

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

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3 4 5 6 7	1 2 3	INTRATHALLINE METABOLITE PROFILES IN THE LICHEN Argopsis friesiana SHAPE 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.*

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).

64 Phenolic compounds in lichens have various roles. Some may deter lichen feeders such as 65 molluscs (Coker 1967; Asplund et al. 2010b), mammals (Cook et al. 2007; Nybakken et al. 2010) 66 and arthropods (Gerson 1973; Wieners et al. 2018). Various physical and chemical factors may 67 influence the attractiveness of lichens for lichenivorous organisms, such as roughness and

growth form, occurrence of defence compounds and N-fixing ability of the lichen (Asplund and Wardle 2016). In studies focussing on interactions between plants and phytophagous animals, different hypotheses have been proposed postulating a trade-off between the cost of operating chemical defence and the benefit of enhancing survival. In his work on plant-herbivore coevolution, McKey (1974) developed the "optimal defence theory" (ODT) which was later applied by Asplund et al. (2010b) to lichen-gastropod interactions. This theory predicts that secondary metabolites in plants are allocated proportionally to the risk of attack for a specific tissue and the value of this tissue in terms of the reduction in fitness that its loss would entail. In many lichens, secondary metabolites occur in higher concentrations in reproductive parts, whether sexual (apothecia) or asexual (isidia, soralia), than in the rest of the thallus, or occur only in these reproductive parts. (Hyvärinen et al. 2000). Rundel (1978) conceptualized another hypothesis, suggesting that the higher the nitrogen content, the higher is the lichen's palatability. Hence, lichens with low nitrogen content and high concentration of secondary metabolites (particularly depsides and depsidones) should suffer low grazing. Rundel's hypothesis was confirmed for most chlorolichens, which were indeed more grazed after non-destructive removal of the secondary metabolites by the acetone-rinsing method (Solhaug and Gauslaa 2001; Asplund and Wardle 2013). Conversely, Rundel's theory was not validated for some cyanolichens which were not consumed although characterised by high nitrogen levels and low content of secondary chemicals. The presence of intracellular repulsive compounds or of metabolites not extractable by acetone (i.e. mycosporines) was hypothesized, due to the persistence of a deterrent effect after acetone rinsing (Solhaug and Gauslaa 2012).

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

 94 particularly widespread on Crozet (Possession Island) and Kerguelen Archipelagos (Charrier et 95 al. 2013). This species provides an appropriate model organism for studying lichen-gastropod 96 interactions. Recently, Gadea et al. (2017) demonstrated the generalist status of *N. hookeri* by a 97 comparative study of snail metabolism after feeding on *Usnea taylorii* (chlorolichen) and 98 *Pseudocyphellaria crocata* (cyanolichen). These authors observed several snail strategies to 99 overcome potentially toxic metabolites present in lichens.

Optimal foraging in generalist species, such as most terrestrial gastropods, requires a balance between the access to useful nutrients and the need to overcome toxic compounds. Therefore, the snail should be able to select lichen parts according to their nutritional quality and to avoid toxic compounds. In this study, we focussed on the cephalolichen Argopsis friesiana Müll. Arg. (Stereocaulaceae), a tufty \leq 4 cm tall species, widespread in low-nutrient, windy fell-fields of Subantarctic islands (Lamb 1974; Galloway 1980). Argopsis friesiana is characterized by a rigid pseudopodetium, cylindric-corraloid phyllocladia, concave-scutelliform apothecia and botryose cephalodia. Its main photobiont is a green alga (Trebouxia s. lat.), its secondary autotrophic partner is the cyanobacterium Stigonema sp., located in the cephalodia. To understand how this snail could benefit from eating this lichen, we examined the influence of lichen chemistry on snail nutrition. For this purpose, we explored how the metabolites were distributed in the different parts of the lichen A. friesiana and how this distribution shaped the snail grazing patterns. Intrathalline sectorization of the symbiotic partners facilitated the separation of A. friesiana into four different parts to be analysed separately. Three hypotheses were tested: The snail (1) avoids lichen parts with high concentrations of secondary metabolites, (2) prefers lichen parts that are rich in essential nutrients (sugars, amino acids, fatty acids, polysaccharides) and (3) prefers thallus parts rich in nitrogen.

