2.54)	41.50 (32.12 - 52.18)	1.31 (1.08 - 1.47)
Argopsin (Argo)	1.14 (0.26 - 2.41)	2.46 (0.57 - 3.69)	7.23(5.60 - 8.75)	0.40 (0.35 - 0.42)
Caloploicin (Calo)	0.54 (0.21 - 1.03)	2.57 (0.86 - 4.82)	6.38 (5.47 - 7.20)	0.55 (0.47 - 0.66)
Free Sugars/Polyols:	Ap	Pf	Ph + Pa	Ce
Arabinose (Aras)	0.31 (0 - 0.58)	5.69 (1.52 - 12.19)	2.81 (1.38 - 4.99)	0.59 (0.48 - 0.71)
Arabitol (Ara)	33.41 (25.82 - 39.24)	89.70 (58.60 - 133.29)	91.86 (87.13 - 100.73)	178.02 (57.6 - 399.02)
Mannitol (Man)	1.08 (1.03 - 1.13)	3.50 (2.63 - 4.76)	1.97 (1.66 - 2.13)	11.13 (1.69 - 29.99)
Myo-inositol (Myo)	0.02 (0 - 0.05)	0.07 (0 - 0.13)	0.10 (0 - 0.17)	0.11 (0 - 0.33)
Sucrose (Sac)	0.04 (0 - 0.13)	-	0.13 (0 - 0.20)	0.28 (0.24 - 0.32)
Sorbitol (Sor)	-	-	0.16 (0 - 0.25)	0.09 (0 - 0.28)
Trehalose (Tre)	-	-	-	0.64 (0.51 - 0.82)
Xylitol (Xyl)	-	0.19 (0 - 0.35)	0.07 (0 - 0.22)	0.41 (0 - 1.24)
Free Amino Acids:	Ap	Pf	Ph + Pa	Ce
α-Alanine (αAla)	0.08 (0.04 - 0.13)	0.19 (0.10 - 0.29)	0.13 (0.12 - 0.15)	0.09 (0.08 - 0.09)
Arginine (Arg)	-	-	-	0.11 (0 - 0.19)
GABA	0.20 (0.17 - 0.25)	0.40 (0.38 - 0.44)	0.44 (0.40 - 0.48)	0.27 (0.23 - 0.30)
Glutamine (Gln)	0.12 (0.07 - 0.16)	-	0.03 (0 - 0.10)	0.16 (0.16 - 0.17)
Glutamate (Glut)	0.14 (0.11 - 0.15)	0.18 (0.06 - 0.30)	0.25 (0.14 - 0.33)	0.34 (0.31 - 0.37)
Glycine (Gly)	0.04 (0 - 0.11)	0.02 (0 - 0.05)	0.02 (0 - 0.05)	-
Lysine (Lys)	-	-	-	0.02 (0 - 0.04)
Phenylalanine (Phe)	-	-	0.05 (0 - 0.16)	0.10 (0 - 0.17)
Proline (Pro)	-	-	0.05 (0.04 - 0.05)	-
Serine (Ser)	0.08 (0 - 0.25)	0.01 (0 - 0.04)	0.15 (0.04 - 0.37)	-
Tryptophan (Trp)	0.10 (0 - 0.17)	0.11 (0 - 0.17)	0.03 (0 - 0.08)	0.15 (0.08 - 0.26)
Fatty Acids:	Ap	Pf	Ph + Pa	Ce
C14:0	0.01 (0.01 - 0.02)	0.03 (0.02 - 0.03)	0.03 (0.02 - 0.04)	0.05 (0.02 - 0.08)
C15:0	0.01 (0 - 0.01)	0.02 (0.02 - 0.02)	0.02 (0.01 - 0.03)	0.02 (0.01 - 0.04)
C16:0	0.14 (0.09 - 0.23)	0.34 (0.32 - 0.36)	0.43 (0.33 - 0.53)	0.46 (0.26 - 0.84)
C17:0	0.01 (0 - 0.01)	0.02 (0.01 - 0.02)	0.02 (0.01 - 0.04)	0.02 (0.01 - 0.05)
C18:0	0.09 (0.06 - 0.14)	0.25 (0.24 - 0.26)	0.29 (0.23 - 0.32)	0.24 (0.14 - 0.40)

C20:0	0.01 (0.01 - 0.02)	0.03 (0.02 - 0.03)	0.03 (0.02 - 0.04)	0.03 (0.01 - 0.05)
C22:0	0.02 (0.01 - 0.05)	0.05 (0.03 - 0.06)	0.07 (0.04 - 0.09)	0.05 (0.03 - 0.09)
