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**Contrasting changes in palatability following senescence of the lichenized
fungi *Lobaria pulmonaria* and *L. scrobiculata***

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1 **Abstract (max 150 words):**

2 Epiphytic lichens can contribute significantly to ecosystem nutrient input because they efficiently
3 accumulate atmospheric mineral nutrients and, in the case of cyanolichens, also fix nitrogen. The
4 rate at which carbon and other nutrients gained by lichens enters the ecosystem is determined by
5 lichen litter decomposability and by invertebrate consumption of lichen litter. In turn, these
6 processes are driven by the secondary compounds present in senesced lichens. Therefore, we
7 explored how lichen palatability and concentrations of secondary compounds change with tissue
8 senescence for *Lobaria pulmonaria*, a green algal lichen with cyanobacterial cephalodia, and *L.*
9 *scrobiculata*, a cyanobacterial lichen. During senescence both lichens lost 38-48% of their stictic
10 acid chemosyndrome, while *m*-scrobiculin and usnic acid in *L. scrobiculata* remained unchanged.
11 Snails preferred senesced rather than fresh *L. pulmonaria*, while senesced *L. scrobiculata* were
12 avoided. This provides evidence that species with labile secondary compounds will have higher
13 turnover rates, through consumption and decomposition, than those producing more stable
14 secondary compounds.

15

16 **Keywords (max 6):** *Cepaea hortensis*; decomposition; gastropods; herbivory; secondary
17 compounds; snails

18 **Introduction**

19 Lichens are symbiotic associations in which a fungal partner (mycobiont) hosts cells of
20 photobionts (green algae and/or cyanobacteria) that provide carbohydrates and, in the case of
21 cyanobacteria, fix atmospheric nitrogen (N₂) (Nash 2008). Such symbiotic relationships shape
22 plant-like life forms that play a prominent role in boreal forest ecosystems. Cyanobacterial N-
23 fixing lichens may contribute significantly to the N cycling of those ecosystems in which they are
24 dominant components (Nash 2008), such as boreal and temperate rainforests where species of
25 *Lobaria*, *Pseudocyphellaria* and *Sticta* occur in abundance as epiphytes (Green *et al.* 1980;
26 Antoine 2004). In addition, green-algal lichens are efficient accumulators of atmospheric
27 nutrients from wet and dry deposition. For example, Knops *et al.* (1996) showed that the green-
28 algal epiphyte *Ramalina menziesii* augments the input of total N, NO₃, organic N, Ca, Mg, Na
29 and Cl in temperate deciduous forests. Further, lichen litter inputs may have significantly higher
30 quantities of N and micronutrients than leaf litter from trees, because nutrients from tree leaves
31 are usually resorbed back to the plant before abscission, while epiphytic lichen tissues fall to the
32 ground with their nutrient concentration largely unchanged (Knops *et al.* 1991).

33
34 The turnover rates of carbon (C) and mineral nutrients entering the ecosystem from epiphytic
35 lichens are determined by their tissue decomposition rates, and how quickly these tissues are
36 consumed by invertebrates such as gastropods, springtails and mites. Both these processes are
37 driven in part by functional characteristics of the thalli, including their concentrations of
38 secondary compounds (Hättenschwiler & Vitousek 2000; Gauslaa 2005). Snails prefer specimens
39 with artificially reduced levels of secondary compounds in both laboratory feeding experiments
40 (e.g. Gauslaa 2005) and natural field conditions (Asplund & Gauslaa 2008). In addition, it is well
41 known from studies on vascular plants that secondary compounds often greatly impair tissue

42 decomposability and the release of nutrients during decomposition (Hättenschwiler & Vitousek
43 2000). However, to understand whether lichen secondary compounds have “afterlife” effects on
44 lichen litter, we need to know the extent to which the lichens retain these compounds during
45 senescence, and the consequences of this retention for breakdown of senesced lichen tissues
46 through decomposition and consumption by invertebrates. In this light, while it is known that
47 gastropods prefer senescent leaves to fresh leaves (Speiser 2001), it is unknown whether this is
48 also the case with lichens.

49
50 In this study we explored how lichen palatability to snails changes during senescence for each of
51 two contrasting epiphytic lichens (the cephalodial green-algal *Lobaria pulmonaria* and the
52 cyanobacterial *L. scrobiculata*), and how these changes correspond to shifts in concentrations of
53 secondary compounds and mineral nutrients. Lichenivorous invertebrates can play an important
54 role in the breakdown of lichen litter (McCune & Daly 1994; De Oliveira *et al.* 2010) and the
55 palatability of senesced lichens may, therefore, potentially affect their contribution to C and
56 nutrient turnover in the ecosystem. As such, our study aims to improve knowledge about how
57 senescence of lichens influences their palatability, and therefore to add to our understanding of
58 how lichens contribute to forest ecosystem processes.