118 MATERIALS AND METHODS

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

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

Lichen Preparation. Argopsis friesiana was cut into four parts as explained in Fig. 1a. The pseudopodetium consists of (i) a central axis, the medulla, made up of compact fungus hyphae (Pf) and (ii) an outer layer with fungi and green algae (Pa). Phyllocladia are the terminal branchlets of pseudopodetia containing algae (Ph). Cyanobacteria live inside numerous black **139 140** cephalodia (Ce), external gall-like structures emerging laterally from the pseudopodetium. Apothecia, the sexual reproductive parts of the lichen, are scattered on the upper surface of the thallus. The underside of the apothecium is similar to the algal layer of the pseudopodetium. For ₅₈ 143 chemical analyses, algal layer and phyllocladia were combined because of their structural similarity. 60 144

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

Level of grazing (LD) was adapted from Nimis and Skert (2006) and measured as 22 153 follows: LD0 intact, LD1= low grazing (<25%), LD2=medium damage (>25-50% consumed) and LD3= high damage (>50%). For pseudopodetia, grazing on the algae-containing cortical layer (Pa) was assessed separately from that on the fungus axis (Pf) and the levels of grazing were defined as: LD0 = algal layer and fungal axis of the pseudopodetia remained intact, LD1 = 31 157 ³³ 158 minimum damage (algal layer consumed <25%, fungal axis intact), LD2 = medium damage (algal layer consumed <50%, fungal axis intact), LD3 = high damage (algal layer Pa and fungal axis Pf consumed, > 50%). To assess the damage to cephalodia and apothecia, a grazing ratio was determined as follows: Number of apothecia or cephalodia with grazing marks / Number of 40 161 apothecia or cephalodia available. To compare grazing on cephalodia and apothecia with damage to algal layer, fungal axis and phyllocladia, the levels described above (LD in %) were utilized.

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Sample Preparation for Chemical Analyses. To determine a sectorial distribution of metabolites, three samples of several A. friesiana thalli (about one gram each) were prepared. Each thallus was cut into the four parts apothecia, cephalodia, fungal axis and algal layers. The 12 samples were ground separately with mortar and pestle in liquid nitrogen. To quantify secondary metabolites, freeze-dried samples (40 mg each) were used. Primary metabolites, including amino acids, free sugars and polyols, were analysed with 10 mg of each thallus part, while 50 mg of each part was needed for quantification of fatty acid and sugar-forming polysaccharides and for determination of nitrogen content.

Secondary Metabolite Profiling. Each part of the air-dried lichen thalli was extracted with 100% acetone (3 x 500 µL). Acetone extracts were solubilized in tetrahydrofuran (THF), filtered and injected (10 µL at 0.5 mg.mL⁻¹) in a Liquid Chromatography - Diode Array Detector (LC-DAD) (Shimadzu®, Marne La Vallée, France, LC-20AD SP, injector SIL-20A HI, column oven CTO-**178** ³³ 179 20A, diode array detector SPD-M20A) and a mass spectrometer (MS) (Advion® expression CMS, Ithaca, USA). Ionization was performed by negative-ion mode electrospray (ESI-). A gradient system was applied: A (0.1% formic acid in water) and B (0.1% formic acid in 40 182 acetonitrile) for 48 min on a C₁₈ column (Phenomenex®, Kinetex 2.6µ C₁₈ 100A, temperature 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 35-42 min, 100% B linear; T 45-48 min, 20% B linear. Linear equations based on the calibration curve were used to quantify atranorin and the results were expressed in mg.g⁻¹ Dry Mass (DM). Atranorin (JB/A/142) used for calibration belong to the library of single lichenic compounds of UMR 6226, being isolated during previous phytochemical investigations. For the other metabolites, also detected by UV on HPLC chromatograms, a relative quantification was performed, by multiplying the area under the curve (AUC) and the extraction yield of the lichen extract. Full scan mass spectra were recorded in negative-ion mode in a mass range of 100 to 1200 Da, applying the following parameters: detector gain 1200, ESI voltage 3.5 kV, capillary **191**

