Changes in functional traits of the terricolous lichen *Peltigera aphthosa* across a retrogressive boreal forest chronosequence

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Abstract: Changes in the functional traits of the terricolous lichen *Peltigera aphthosa* with declining soil fertility during ecosystem retrogression were investigated. A well-documented retrogressive chronosequence of 28 forested islands in northern Sweden that differ greatly in fire history and which spans 5000 years was used. The abundance of cephalodia increased, indicative of higher N_2-fixation rates resulting from lower N availability. Thallus $\delta^{13}C$ values increased with ageing soils, in line with declining $\delta^{13}C$ values of the humus substratum along this gradient. However, $\delta^{13}C$ values were also driven by variation in factors that were at least partly independent of soil ageing. As such, $\delta^{13}C$ values were mostly related to specific thallus mass (STM), possibly because a higher STM gives a thicker cortical layer and thus greater resistance to CO_2 diffusion, leading to higher $\delta^{13}C$ values. STM and other measured traits (i.e. thallus N, P, secondary compounds and water-holding capacity) were unresponsive to the gradient, despite these traits being very responsive to the same gradient in epiphytic lichen species.

Key words: lichenized fungi, nitrogen, phosphorus, secondary compounds, soil fertility, specific thallus mass, stable isotopes, water-holding capacity

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Introduction

Ecosystem succession is characterized by an initial build-up phase during which nutrients accumulate, resulting in a maximum biomass phase (Odum 1969; Grime 2001; Walker & del Moral 2003). However, in the long-term absence of major disturbance (normally in the order of millennia), ecosystem retrogression occurs, which involves increasing nutrient limitation and a decline in plant productivity and standing biomass, as well as corresponding impairment of belowground processes (Walker *et al.* 1983; Wardle *et al.* 2004; Peltzer *et al.* 2010). Several recent studies on chronosequences that have included retrogressed stages have shown distinct responses in plant functional traits as soil fertility declines over time (Cordell *et al.* 2001; Richardson *et al.* 2005; Crutsinger *et al.* 2008; Bansal *et al.* 2012; Wardle *et al.* 2012; Lagerström *et al.* 2013). In light of this, there is a growing interest in understanding the variation in functional traits across environmental gradients because of the role traits play in community assembly, niche overlap and ecosystem processes (Bolnick *et al.* 2011). Until recently, studies on functional trait variability have focused on interspecific variation, and the importance of intraspecific trait variability has been recognized as an important ecological driver only in the past few years (Albert *et al.* 2010; Messier *et al.* 2010; Kichenin *et al.* 2013). We have recently shown that functional traits of epiphytic lichens are highly responsive to ecosystem retrogression, and at the within-species level are much more responsive than those of vascular plant species (Asplund *et al.* 2012; Asplund & Wardle 2014).

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However, whether functional traits of terricolous lichens show comparable responses to ecosystem retrogression remains unknown.

We used a previously established, fire-driven boreal forest chronosequence spanning 5000 years to evaluate how functional traits of the terricolous lichen *Peltigera aphthosa* (L.) Willd. vary with ecosystem retrogression. The study system is well established as a chronosequence because all major driving factors other than chronosequence stage are consistent across all units and confounding factors are therefore largely lacking (Wardle *et al.* 1997, 2012). As such, this particular chronosequence has been extensively used to test how ecosystem retrogression affects various ecosystem processes, as well as the functional traits of both plants and epiphytic lichens (Wardle *et al.* 2004, 2012; Wardle & Zackrisson 2005; Clemmensen *et al.* 2013; Lagerström *et al.* 2013; Asplund & Wardle 2014). First, we hypothesized that with decreasing soil fertility, and as retrogression proceeds, there will be a shift in lichen thallus traits from those associated with rapid resource capture of soil nutrients and light to those linked to resource conservation, in a similar manner to that well known to occur for vascular plant leaf traits (Grime 1979; Aerts & Chapin 2000). As such, we predicted increasing specific thallus mass (STM) and carbon-based secondary compounds (CBSCs), and decreasing thallus nitrogen (N) and phosphorus (P) during retrogression. Secondly, we hypothesized that as the availability of soil N to living organisms declines during retrogression (Wardle *et al.* 1997), *P. aphthosa* will have higher densities of cephalodia (containing N_2-fixing *Nostoc* sp.) to allow greater biological N_2-fixation and thus compensate for lower N availability, and that *P. aphthosa* thalli will therefore have higher $\delta^{15}N$ values (Brenner *et al.* 2001). Thirdly, we hypothesized that thallus $\delta^{13}C$ values will increase with ecosystem retrogression as a consequence of the higher $\delta^{13}C$ values of their substratum (Hyodo & Wardle 2009). We note that the lichen functional traits we measured may also be influenced by factors that are at least partly independent of island size or retrogressive stage. As these factors may also contribute to the understanding of trait variation in this system, they were also considered.