59

60 **Materials and methods**

61 Our study focuses on *Lobaria pulmonaria* and the closely related *Lobaria scrobiculata*. Both
62 these N₂-fixing species are common in temperate and boreal rainforests but rather uncommon
63 elsewhere. The main functional differences between them are the types of photobionts; *L.*
64 *scrobiculata* has a cyanobacterial photobiont while *L. pulmonaria* has a green-algal photobiont
65 and cyanobacteria in internal cephalodia. Both species exhibit the stictic acid chemosyndrome,

66 with the substances stictic, constictic, norstictic acids and other minor derivatives (Jørgensen
67 2007). In addition, *L. scrobiculata* produces *m*-scrobiculin and usnic acid (Jørgensen 2007). The
68 invertebrate herbivore used in our study is the 14-22 mm wide *Cepaea hortensis*, which is a
69 common and widespread broad generalist snail that climbs trees to feed on epiphytic lichens,
70 including both of our studied lichen species (Asplund *et al.* 2010a).

71
72 Mature thalli of *L. pulmonaria* and *L. scrobiculata* were collected from four neighbouring *Salix*
73 *caprea* trunks in an open *Picea abies* forest at Horka (64°26'N, 11°47 'E, 30 m a.s.l., Overhalla,
74 Nord-Trøndelag, W Norway) in May 2011. The thalli were stored dry in the freezer until the start
75 of the experiment; freezing is the recommended means of long-term storage of viable thalli for
76 experimental studies (Honegger 2003). For each species, half of the collected thallus material
77 was put in one 1.5 mm mesh litter bag of 30 × 30 cm and placed on the forest floor to engender
78 tissue senescence. This involved placing these bags among *Populus tremula* litter in an old
79 growth *Picea abies* forest at Kollåsen (59°45'N 10°57'E, 200 m.a.s.l., Ski, Akershus, SE
80 Norway) on August 25 2011, where both species occur naturally. The other half of the thallus
81 material was stored in a freezer until the start of the feeding experiment. The senesced lichens in
82 the litter bags were brought into the lab after 6 weeks of undergoing senescence in the field, and
83 left to dry at room temperature. Senesced thalli showed reduced maximal photosystem II
84 efficiency when measured as described by Solhaug *et al.* (2003) using a portable, modulated
85 fluorometer (PAM-2000, Walz, Effeltrich, Germany). As such, mean ± SE values of F_V/F_M for
86 senesced and living thalli, respectively, were 0.43 ± 0.11 and 0.69 ± 0.01 for *L. pulmonaria*, and
87 0.23 ± 0.07 and 0.58 ± 0.01 for *L. scrobiculata*. For the senesced lichens, photobionts of both
88 species had turned more or less brown and the red-tinged mycobionts produced a red leachate
89 when moistened. As such, we assume that both bionts were dying at this stage. For each species,

90 15 lobes of lichen thallus material from each of the two treatments (senesced thalli from litter
91 bags, and living thalli from freezer) were then randomly selected from the available material.
92 Each of these lobes was air dried and weighed (± 0.1 mg). For each species, 15 plastic boxes
93 (each measuring $10 \times 7 \times 6$ cm) were set up, and one senesced and one living lobe were placed in
94 each box; the lichens were sprayed with 3 ml water and 4 randomly selected snails were placed in
95 10 of the 15 boxes for each lichen species. These boxes were then closed with a perforated lid
96 and left for 24 hr at room temperature and natural day light but not in direct sunlight (as
97 described by Gauslaa 2005). The five boxes without snails were used to control for any non-snail
98 related changes in air-dry weight. After the 24 hr feeding experiment the lobes were left to air dry
99 and subsequently re-weighed. There were no significant differences in air-dry weight change
100 between the senesced and living thalli in the control boxes. Preference, *sensu* Lockwood (1998),
101 was calculated as the biomass consumed of one lobe divided by the pooled consumption of both
102 lobes in the box (hereafter referred to as feeding preference, expressed as a percentage). Thus,
103 when the snails consume equal amounts of each lobe the preference will be 50 % for both lobes.