voltage 180 V, source voltage 20 V, source voltage dynamic 20 V, nebulizer gas pressure 60
psig, desolvation flow gas rate 4 L.min⁻¹, capillary temperature 250°C and gas temperature
20°C. Data processing and evaluation for MS measurement were performed with the Data and
Mass Express 2.2.29.2 software (Advion). As a complement to LC-DAD-MS experiments, in situ
analyses of each part of the lichen were made using the ambient air source DART-HRMS,
enabling the chemical profiling of unprocessed pieces of thallus (Le Pogam et al. 2015, 2016);
more detailed protocol in Online Resource 2). The identification of metabolites was done against
compounds formerly identified within the genus *Argopsis* and the family Stereocaulaceae (Bodo
and Molho 1974; Lamb 1974; Galloway 1980).

Primary Metabolite Profiling. Soluble carbohydrates, polyols and amino acids were extracted from each part of the lichen, following the methodology of Gravot et al. (2010). Twenty mM of adonitol (soluble carbohydrates and polyols) and 10mM of 3-aminobutyric acid (BABA) (amino acids) were used as internal standards. Soluble carbohydrates and polyols were profiled by a Gas Chromatography - Flame Ionization Detector (GC-FID) (Thermo-Fisher Scientific, Waltham, CA, USA) according to Adams et al. (1999) and Lugan et al. (2009). Amino acids were detected with an Ultra High Performance Liquid Chromatography – Diode Array Detector (UPLC-DAD, Waters), by using an AccQ-Tag Ultra derivatization kit (Waters Corporation, Milford, MA, USA) following the protocol described in Gravot et al. (2010). The metabolites were identified from their retention time by comparison with external standards. Concentrations (mg.g⁻¹ DM) were obtained on the basis of internal standards.

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

internal standard. The organic phase containing the total lipids was recovered and evaporated to dryness under a nitrogen flow. Sample derivatization and GC-FID/GC-MS (Agilent 6890N, Toulouse, France) analyses were conducted using the protocol of Vu et al. (2016). Identification of fatty acid methyl esters (FAMEs) was based on the retention times. The quantity of total FAs (in mg.g⁻¹ DM) was calculated using the internal standard.

Extraction of Sugar-Forming Polysaccharides. Approximately 50 mg of each dried lichen tissue were reduced to a fine powder with a pestle and transferred in 22 mL cells of an accelerated solvent extraction unit ASE® 350 (ASE[™] 350, Thermo Scientific[™] Dionex[™]), dried at 40°C overnight under vacuum over P_2O_5 , prior to weighing. Alcohol-insoluble residues (AIRs) were obtained by using 80% ethanol at 2 mL min⁻¹ flow. The conditions for the ASE extraction were set at 100°C for 20 min, followed by rinse volume of 150%, and a purge time (N_2) of 30 s. Cells of the ASE® 350 containing AIRs were dried at 40°C overnight under vacuum over P_2O_5 , prior to weighing, in order to determine the extraction yield.

Identification and quantification of polysaccharide neutral sugars were performed by GC after sulfuric acid degradation (Hoebler et al. 1989). Five mg of AIR were dispersed in 13 M sulfuric acid for 30 min at 30°C, then hydrolysed in 1 M sulfuric acid (2 h, 100°C). Sugars were converted to alditol acetates according to Blakeney et al. (1983) and chromatographed on a TG-225 GC Column (30 x 0.32 mm ID) using TRACE[™] Ultra Gas Chromatograph (Thermo Scientific™; Waltham, CA, USA; temperature 205°C, carrier gas H₂). Inositol was used as internal standard. The quantity of each sugar (in mg.g⁻¹ DM) was determined, using the calibration curves drawn from standard solutions.

Total Nitrogen (TN) Measurement. TN (in % of DM) was determined with an automated dry combustion method (Dumas method; Rhee 2001) using an elemental analyser (vario MICRO cube, Elementar Analysensysteme, Hanau, Germany).