Because of the role lichen functional traits play in regulating lichen tissue decomposition rates and consumption by invertebrates, and thus in the turnover rates of carbon and nutrients in the ecosystem (Lang *et al.* 2009; Asplund & Wardle 2013), our study contributes to the understanding of how long-term ecosystem development may influence the role of lichens in community and ecosystem processes (Knops *et al.* 1996; Nash 2008).

Material and Methods

We used 28 forested islands located in two neighbouring lakes, Hornavan and Uddjaur, in the northern boreal zone of Sweden (65°55'N, 17°43'E to 66°09'N, 17°55'E). These islands, consisting of moraine deposits, vary in size from 0·02 to 15 ha and are grouped into three size classes: 10 'large' (>1·0 ha), 9 'medium' (0·1 to 1·0 ha) and 9 'small' (<0·1 ha) (Wardle *et al.* 2003, 2012). Fire history is dependent on island size since larger islands are more often hit by lightning. As such, the mean time since fire for the large, medium and small islands is 585, 2180 and 3250 years respectively, according to ^{14}C dating of charcoal particles in the humus layer (Wardle *et al.* 1997, 2003). With increasing time since fire and decreasing island size, the islands enter a state of retrogression, with lower soil fertility, plant biomass and productivity. Further details about the environmental properties for each of the three island size classes are given in the Appendix. In this system, we collected thalli from the lichen *P. aphthosa*, growing on soil among feather mosses. This green-algal lichen supports colonies of N_2-fixing *Nostoc* sp. in cephalodia which can be seen as black dots on the thallus surface. This species was chosen for the present study because it is the only terricolous lichen that occurs across the entire chronosequence.

For each island, lobes from ten thalli were collected in August 2012 in the vicinity of plots used for previous work (Wardle *et al.* 2003, 2012), from which most of the additional data used in the present study are derived (Appendix). Plots were located at comparable distances from the shore to minimize the potential of confounding factors covarying with island size, such as edge effects and macroclimate (Wardle *et al.* 2003). The thalli were not collected directly beneath tree canopies.

The collected lichens were hydrated by spraying deionized water on the upper surface in the laboratory. From each of the ten well-moistened lobes per island, one disc with an area of 1·18 cm^2 was taken out with a cork borer. Each disc was then photographed in the fully hydrated state with a Konica Minolta Dynax 7D digital

