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

Johan ASPLUND[1,2]* and David A WARDLE[1]

[1]Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden.

[2]Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, NO-1432 Ås, Norway.

*Corresponding Author:

johan.asplund@slu.se

Phone: +47 47 357 127

Fax: +46 90 786 8163

Asplund J, Wardle DA, 2012. Contrasting changes in palatability following senescence of the lichenized fungi *Lobaria pulmonaria* and *L. scrobiculata*. *Fungal Ecology* **5**: 710–713. Publisher (Elsevier) PDF available at http://dx.doi.org/10.1016/j.funeco.2012.06.004

Asplund J, Wardle DA, 2012. Contrasting changes in palatability following senescence of the lichenized fungi *Lobaria pulmonaria* and *L. scrobiculata*. *Fungal Ecology* **5**: 710–713. Publisher (Elsevier) PDF available at http://dx.doi.org/10.1016/j.funeco.2012.06.004

Abstract (max 150 words):

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

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

Introduction

Lichens are symbiotic associations in which a fungal partner (mycobiont) hosts cells of photobionts (green algae and/or cyanobacteria) that provide carbohydrates and, in the case of cyanobacteria, fix atmospheric nitrogen (N_2) (Nash 2008). Such symbiotic relationships shape plant-like life forms that play a prominent role in boreal forest ecosystems. Cyanobacterial N-fixing lichens may contribute significantly to the N cycling of those ecosystems in which they are dominant components (Nash 2008), such as boreal and temperate rainforests where species of *Lobaria*, *Pseudocyphellaria* and *Sticta* occur in abundance as epiphytes (Green *et al.* 1980; Antoine 2004). In addition, green-algal lichens are efficient accumulators of atmospheric nutrients from wet and dry deposition. For example, Knops *et al*. (1996) showed that the green-algal epiphyte *Ramalina menziesii* augments the input of total N, NO_3, organic N, Ca, Mg, Na and Cl in temperate deciduous forests. Further, lichen litter inputs may have significantly higher quantities of N and micronutrients than leaf litter from trees, because nutrients from tree leaves are usually resorbed back to the plant before abscission, while epiphytic lichen tissues fall to the ground with their nutrient concentration largely unchanged (Knops *et al.* 1991).

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

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

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

Materials and methods

Our study focuses on *Lobaria pulmonaria* and the closely related *Lobaria scrobiculata*. Both these N_2-fixing species are common in temperate and boreal rainforests but rather uncommon elsewhere. The main functional differences between them are the types of photobionts; *L. scrobiculata* has a cyanobacterial photobiont while *L. pulmonaria* has a green-algal photobiont and cyanobacteria in internal cephalodia. Both species exhibit the stictic acid chemosyndrome,

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

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

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

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

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

Results and Discussion

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

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

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

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

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

Acknowledgement

This work was funded by a grant to JA from the Swedish Research Council (Vetenskapsrådet). We thank Ulla Kaasalainen for helpful discussions.

References

Antoine ME, 2004. An ecophysiological approach to quantifying nitrogen fixation by *Lobaria oregana*. *Bryologist* **107**: 82–87.

Asplund J, 2011. Chemical races of *Lobaria pulmonaria* differ in palatability to gastropods. *Lichenologist* **43**: 491–494.

Asplund J, Gauslaa Y, 2008. Mollusc grazing limits growth and early development of the old forest lichen *Lobaria pulmonaria* in broadleaved deciduous forests. *Oecologia* **155**: 93–99.

184 Asplund J, Larsson P, Vatne S, Gauslaa Y, 2010a. Gastropod grazing shapes the vertical
185 distribution of epiphytic lichens in forest canopies. *Journal of Ecology* **98**: 218–225.

186 Asplund J, Solhaug KA, Gauslaa Y, 2010b. Optimal defense - snails avoid reproductive parts of
187 the lichen *Lobaria scrobiculata* due to internal defense allocation. *Ecology* **91**: 3100–
188 3105.

189 Blakemore LC, Searle PL, Daly BK, 1987. Methods for chemical analysis of soils. *New Zealand*
190 *Soil Bureau scientific report* **80**: 1–103.

191 Cooper G, Carroll GC, 1978. Ribitol as a major component of water-soluble leachates from
192 *Lobaria oregana*. *Bryologist* **81**: 568–572.

193 Dubay SA, Hayward GD, Martínez del Rio C, 2008. Nutritional value and diet preference of
194 arboreal lichens and hypogeous fungi for small mammals in the Rocky Mountains.
195 *Canadian Journal of Zoology* **86**: 851–862.

196 Dudley SA, Lechowicz MJ, 1987. Losses of polyol through leaching in subarctic lichens. *Plant*
197 *Physiology* **83**: 813–815.

198 Gauslaa Y, 2005. Lichen palatability depends on investments in herbivore defence. *Oecologia*
199 **143**: 94–105.

200 Green TGA, Horstmann J, Bonnett H, Wilkins A, Silvester WB, 1980. Nitrogen fixation by
201 members of the Stictaceae (Lichenes) of New Zealand. *New Phytologist* **84**: 339–348.