Statistical Analyses. A Fisher's exact test was applied to LD values obtained for damage to algal layer and fungal axis of the pseudopodetia, phyllocladia, cephalodia and apothecia, in order to compare the observed frequencies among lichen parts. In the text, mean ± standard deviation is given. A Spearman correlation was used to compare LD values with the number of cephalodia or apothecia carried out by the thallus.

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

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

RESULTS

Snail Feeding Preferences. Snails from Mascarin site had shell sizes of 4.9 ± 0.5 mm (mean \pm s.d.). Shell size did not differ among groups (n = 15 x 5 individuals per group; ANOVA, $F_{14.60}$ = 0.415, P = 0.964).

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

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

Metabolite Profiling of A. friesiana. The chemical profiles in secondary metabolites of each part of the lichen A. friesiana were established by LC-DAD-ESI-MS from their acetone extracts. Three main compounds were identified: the depside atranorin (m/z 373) and two chlorinated compounds (Table 1, Fig. S2). These two chlorodepsidones were identified as the dichlorinated argopsin (m/z 395) and as the trichlorinated caloploicin (m/z 401) through isolation and spectroscopic data analysis (Purification protocol and NMR data are given in Online Resource 2, Fig. S3-S4, Table S1-S2). In situ DART-HRMS analysis confirmed identification of chlorinated compounds detected by LC-DAD-MS from unprocessed pieces of lichens through exact mass measurements (Online Resource 2, Table S3). Algal layers had the highest concentration of secondary metabolites, particularly for atranorin, reaching $10.6 \pm 2.3 \text{ mg.g}^{-1}$ DM. In comparison, 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 pseudopodetia, the medullar caloploicin with argopsin accounted for about two-thirds of the secondary metabolites, while atranorin alone approximately reached this proportion in apothecia and in green algae-containing tissues (Table S4).

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

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, FAunsat (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 (FAsat 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 (FAsat, 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).

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

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

Cephalodia and algal layers were selected by N. hookeri when given a choice among the four parts of A. friesiana. These lichen parts were characterized by their richness in some nutrients: (i) polyols and free sugars, among which trehalose was encountered in cephalodia only, (ii) amino acids, among which proline was exclusive of algal layers. Avoidance of apothecia 13 351 by the snail might be linked to their low content of phagostimulant compounds. However, we cannot explain why the snails did not consume the fungal axis of pseudopodetia. Although rich in arabitol, the fungal axis was characterized by a lack of diversity in nutrients compared to that found in apothecia. Moreover, the central axis made of compact hyphae, as well as apothecia, may be resistant to grazing. Indeed, the radular teeth of N. hookeri are smooth and more or less 22 355 bulging or flat, features of non-specialized radulae potentially sensitive to rough (apothecia) and fibrous (fungal axis) textures (Fig. S8). By contrast, the soft and gelatinous structure of the cephalodia could promote grazing. This observation is in line with the study of Baur et al. (2000), who compared the ultrastructure of snail radulae and concluded that hook-shaped teeth, such as 31 359 those of *Chondrina clienta*, are necessary to scrape efficiently on crustose lichens.

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

₅₈ 371 The glucose residues in polysaccharides, although they were half as abundant in cephalodia as in other parts, they suggested the presence of glucans, probably isolichenan (α -60 372

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glucan), as found for Stereocaulon ramulosum (Baron et al. 1991). The abundance of galactose and mannose units in the cephalodia could reflect the synthesis of galactomannan, a hypothesis supported by the description of these heteropolymers in Stereocaulon paschale (Baron et al. 1989). Because lichenan, mannan and galactomannan degrading-enzymes occur in land snails (Flari et al. 1995; Charrier and Rouland 2001), N. hookeri can probably exploit this source of glycosidic residues and thereby meet its energy needs. Indeed, gastropod metabolism is considered to be polysaccharide-orientated as energy is mainly provided by glycogen storage (Nicolai et al. 2012). Sugars and amino acids are also needed to synthetize mucus proteoglycans for snail locomotion, adhesion, body hydration and chemical communication (Ng et al. 2013). Because mucus production requires around 20% of ingested energy (Denny 1980), the consumption of food rich in potential mucin-like compounds would be beneficial to snail survival.