SLR (Konica, Tokyo, Japan) fitted with a Sigma 105 mm f2.8 DG EX macro lens (Sigma, Kawasaki, Japan), following Asplund *et al.* (2010), and the percent area cover of cephalodia (cephalodia abundance) was determined using ImageJ 1.46 (National Institutes of Health, Bethesda, Maryland). Water-holding capacity (WHC) and specific thallus mass [STM, the lichen equivalent of plant-specific leaf mass (i.e. reciprocal of specific leaf area)] of each disc was then determined following Gauslaa & Coxson (2011). Next, the fully hydrated discs were gently blotted and wet mass (WM) was recorded (± 0.1 mg). Discs were subsequently oven-dried (24 h at 70°C) and dry mass (DM) was recorded. From these measurements, STM was computed as DM/area and WHC as (WM – DW)/area. Blotting, as opposed to only shaking the lichen thallus prior to determining WM, removes zero potential surface water (Snelgar & Green 1981). However, the amount of water stored on the thallus surface and measured on shaken thalli is not always constant between measurements and therefore difficult to estimate correctly; for this reason, we utilized blotting as the preferred method in our study. For each island, the remaining lichen material was collectively ground in a ball mill for chemical analyses. Concentrations of N and P were determined by Kjeldahl digestion followed by automatic colorimetric methods (Blakemore *et al.* 1987). To analyze concentrations of CBSCs, *c.* 30 mg of the powder was extracted for three 45 min intervals in acetone. The combined supernatants were evaporated to dryness and dissolved in 1–2 ml 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). Compound identification was based on retention times, online UV spectra and co-chromatography of commercial standards.

For lichen material from each island, stable N and C isotope ratios were measured on ground and dried (70°C, 18 h) material contained in folded tin capsules using an isotope ratio mass spectrometer (Delta V®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with an elemental analyzer (Flash EA 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The natural abundances of ^{13}C and ^{15}N were expressed in per mil (‰) deviation from international standards (VPDB and atmospheric N for C and N, respectively); δ^{13}C and δ^{15}N was calculated as ($R_{sample}/R_{standard} - 1$) $\times$ 1000, where R in δ^{13}C and δ^{15}N is ^{13}C/^{12}C and ^{15}N/^{14}N, respectively.

Individual islands served as the units of replication. One-way ANOVAs were used to test for the effect of island size class on each functional trait measured. When island size effects were significant, means across island size classes were compared by post-hoc comparison (Tukey's test) at $P < 0.05$. Relationships between lichen functional traits and previously measured environmental variables (island size, light transmission through the canopy, soil total N, soil N : P ratio, soil mineral N and soil mineral P), and among the functional traits, were analyzed with Spearman's rank correlation coefficients among the 28 islands. All statistical analyses were performed in R 2.15.2.

Results and Discussion

Our first hypothesis, which predicted that as retrogression proceeds and soil fertility declines there will be a shift in lichen thallus traits from those associated with rapid resource capture of soil nutrients and light to those linked to resource conservation, was not supported. As such, thallus concentrations of N, P, STM, WHC, and the CBSCs gyrophoric acid and methylgyrophorate (the only two CBSCs that were detected), did not respond to island size (Fig. 1A–G). These findings contrast with previous work on epiphytic lichens growing on *Betula pubescens* in the same study system, for which thalli had much higher nutrient concentrations and STM on small, less fertile islands (Asplund *et al.* 2012; Asplund & Wardle 2014). For thallus N, this unresponsiveness could be explained by *P. aphthosa* regulating its N status through N_2-fixation (Dahlman & Palmqvist 2003). While STM did not respond to island size class, we did find that STM increased with declining soil mineral N (Table 1). This suggests that the high level of variation in soil N availability between islands that is independent of island size or stage of retrogression may exert an effect on STM (Appendix).