202 Hättenschwiler S, Vitousek PM, 2000. The role of polyphenols in terrestrial ecosystem nutrient
203 cycling. *Trends in Ecology and Evolution* **15**: 238–242.

Holub SM, Lajtha K, 2003. Mass loss and nitrogen dynamics during the decomposition of a ^{15}N-labeled N_2-fixing epiphytic lichen, *Lobaria oregana*. *Canadian Journal of Botany* **81**: 698–705.

Honegger R, 2003. The impact of different long-term storage conditions on the viability of lichen-forming ascomycetes and their green algal photobiont, *Trebouxia* spp. *Plant Biology* **5**: 324–330.

Jørgensen PM, 2007. Lobariaceae. *Nordic Lichen Flora* **3**: 77–86.

Kaasalainen U, Fewer DP, Jokela J, Wahlsten M, Sivonen K, Rikkinen J, 2012. Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis. *Proceedings of the National Academy of Sciences* **109**: 5886–5891.

Killingbeck KT, 1996. Nutrients in senesced leaves: keys to the search for potential resorption and resorption proficiency. *Ecology* **77**: 1716–1727.

Knops JMH, Nash III TH, Boucher VL, Schlesinger WH, 1991. Mineral cycling and epiphytic lichens: Implications at the ecosystem level. *Lichenologist* **23**: 309–321.

Knops JMH, Nash III TH, Schlesinger WH, 1996. The influence of epiphytic lichens on the nutrient cycling of an oak woodland. *Ecological Monographs* **66**: 159–179.

Lockwood JR, 1998. On the statistical analysis of multiple-choice feeding preference experiments. *Oecologia* **116**: 475–481.

McCune B, Daly WJ, 1994. Consumption and decomposition of lichen litter in a temperate coniferous rain-forest. *Lichenologist* **26**: 67–71.

Nash III TH, 2008. Nitrogen, its metabolism and potential contribution to ecosystems. In: Nash III TH (Ed.), *Lichen Biology*, Cambridge University Press, Cambridge, pp. 216–233.

Nash III TH, 2008. *Lichen Biology*. Cambridge University Press, Cambridge.

Newman RM, Hanscom Z, Kerfoot WC, 1992. The watercress glucosinolate-myrosinase system: a feeding deterrent to caddisflies, snails and amphipods. *Oecologia* **92**: 1–7.

Nybakken L, Asplund J, Solhaug KA, Gauslaa Y, 2007. Forest successional stage affects the cortical secondary chemistry of three old forest lichens. *Journal of Chemical Ecology* **33**: 1607–1618.

Oliveira T De, Hättenschwiler S, Handa IT, 2010. Snail and millipede complementarity in decomposing Mediterranean forest leaf litter mixtures. *Functional Ecology* **24**: 937–946.

Solhaug KA, Gauslaa Y, Nybakken L, Bilger W, 2003. UV-induction of sun-screening pigments in lichens. *New Phytologist* **158**: 91–100.

Speiser B, 2001. Food and feeding behaviour. In: Barker GM (Ed.), *The Biology of Terrestrial Molluscs*, CABI, Oxon, pp. 259–288.

Swift MJ, Heal OW, Anderson JM, 1979. *Decomposition in Terrestrial Ecosystems*. Blackwell, Oxford.

Watanabe MF, Tsuji K, Watanabe Y, Harada K, Suzuki M, 2006. Release of heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*. *Natural Toxins* **1**: 48–53.

244 **Table 1**. Concentrations (mean ± 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.

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

248

249

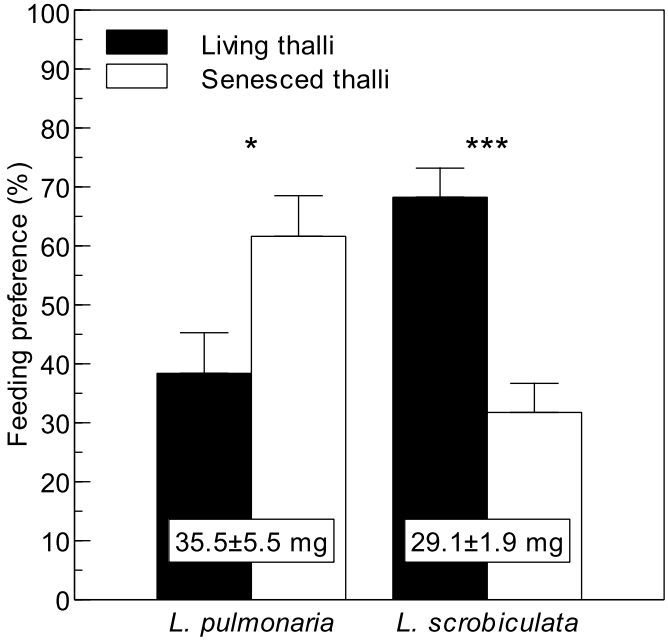

Figure 1. Feeding preference (expressed as percentage of total consumption, mean + SE) of the snail *Cepaea hortensis* when given the choice between living and senesced thalli of either *Lobaria pulmonaria* or *L. scrobiculata*. * and *** denotes $p<0.05$ and $p<0.001$, respectively. T-test was used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