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

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

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

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

Conflict of Interest: The authors declare that they have no conflict of interest.

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feeding (a-2 and b-2). The black line shows the transversal section of a pseudopodetium to discriminate between the cortical 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 (FAsat) 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.

SUPPLEMENTARY DATA

The following supplementary data are related to this article: see Supplementary data file

Online resource 1. Fig. S1: Study sites on Possession Island, which belongs to Crozet Archipelago (45° 30' – 46° 30' S; 50° 00' – 52° 30' E) in the Subantarctic region.

Online Resource 2: Chemical and spectroscopic data. Fig. S2: Three main secondary metabolites of Argopsis friesiana: the chlorodepsidones argopsin (1) and caloploicin (2) and the **630**

depside atranorine (3). Fig. S3: NMR spectra (¹H, 300 MHz and ¹³C, 75 MHz) of argopsin in CDCl₃ and argopsin formula, Table S1: ¹H NMR (300 MHz) and ¹³C NMR (75 MHz) data for argopsin. Data recorded inCDCl₃, δ in ppm; Fig. S4: NMR spectra (¹H, 500 MHz and ¹³C, 125 MHz) of caloploicin in acetone (*d*₆-acetone) and caloploicin formula, Table S2: ¹H NMR (500 MHz, cryo) and ¹³C NMR (125 MHz) data for caloploicin. Data recorded in acetone-*d*6, δ in ppm, Table S3: Results of exact mass measurements in negative-ion mode DART-HRMS of the molecules retrieved in *Argopsis friesiana*, Table S4: Each secondary metabolite in the four lichen parts, calculated as a percentage of all the compounds detected in this chemical group.

Online Resource 3: Multivariate analysis supplementary data. Fig. S5: Projection to the axis PC1
 of the metabolites analysed by PPLS-DA. Fig. S6: Projection to the axis PC2 of the metabolites
 analysed by PPLS-DA.; Fig. S7: Projection to the axis PC3 of the metabolites analysed by
 PPLS-DA. Table S5: Correlated compounds (> 95%) in the PPLS-DA analysis from the data set
 of the compounds identified in the lichen *A. friesiana*.

Online Resource 4: Fig. S8: Scanning Electron Microscopy of the radula of *Notodiscus hookeri*,
 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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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): secondarymetabolites, free sugars and polyols, amino acids, fatty acids and sugar forming polysaccharides. Values are means (n=3replicates) in mg.g⁻¹ dry mass with (*minimum - maximum*) in brackets, except the secondary metabolites, which areexpressed in Area Under the Curve (samples: 0.5 mg.ml⁻¹) *extraction yield (x10⁵). Total polysaccharides (Total PS) and totalnitrogen (TN) are expressed in percentage of DM. The sign '-' means that the metabolite was not detected.