We found no relationship between STM and light (Table 2). This contrasts with earlier studies indicating that light can promote STM of lichens by causing increased desiccation and thereby decreased hyphal expansion (leading to a decrease in surface area) but an increase in mass within the area that the lichens occupy (Snelgar & Green 1981; Dahlman & Palmqvist 2003; Solhaug *et al.* 2009; Asplund *et al.* 2012). For instance, epiphytic lichens in this study system tend to have higher STM on those islands that have high light transmission through the canopy (Asplund *et al.* 2012; Asplund & Wardle 2014). However, *P. aphthosa* has a higher water-holding capacity than do the main
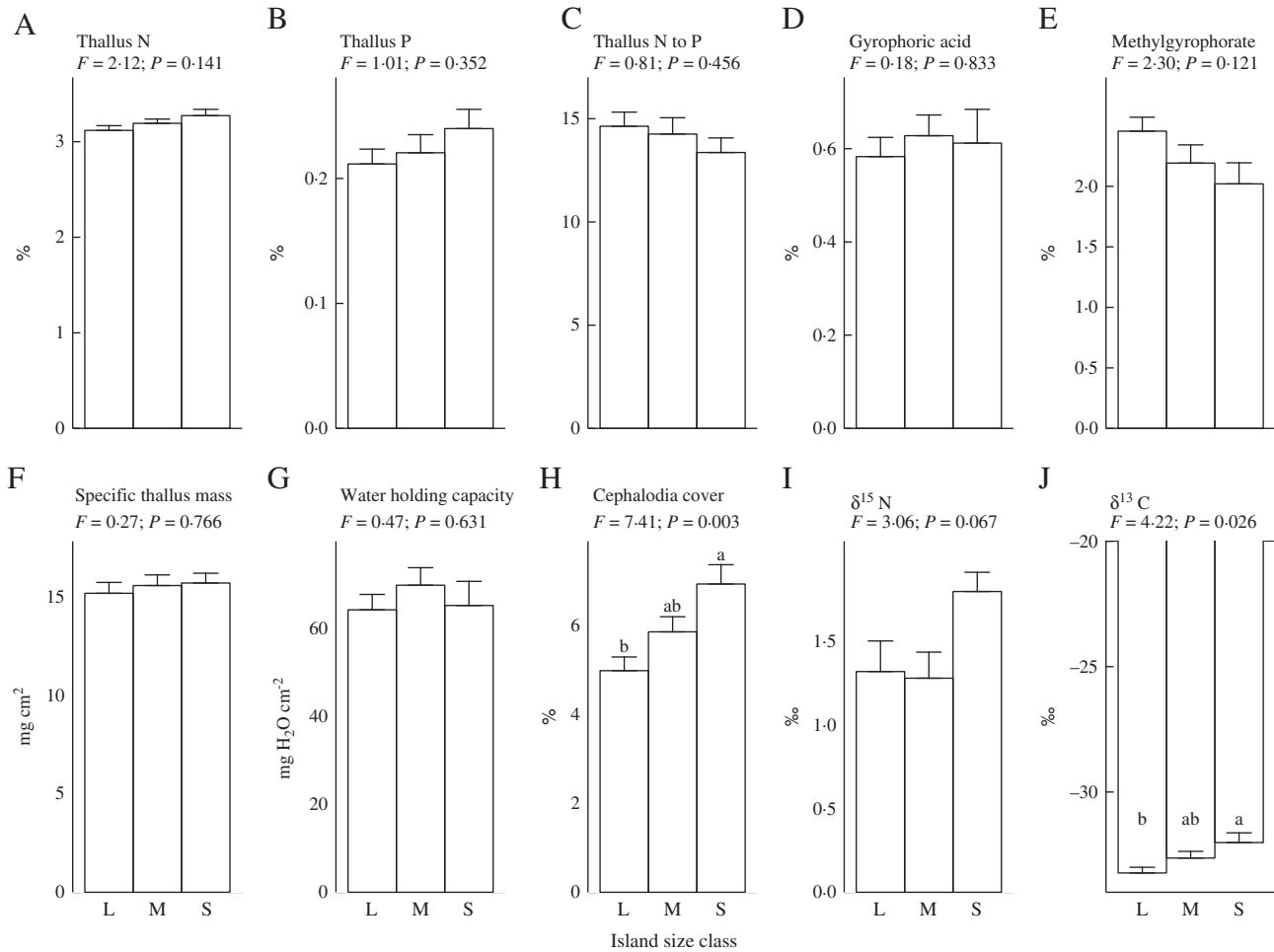
A