C23:0	0.01 (0 - 0.01)	0.02 (0.01 - 0.02)	0.02 (0 - 0.03)	0.01 (0 - 0.03)
C24:0	0.01 (0 - 0.02)	0.03 (0.02 - 0.04)	0.05 (0.04 - 0.06)	0.03 (0.01 - 0.05)
Total Saturated (FA _{sat})	0.30 (0.19 - 0.51)	0.78 (0.75 - 0.81)	0.96 (0.81 - 1.14)	0.90 (0.51 - 1.63)
C16:3 n-3	-	-	0.01 (0.01 - 0.02)	-
C18:3 n-3	0.01 (0.01 - 0.01)	-	0.13 (0.09 - 0.15)	0.10 (0.06 - 0.15)
C16:2 n-6	-	-	-	0.01 (0.01 - 0.02)
C18:2 n-6	0.26 (0.24 - 0.27)	0.95 (0.86 - 1.01)	1.15 (0.95 - 1.45)	0.45 (0.37 - 0.59)
C16:1 n-7	0.01 (0.01 - 0.01)	-	0.03 (0.02 - 0.03)	0.06 (0.05 - 0.09)
C18:1 n-7	0.01 (0.01 - 0.01)	0.02 (0.02 - 0.02)	0.04 (0.03 - 0.04)	0.07 (0.05 - 0.10)
C16:1 n-9	0.01 (0.01 - 0.01)	0.03 (0.02 - 0.04)	0.03 (0.02 - 0.04)	0.02 (0.01 - 0.04)
C18:1 n-9	0.07 (0.06 - 0.07)	0.14 (0.12 - 0.16)	0.16 (0.13 - 0.19)	0.10 (0.07 - 0.15)
FA _{unsat} (n-3)	0.01 (0.01 - 0.01)	-	0.14 (0.11 - 0.16)	0.10 (0.06 - 0.15)
FA _{unsat} (n-6)	0.26 (0.24 - 0.27)	0.95 (0.86 - 1.01)	1.15 (0.95 - 1.45)	0.46 (0.38 - 0.61)
FA _{unsat} (n-7)	0.02 (0.02 - 0.02)	0.03 (0.02 - 0.03)	0.07 (0.06 - 0.08)	0.13 (0.10 - 0.18)
FA _{unsat} (n-9)	0.08 (0.07 - 0.08)	0.17 (0.14 - 0.18)	0.19 (0.16 - 0.22)	0.13 (0.09 - 0.19)
Total Unsaturated (FA _{unsat})	0.36 (0.33 - 0.38)	1.15 (1.05 - 1.18)	1.55 (1.28 - 1.90)	0.81 (0.65 - 1.13)
Total FA	0.66 (0.56 - 0.83)	1.93 (1.84 - 2.01)	2.50 (2.08 - 2.83)	1.71 (1.16 - 2.76)
Sugars Forming Polysaccharides:	Ap	Pf	Ph + Pa	Ce
Arabinose (PS_Ara)	21.06 (15.64 - 24.66)	17.33 (13.04 - 23.14)	10.85 (10.06 - 11.55)	23.98 (18.00 - 30.12)
Fucose (PS_Fuc)	-	-	-	5.41 (3.63 - 7.54)
Galactose (PS_Gals)	63.48 (60.25 - 68.95)	55.37 (48.63 - 61.75)	56.35 (55.56 - 57.29)	104.80 (89.72-116.90)
Glucose (PS_Glc)	620.27 (580.91 - 654.66)	718.34 (678.57 - 785.53)	667.19 (630.97 - 705.97)	316.64 (290.01 - 337.77)
Mannose (PS_Mans)	116.54 (112.02 - 122.58)	77.24 (66.43 - 85.07)	74.41 (73.73 - 75.31)	182.82 (150.65 - 201.89)
Rhamnose (PS_Rhas)	4.15 (3.89 - 4.54)	4.02 (3.66 - 4.48)	3.62 (2.88 - 4.06)	6.53 (5.00 - 7.29)
Xylose (PS_Xyls)	9.76 (7.93 - 11.16)	7.00 (4.20 - 10.39)	5.36 (4.99 - 5.78)	10.04 (8.15 - 12.60)
Total PS (%)	72.39 (67.83 - 76.95)	71.94 (66.17 - 77.71)	66.55 (63.64 - 69.46)	54.27 (50.60 - 57.63)
Total Nitrogen (TN) (%)	0.68 (0.57 - 0.85)	0.37 (0.36 - 0.38)	0.66 (0.63 - 0.72)	1.62 (1.46 - 1.78)