104
105 For each species, ten lobes of senescent and of fresh material, each adjacent to the lobes selected
106 for use in the feeding experiment, were ground to powder in a ball mill. Approximately 20 mg of
107 the powder was extracted for four 30 min intervals in 2 ml acetone. The combined supernatants
108 were evaporated to dryness and dissolved in 500-1000 μ l acetone. The extracted compounds were
109 then quantified by HPLC using an ODS Hypersil column, 50×4.6 mm using 0.25%
110 orthophosphoric acid and 1.5% tetrahydrofuran in Millipore (Millipore, Billerica, Massachusetts,
111 USA) water (A) and 100% methanol (B) as mobile phases at 2 ml min^{-1} , and UV detection at 245
112 nm (following Nybakken *et al.* 2007). In addition, the *L. scrobiculata* extracts were run through a
113 250×4.6 mm ODS Hypersil column (at 1 ml min^{-1}) to separate *m*-scrobiculin from usnic acid.

114 Compound identification was based on retention times, online UV spectra and co-
115 chromatography of commercial standards. Nitrogen and phosphorus concentrations were
116 determined by Kjeldahl digestion of a subsample of each lobe (n=5 for each species x treatment
117 combination) followed by automatic colorimetric methods (Blakemore *et al.* 1987).

118

119 **Results and Discussion**

120 Senescence alters the palatability to snails of each of the two closely related lichen species but in
121 contrasting directions (Fig 1). Senesced thalli of the cephalodial green-algal *L. pulmonaria* were
122 more preferred than living thalli (Fig 1). The increasing palatability of *L. pulmonaria* thalli
123 during senescence was concomitant with decreasing concentrations of secondary compounds
124 such as the stictic acid chemosyndrome (Table 1), which has repeatedly been shown to deter
125 snails (Gauslaa 2005; Asplund & Gauslaa 2008; Asplund 2011). In contrast, senesced thalli of the
126 cyanobacterial *L. scrobiculata* were consumed less by the snails than were the living thalli (Fig
127 1). In addition to the stictic acid chemosyndrome, *L. scrobiculata* also produces usnic acid and *m*-
128 scrobiculin that did not decline in concentration during the senescence process (Table 1). In this
129 light, laboratory feeding experiments have shown that *m*-scrobiculin is a very effective
130 lichenivore deterrent (Asplund *et al.* 2010b). Thus, even though the total concentration of
131 secondary compounds was slightly reduced in *L. scrobiculata* during senescence, previous results
132 suggest that the lichen should have remained sufficiently defended due to the unchanged
133 concentrations of *m*-scrobiculin. However, concentrations of secondary compounds cannot
134 explain why the senesced *L. scrobiculata* were avoided by the snails, and this avoidance could
135 instead be due to the loss of easily utilized carbohydrates during the senescence process (Cooper
136 & Carroll 1978; Dudley & Lechowicz 1987). As such, it has been suggested that invertebrate
137 preferences for lichens are primarily based on their concentrations of easily digestible

138 carbohydrates rather than nutrients (Dubay *et al.* 2008). Further, *L. scrobiculata* may produce
139 cyanotoxins (Kaasalainen *et al.* 2012), which are quickly released from the cyanobacterial cells
140 during lysis (Watanabe *et al.* 2006). If these toxins remain in the thallus and are not leached out,
141 they may be consumed by lichenivores. However, globally only 12 % of cyanobacteria associated
142 with lichens have the biosynthetic genes for producing cyanotoxins (Kaasalainen *et al.* 2012), and
143 it is therefore uncertain as to whether the lichens used in our study actually produce toxins.

144
145 We found higher concentrations of N and unchanged concentrations of P in senesced compared
146 to living thalli (Table 1). Since the entire lichen thallus falls to the ground and senesces, there is
147 no loss of mineral nutrients in tissues resulting from nutrient resorption, unlike the situation
148 frequently observed in vascular plants (Knops *et al.* 1991; Killingbeck 1996). Instead, N in
149 senescing thallus tissues appears to be stable, and initial mass loss during senescence is due to
150 loss of primary and secondary C-based compounds, resulting in increased N-concentrations at
151 least in the first few weeks. In an *in situ* decomposition study of *Lobaria oregana*, no significant
152 net N loss occurred until 17 % of the initial mass was lost (Holub & Lajtha 2003). The high N
153 concentration of lichen litter compared with leaf litter suggests that lichen thalli may be an
154 important source of N in the ecosystem even when they have a lower total biomass relative to that
155 of leaves (reviewed by Nash 2008). Even though senesced plant leaves are low in N, they are
156 often favoured by gastropods (Speiser 2001) as a consequence of substantial reductions in
157 defence compounds during senescence (e.g. Newman *et al.* 1992). Thus, the palatability of
158 senesced *versus* living leaf material is driven more by secondary compounds than by nutrients.