Thallus N
$F = 2.12; P = 0.141$

%

3

2

1

0

B

Thallus P
$F = 1.01; P = 0.352$

%

0.2

0.1

0.0

C

Thallus N to P
$F = 0.81; P = 0.456$

15

10

5

0

D

Gyrophoric acid
$F = 0.18; P = 0.833$

%

0.6

0.4

0.2

0.0

E

Methylgyrophorate
$F = 2.30; P = 0.121$

%

2.0

1.5

1.0

0.5

0.0

F

Specific thallus mass
$F = 0.27; P = 0.766$

mg cm^{-2}

15

10

5

0

L M S

G

Water holding capacity
$F = 0.47; P = 0.631$

mg H$_2$O cm^{-2}

60

40

20

0

L M S

H

Cephalodia cover
$F = 7.41; P = 0.003$

%

a
ab
b

6

4

2

0

L M S

I

δ^{15}N
$F = 3.06; P = 0.067$

‰

1.5

1.0

0.5

0.0

L M S

J

δ^{13}C
$F = 4.22; P = 0.026$

‰

−20

−25

−30

b   ab   a

L M S

Island size class

FIG. 1. Relationships between functional traits of thalli of the terricolous lichen *Peltigera aphthosa* and island size class (L = large, M = medium and S = small). Mean values are plotted ±1 SE. *F* and *P*-values are derived from one-way ANOVAs (df = 2 and 25, respectively). Within each panel, letters on top of bars denote significant differences ($P < 0.05$, Tukey's *post hoc* test). 'Cephalodia cover' = percent area covered with cephalodia, N = nitrogen, P = phosphorus.

TABLE 1. *Relationships between lichen traits in* Peltigera aphthosa *and environmental variables collected from 28 islands. Values given are Spearman rank correlation coefficients.*

	Thallus N	Thallus P	Thallus N:P	Gyrophoric acid	Methylgyrophorate	STM	WHC	Cephalodia	$\delta^{13}C$	$\delta^{15}N$
Island Size	−0·305	−0·147	0·063	−0·139	0·345	−0·131	0·073	**−0·539****	**−0·393***	**−0·424***
Light	0·149	−0·346	**0·415***	0·068	0·001	0·229	−0·026	0·127	0·272	0·227
Soil total N	0·149	0·311	−0·260	−0·049	−0·312	0·062	−0·106	0·370	**0·415***	0·235
Soil N to P	0·137	0·189	0·038	0·105	−0·338	0·036	−0·177	0·323	0·330	**0·391***
Soil Mineral N	−0·285	0·357	**−0·433***	0·066	0·147	**−0·491****	−0·299	0·137	**−0·376***	−0·176
Soil Mineral P	−0·278	−0·113	0·023	−0·003	0·334	−0·329	−0·060	−0·131	**−0·425***	**−0·444***

Light = light transmission through the canopy, N = nitrogen, P = phosphorus, STM = specific thallus mass, WHC = water-holding capacity, 'cephalodia' = percent area covered with cephalodia. Bold values indicate significant correlations ($P < 0.05$) and * and ** indicate significant relationships at $P = 0.05$ and 0.01, respectively.

species of epiphytic green-algal lichens in this system (Asplund & Wardle 2013), and by growing on the ground amongst poikilohydric mosses it is less exposed to desiccation compared with epiphytes (Colesie *et al.* 2012). Taking both these factors into account, *P. aphthosa* is expected to be less responsive in terms of its STM, relative to changes in light availability.