rea x 10°): Ap	Pţ	rn + Pa	Le			
3.20 (1.23 - 6.25)	1.87 (1.16 - 2.54)	41.50 <i>(32.12 - 52.18)</i>	1.31 (1.08 - 1.47)			
1.14 (0.26 - 2.41)	2.46 (0.57 - 3.69)	7.23(5.60 - 8.75)	0.40 (0.35 - 0.42)			
0.54 (0.21 - 1.03)	2.57 (0.86 - 4.82)	6.38 (5.47 - 7.20)	0.55 <i>(0.47 - 0.66)</i>			
Ар	Pf	Ph + Pa	Ce			
0.31 <i>(0 - 0.58)</i>	5.69 (1.52 - 12.19)	2.81 (1.38 - 4.99)	0.59 (0.48 - 0.71)			
33.41 (25.82 - 39.24)	89.70 <i>(58.60 - 133.29)</i>	91.86 (87.13 - 100.73)	178.02 (57.6 -399.02)			
1.08 (1.03 - 1.13)	3.50 <i>(2.63 - 4.76)</i>	1.97 (1.66 - 2.13)	11.13 <i>(1.69 - 29.99)</i>			
0.02 <i>(0 - 0.05)</i>	0.07 (0 - 0.13)	0.10 <i>(0 - 0.17)</i>	0.11 <i>(0 - 0.33)</i>			
0.04 (0 - 0.13)	-	0.13 <i>(0 - 0.20)</i>	0.28 (0.24 - 0.32)			
-	-	0.16 <i>(0 - 0.25)</i>	0.09 <i>(0 - 0.28)</i>			
-	-	-	0.64 (0.51 - 0.82)			
-	0.19 <i>(0 - 0.35)</i>	0.07 <i>(0 - 0.22)</i>	0.41 (0 - 1.24)			
Ар	Pf	Ph + Pa	Ce			
0.08 (0.04 - 0.13)	0.19 (0.10 – 0.29)	0.13 (0.12 - 0.15)	0.09 <i>(0.08 - 0.09)</i>			
-	-	-	0.11 (0 - 0.19)			
0.20 (0.17 - 0.25)	0.40 (0.38 – 0.44)	0.44 (0.40 - 0.48)	0.27 (0.23 - 0.30)			
0.12 (0.07 - 0.16)	-	0.03 (0 - 0.10)	0.16 (0.16 - 0.17)			
0.14 (0.11 - 0.15)	0.18 (0.06 – 0.30)	0.25 (0.14 - 0.33)	0.34 (0.31 - 0.37)			
0.04 (0 - 0.11)	0.02 (0 -0.05)	0.02 (0 -0.05)	-			
-	-	-	0.02 (0 - 0.04)			
-	-	0.05 (0 - 0.16)	0.10 (0 - 0.17)			
-	-	0.05 (0.04 - 0.05)	-			
0.08 (0 - 0.25)	0.01 (0 - 0.04)	0.15 (0.04 - 0.37)	-			
0.10 (0 - 0.17)	0.11 (0 - 0.17)	0.03 <i>(0 - 0.08)</i>	0.15 (0.08 - 0.26)			
Ар	Pf	Ph + Pa	Ce			
0.01 (0.01 - 0.02)	0.03 (0.02 - 0.03)	0.03 (0.02 - 0.04)	0.05 (0.02 - 0.08)			
0.01 (0 - 0.01)	0.02 (0.02 - 0.02)	0.02 (0.01 - 0.03)	0.02 (0.01 - 0.04)			
0.14 (0.09 - 0.23)	0.34 <i>(0.32 - 0.36)</i>	0.43 (0.33 - 0.53)	0.46 (0.26 - 0.84)			
0.01 (0 - 0.01)	0.02 (0.01 - 0.02)	0.02 (0.01 - 0.04)	0.02 (0.01 - 0.05)			
0.09 (0.06 - 0.14)	0.25 (0.24 - 0.26)	0.29 (0.23 - 0.32)	0.24 (0.14 - 0.40)			
	rea x 10 ⁵): Ap 3.20 (1.23 - 6.25) 1.14 (0.26 - 2.41) 0.54 (0.21 - 1.03) Ap 0.31 (0 - 0.58) 33.41 (25.82 - 39.24) 1.08 (1.03 - 1.13) 0.02 (0 - 0.05) 0.04 (0 - 0.13) - - - - 0.20 (0.17 - 0.25) 0.12 (0.07 - 0.16) 0.14 (0.11 - 0.15) 0.04 (0 - 0.11) - - 0.08 (0 - 0.25) 0.10 (0 - 0.17) Ap 0.01 (0 - 0.25) 0.10 (0 - 0.17) Ap	Pf 3.20 (1.23 - 6.25) 1.87 (1.16 - 2.54) 1.14 (0.26 - 2.41) 2.46 (0.57 - 3.69) 0.54 (0.21 - 1.03) 2.57 (0.86 - 4.82) Ap Pf 0.31 (0 - 0.58) 5.69 (1.52 - 12.19) 33.41 (25.82 - 39.24) 89.70 (58.60 - 133.29) 1.08 (1.03 - 1.13) 3.50 (2.63 - 4.76) 0.02 (0 - 0.05) 0.07 (0 - 0.13) 0.04 (0 - 0.13) - - - 0.19 (0 - 0.35) Ap Pf 0.08 (0.04 - 0.13) 0.19 (0 - 0.35) - - - 0.20 (0.17 - 0.25) 0.40 (0.38 - 0.44) 0.12 (0.07 - 0.16) - - - 0.14 (0.11 - 0.15) 0.18 (0.06 - 0.30) 0.04 (0 - 0.11) 0.02 (0 -0.05) - - - - 0.08 (0 - 0.25) 0.01 (0 - 0.04) 0.02 (0 - 0.17) 0.118 (0.06 - 0.30) 0.04 (0 - 0.11) 0.02 (0 - 0.05) - - - - 0.	Pf Ph + Pa $3.20 (1.23 - 6.25)$ $1.87 (1.16 - 2.54)$ $41.50 (32.12 - 52.18)$ $1.14 (0.26 - 2.41)$ $2.46 (0.57 - 3.69)$ $7.23(5.60 - 8.75)$ $0.54 (0.21 - 1.03)$ $2.57 (0.86 - 4.82)$ $6.38 (5.47 - 7.20)$ Ap Pf Ph + Pa $0.31 (0 - 0.58)$ $5.69 (1.52 - 12.19)$ $2.81 (1.38 - 4.99)$ $33.41 (25.82 - 39.24)$ $89.70 (58.60 - 133.29)$ $91.86 (87.13 - 100.73)$ $1.08 (1.03 - 1.13)$ $3.50 (2.63 - 4.76)$ $1.97 (1.66 - 2.13)$ $0.02 (0 - 0.05)$ $0.07 (0 - 0.13)$ $0.10 (0 - 0.17)$ $0.04 (0 - 0.13)$ - $0.13 (0 - 0.29)$ $ 0.19 (0 - 0.35)$ $0.07 (0 - 0.22)$ Ap Pf Ph + Pa $0.08 (0.04 - 0.13)$ $0.19 (0.10 - 0.29)$ $0.13 (0.12 - 0.15)$ $ 0.20 (0.17 - 0.25)$ $0.40 (0.38 - 0.44)$ $0.44 (0.40 - 0.48)$ $0.112 (0.07 - 0.16)$ $ 0.03 (0 - 0.10)$ $0.12 (0.07 - 0.16)$ $ 0.05 (0 - 0.16)$ $ -$			