159
160 Our results show compound-specific variation in the stability of lichen secondary compounds
161 during senescence, and that the change in palatability during senescence is species-specific.

162 These species differences could potentially have large effects on the rate at which C and mineral
163 nutrients in lichen tissues are released back into the ecosystem through invertebrate activity
164 (McCune & Daly 1994). In other words, lichens that produce secondary compounds, e.g. stictic
165 acid, which are quickly lost during senescence will have higher turnover rates (through both
166 consumption and decomposition) than those producing more stable secondary compounds. Litter
167 decomposition is a major driver of nutrient cycling in ecosystems, and key macrofaunal groups
168 such as gastropods play a fundamental role in this process (Swift *et al.* 1979; De Oliveira *et al.*
169 2010). Thus, understanding the controls of palatability of lichen tissue as it undergoes senescence
170 contributes to our knowledge of the role lichens play in affecting ecosystem processes.

171

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175

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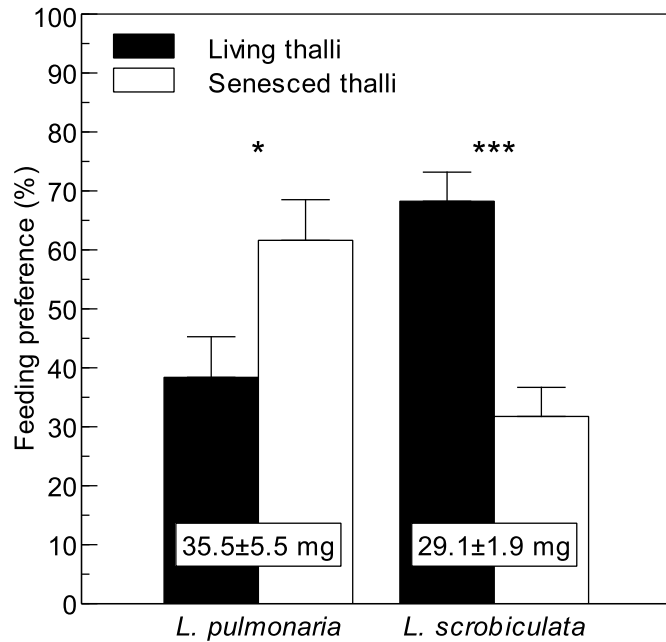
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- 243

244 **Table 1.** Concentrations (mean \pm SE) of secondary compounds (n=10), nitrogen and phosphorus
 245 (n=5) in living and senesced thalli of *Lobaria pulmonaria* and *L. scrobiculata*. *P*-values are from
 246 *t*-tests except where denoted with ^a where a Wilcoxon rank sum test was performed because
 247 assumptions for parametric data analysis could not be satisfied.

	<i>Lobaria pulmonaria</i>			<i>Lobaria scrobiculata</i>		
	Living	Senesced	<i>P</i>	Living	Senesced	<i>P</i>
Stictic acid chemosyndrome (mg g ⁻¹)	39.0 \pm 2.0	20.4 \pm 3.9	<0.001	31.1 \pm 2.2	19.4 \pm 3.5	<0.05
<i>m</i> -Scrobiculin (mg g ⁻¹)	-	-	-	7.3 \pm 1.1	8.6 \pm 1.8	ns
Usnic acid (mg g ⁻¹)	-	-	-	6.0 \pm 0.5	7.0 \pm 1.8	ns
Total CBSCs (mg g ⁻¹)	39.0 \pm 2.0	20.4 \pm 3.9	<0.001	44.4 \pm 2.0	35.0 \pm 4.6	<0.05 ^a
Nitrogen (%)	2.1 \pm 0.08	2.4 \pm 0.08	<0.05	2.6 \pm 0.07	3.0 \pm 0.03	<0.01
Phosphorus (%)	0.16 \pm 0.03	0.15 \pm 0.02	ns	0.29 \pm 0.05	0.32 \pm 0.04	ns

248

249



250
251 **Figure 1.** Feeding preference (expressed as percentage of total consumption, mean + SE) of the
252 snail *Cepaea hortensis* when given the choice between living and senesced thalli of either
253 *Lobaria pulmonaria* or *L. scrobiculata*. * and *** denotes $p < 0.05$ and $p < 0.001$, respectively. T-
254 test was used for *L. pulmonaria* and the Wilcoxon rank sum test was used for *L. scrobiculata*
255 because assumptions for parametric analysis could not be satisfied. Values in boxes represent
256 total consumption (mean ± SE).