In line with our second hypothesis, we found the relative abundance of cephalodia to increase with decreasing island size (Fig. 1H). This suggests a higher N_2-fixing ability of *P. aphthosa* on small islands. We also found thallus N concentration to increase with cephalodia abundance (Table 2). Our findings are consistent with earlier work on these islands showing that with decreasing island size, cyanobacteria associated with feather mosses also show increasing N_2-fixation rates (Lagerström *et al.* 2007), and that vascular plants and mosses show increasing $\delta^{15}N$ values (Hyodo & Wardle 2009; Bansal *et al.* 2012). One likely explanation for the pattern observed is that reduced N availability on small retrogressed islands (Wardle *et al.* 1997) promotes the production of cephalodia in order to enable higher N_2-fixation rates. In line with this, NH_4^+ is known to impede N_2-fixation for *P. aphthosa* (Rai *et al.* 1981; Hällbom & Bergman 1983). However, we found no significant correlations between individual soil nutrients and cephalodia abundance. Island size, as a proxy for retrogression stage, integrates all variables that change as time since fire disturbance increases and retrogression proceeds (Wardle *et al.* 2012), and is therefore a more robust explanatory variable than individual soil variables are for understanding responses to ecosystem retrogression. In light of this, it is not surprising to find significant effects of island size but not of individual soil nutrients. Furthermore, there was a positive relationship between relative abundance of cephalodia and thallus P concentration (Table 2). Nitrogen-fixing activity by symbiotic cyanobacteria in lichens is strongly regulated by P supply (Crittenden *et al.* 1994; Kurina & Vitousek 1999), and Raggio *et al.* (2012) observed positive relationships between lichen thallus P

TABLE 2. Relationships between lichen traits in Peltigera aphthosa collected from 28 islands. Values given are Spearman rank correlation coefficients.

	Thallus N	Thallus P	Gyrophoric acid	Methylgyrophorate	STM	WHC	Cephalodia	$\delta^{13}C$
Thallus P	**0·685***							
Gyrophoric acid	−0·160	−0·276						
Methylgyrophorate	−0·166	−0·330	0·239					
STM	−0·151	−0·285	0·174	−0·290				
WHC	−0·068	−0·003	−0·113	**−0·380***	0·349			
Cephalodia	**0·448***	**0·457***	−0·291	−0·194	−0·192	0·041		
$\delta^{13}C$	0·097	−0·204	0·141	−0·373	**0·637***	**0·518****	0·200	
$\delta^{15}N$	**0·551****	0·241	0·321	0·039	−0·019	−0·372	0·102	0·057

N = nitrogen, P = phosphorus, STM = specific thallus mass, WHC = water-holding capacity, 'cephalodia' = percent area covered with cephalodia. Bold values indicate significant correlations ($P < 0.05$) and *, ** and *** indicate significant relationships at $P = 0.05$, 0.01 and 0.001, respectively.

and acetylene reduction (which is indicative of biological N_2 fixation).

We predicted that lichens on small islands should have higher $\delta^{15}N$ values due to increased N_2-fixation. We found that thallus $\delta^{15}N$ values were negatively related to island size (Table 1) and were higher on small islands although this difference was not statistically significant (Fig. 1I). However, increased biological N_2-fixation should result in $\delta^{15}N$ values increasing from negative towards zero, and not increasing to more positive values in the manner that we observed here. This is because the $\delta^{15}N$ of atmospheric N_2 is zero, and that of N in precipitation is generally below zero (i.e. 0 to −10‰) (Nadelhoffer & Fry 1994). Furthermore, we found no significant relationship between density of cephalodia (where N_2-fixation is located) and $\delta^{15}N$ (Table 2). An alternative explanation for the increasing $\delta^{15}N$ values observed with decreasing island size is that the lichens may increasingly use organic N relative to inorganic N. Due to discrimination against ^{15}N during mineralization, inorganic N should be more depleted in ^{15}N than organic N (Nadelhoffer & Fry 1994) and, in our study system, availability of inorganic N declines while dissolved organic N increases with declining island size (Wardle & Zackrisson 2005).

We found support for our third hypothesis that thallus $\delta^{13}C$ should increase as ecosystem retrogression proceeded (Fig. 1J). This is in line with what we know from vascular plants and mosses in this system (Hyodo & Wardle 2009; Bansal et al. 2012). A possible explanation is that ground-dwelling lichens potentially assimilate CO_2 from respiration of C derived from the humus substratum, which may influence $\delta^{13}C$ values of the thalli (Broadmeadow et al. 1992; Tarnawski et al. 1994; Máguas et al. 1995; Lakatos et al. 2007; Colesie et al. 2012), and that as retrogression proceeds, humus $\delta^{13}C$ increases (Hyodo & Wardle 2009). It has also previously been shown that $\delta^{13}C$ values in lichens are dependent on the substratum upon which they grow (Beck & Mayr 2012). In support of this explanation, we found a positive relationship between humus $\delta^{13}C$ (using data from