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 <i>(0.03 - 0.06)</i>	0.07 <i>(0.04 - 0.09)</i>	0.05 <i>(0.03 - 0.09)</i>
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 <i>(0.95 - 1.45)</i>	0.45 <i>(0.37 - 0.59)</i>
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 <i>(0.06 - 0.07)</i>	0.14 (0.12 - 0.16)	0.16 <i>(0.13 - 0.19)</i>	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 <i>(0.95 - 1.45)</i>	0.46 (0.38 -0.61)
FA _{unsat} (n-7)	0.02 (0.02 - 0.02)	0.03 (0.02 -0.03)	0.07 <i>(0.06 - 0.08)</i>	0.13 (0.10 - 0.18)
FA _{unsat} (n-9)	0.08 <i>(0.07 - 0.08)</i>	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 Polysacc	harides: Ap	Pf	Ph + Pa	Ce
Arabinose (PS_Ara)	21.06 (15.64 - 24.66)	17.33 (13.04 - 23.14)	10.85 <i>(10.06 - 11.55)</i>	23.98 (18.00 - 30.12)
Fucose (PS_Fuc)	-	-	-	5.41 (3.63 - 7.54)
Galactose (PS_Gals)	63.48 <i>(60.25 - 68.95)</i>	55.37 (48.63 - 61.75)	56.35 <i>(55.56 - 57.29)</i>	104.80 (89.72-116.90)
Glucose (PS_Glc)	620.27 (580.91 - 654.66)	718.34 (678.57 - 785.53)	667.19 <i>(630.97 - 705.97)</i>	316.64 <i>(290.01 - 337.77)</i>
Mannose (PS_Mans)	116.54 <i>(112.02 - 122.58)</i>	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 <i>(5.00 - 7.29)</i>
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)

Supplementary Material