Hyodo & Wardle 2009) and *P. aphthosa* δ^{13}C (Pearson correlation $r = 0.374$, $P < 0.05$). Light has an important influence on the internal CO_2 concentration of active lichens and therefore δ^{13}C discrimination (Farquhar *et al*. 1989; Palmqvist 2000). However, we found no relationship between light and δ^{13}C values, suggesting that the observed increase in δ^{13}C with ecosystem retrogression is not driven by differences in light availability between the islands. Furthermore, we found *P. aphthosa* δ^{13}C to show a positive relationship with both STM and WHC (Table 2). However, these relationships do not explain why δ^{13}C increases with ecosystem retrogression, because both STM and WHC were both independent of island size. This suggests that variation in factors that are unrelated to ecosystem retrogression and island size are also important drivers of δ^{13}C. These patterns probably emerged because greater thallus thickness (i.e. higher STM) increases the water-holding capacity, which in turn results in higher CO_2 diffusion resistance, thereby promoting δ^{13}C (Lange *et al*. 1988; Lange & Green 1996; Lakatos *et al*. 2007; Gauslaa & Coxson 2011). Furthermore, higher STM also results in a thicker cortical layer which increases CO_2 diffusion resistance (Máguas & Brugnoli 1996; Gauslaa & Coxson 2011).

Conclusions

While there is an extensive literature on how functional traits of vascular plants drive key ecological processes such as decomposition, herbivory, and the cycling of carbon and nutrients, functional traits of lichens may also have a major, though often overlooked impact on these processes (Lang *et al*. 2009; Asplund & Wardle 2013; Colesie *et al*. 2014). As such, an improved knowledge of how functional traits of lichens change along environmental gradients may help us to better understand how environmental changes have an impact on ecosystem processes. We have studied how functional traits of a N_2-fixing ground-dwelling lichen, *P. aphthosa,* respond to a strong gradient in soil fertility driven by ecosystem

retrogression. Although previous work has revealed that traits of epiphytic lichens and of vascular plants respond strongly to this gradient (Asplund *et al*. 2012; Asplund & Wardle 2014; Kumordzi *et al*. 2014), we found that most traits of *P. aphthosa* are relatively unresponsive. Furthermore, most traits were unaffected by changes in light availability across the gradient, which contrasts with what has been previously shown for responses of epiphytic lichens in this system (Asplund *et al*. 2012; Asplund & Wardle 2014). These contrasting findings between epiphytic and ground-dwelling lichens highlight the importance of studying different functional groups of primary producers along the same gradient.

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Appendix. Ecosystem properties and island size

Changes in selected ecosystem properties across the island size gradient. Values are means with standard errors in brackets. Within each row numbers followed by the same letter are not statistically significant at $P = 0.05$ (Tukey's test following one-way ANOVA). Data from Wardle *et al.* (1997, 2003, 2004) and Hyodo & Wardle (2009).

Ecosystem property	Island size		
	Small	Medium	Large
Island size (ha)	0·06 (0·01) c	0·37 (0·08) b	4·8 (1·60) a
Humus $\delta^{13}C$	−27·6 (0·13) a	−28·0 (0·10) b	−28·2 (0·13) b
Light transmission through forest canopy (%)	67·0 (2·3) a	46·8 (4·1) b	55·9 (4·5) ab
Soil N (%)	1·63 (0·05) a	1·43 (0·06) ab	1·24 (0·05) b
Soil N to P ratio	22·6 (0·9) a	18·6 (0·9) b	15·5 (0·5) c
Soil mineral N ($\mu g\ g^{-1}$)	14·4 (2·2)	19·9 (3·9)	21·6 (5·7)
Soil mineral P ($\mu g\ g^{-1}$)	25·8 (2·2) b	36·8 (4·7) ab	43·6 (4·9) a