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Effects of nutrient addition on leaf chemistry, morphology, and photosynthetic capacity of three bog shrubs

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Abstract Plants in nutrient-poor environments typically have low foliar nitrogen (N) concentrations, long-lived tissues with leaf traits designed to use nutrients efficiently, and low rates of photosynthesis. We postulated that increasing N availability due to atmospheric deposition would increase photosynthetic capacity, foliar N, and specific leaf area (SLA) of bog shrubs. We measured photosynthesis, foliar chemistry and leaf morphology in three ericaceous shrubs (*Vaccinium myrtilloides*, *Ledum groenlandicum* and *Chamaedaphne calyculata*) in a long-term fertilization experiment at Mer Bleue bog, Ontario, Canada, with a background deposition of 0.8 g N m^{-2} a^{-1}. While biomass and chlorophyll concentrations increased in the highest nutrient treatment for *C. calyculata*, we found no change in the rates of light-saturated photosynthesis (A_{max}), carboxylation (V_{cmax}), or SLA with nutrient (N with and without PK) addition, with the exception of a weak positive correlation between foliar N and A_{max} for *C. calyculata*, and higher V_{cmax} in *L. groenlandicum* with low nutrient addition. We found negative correlations between photosynthetic N use efficiency (PNUE) and foliar N, accompanied by a species-specific increase in one or more amino acids, which may be a sign of excess N availability and/or a mechanism to reduce ammonium (NH_4) toxicity. We also observed a decrease in foliar soluble Ca and Mg concentrations, essential minerals for plant growth, but no change in polyamines, indicators of physiological stress under conditions of high N accumulation. These results suggest that plants adapted to low-nutrient environments do not shift their resource allocation to photosynthetic processes, even after reaching N sufficiency, but instead store the excess N in organic compounds for future use. In the long term, bog species may not be able to take advantage of elevated nutrients, resulting in them being replaced by species that are better adapted to a higher nutrient environment.

Keywords N deposition · Nutrient use efficiency · Amino acids · Ammonium toxicity · Peatland · Polyamines

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Introduction

The anthropogenic release of nitrogen (N) into the atmosphere has accelerated N cycling globally (Galloway et al. 2004). Atmospheric N deposition has the potential to enhance plant productivity in N-limited ecosystems (Zaehle et al. 2010 and references therein). However, the rate and duration of N deposition impact cellular and soil N status, and deposition rates can exceed the capacity for N uptake by plants (Aber et al. 2003). The acidifying effect of precipitated NO_3, and the consequent leaching of vital cations such as calcium (Ca) and magnesium (Mg), can lead to nutrient imbalances and ecosystem decline (Ågren and Bosatta 1988; Aber et al. 1998, 2003; Fenn et al. 1998; Bauer et al. 2001). The response to additional N supply depends largely on the physiological adaptations of individual plant species. In peatlands, particularly nutrient-poor bogs, atmospheric N deposition changes plant community composition, enhancing vascular plant growth to the detriment of the moss layer (Bubier et al. 2007). However, mixed results have been reported with respect to changes in whole ecosystem productivity, arising from differences in species composition, physiology and resource utilization (Heijmans et al. 2001; Tomassen et al. 2003; Bragazza et al. 2004; Limpens et al. 2008; Juutinen et al. 2010). Will atmospheric N deposition alleviate the nutrient stress of bog plants and allow species to become more productive, thus sequestering more carbon (C)? Are these species able to shift their allocation strategies and life history traits? Will these species be able to produce more leaves or increase rates of photosynthesis and shift to lower nutrient conservation?

Plants in nutrient-poor environments such as bogs and arctic tundra are adapted in several ways to the slow turnover of N, phosphorus (P) and potassium (K). For example, ericaceous evergreen shrubs make long-lived tissues, including woody stems and roots, as well as leaves that live for 1–4 years (Eckstein et al. 1999; Burns 2004; Wright et al. 2004). Moreover, producing thick, waxy leaves is a strategy to conserve nutrients, prevent frost damage, and reduce water loss with high re-absorption from senescing leaves (Aerts 1995; Burns 2004; Wright et al. 2004). However, nutrient use efficiency requires important trade-offs with productivity, resulting in slow growth rates and lower foliar N concentrations, along with lower rates of maximum photosynthesis (Berendse and Aerts 1987; Oberbauer and Oechel 1989; Reich et al. 1998; Shipley et al. 2006). Theoretically, the light-saturated rate of photosynthesis (A_{max}) increases with increasing foliar N allocation to proteins, particularly to ribulose-1,5-bisphosphate carboxylase (Rubisco), which determines the rate of carboxylation (V_{cmax}) (Bowes 1991). Nutrient addition can also lead to enhanced growth, producing more leaves, and competition for light can result in increased leaf area per leaf mass (specific leaf area, SLA) (Shaver et al. 2000; Niinemets and Kull 2003; Burns 2004).

However, there is evidence that plants can partition excess N such that photosynthesis is downregulated rather than enhanced after the overall nutrient balance reaches a critical threshold (Bauer et al. 2004). For example, in peatlands, Bragazza et al. (2004) and Granath et al. (2009a) found that 1–1.5 g N m^{-2} a^{-1} was the optimal level for *Sphagnum* moss photosynthesis, with lower growth rates at higher N deposition levels. An overload of N, particularly ammonium (NH_4), can be toxic to plant cells, and the presence of amino acids and polyamines (PAs) in high concentrations could explain the strategy by which plants cope with excess N if it is not invested into the primary processes of CO_2 assimilation. While several studies have examined the role of these N-rich organic compounds in forest plant communities, few have examined their role in peatland plants, and most of those studies have focused on *Sphagnum* mosses rather than vascular plants (e.g., Limpens and Berendse 2003; Tomassen et al. 2003; Wiedermann et al. 2009).

In the Mer Bleue bog fertilization study, we have found increases in dwarf shrub growth and a loss of moss biomass, but either no change or decreases in ecosystem photosynthesis rates in response to 9 years of nutrient (N and NPK) addition (Juutinen et al. 2010). The shift in community composition along with changes in plant physiology could explain the ecosystem response. The goals of the current study were to examine the physiological responses of the dominant bog shrubs at Mer Bleue to increased nutrient availability. Our research questions were as follows. (1) How does nutrient addition affect the leaf chemistry and morphology, stress-related metabolism, photosynthetic capacity, and photosynthetic N use efficiency (PNUE) of the ericaceous shrubs *Chamaedaphne calyculata* Moench, *Ledum groenlandicum* Oeder, and *Vaccinium myrtilloides* Michx? (2) How do leaf traits relate to abundance of these species over the duration of the experiment? We hypothesized that nutrient (N and NPK) addition would increase SLA, foliar N, chlorophyll, photosynthetic capacity and PNUE. To address these issues, we studied shrub abundance, leaf dimensions, foliar concentrations of nutrients, soluble ions, free amino acids, free polyamines (PAs), soluble proteins, chlorophyll, and photosynthetic parameters [light saturated net photosynthesis (A_{max}), maximum carboxylation capacity (V_{cmax}), electron transport (J_{max}), and triose phosphate utilization (TPU)], under different nutrient treatments.

Materials and methods

Site description and experimental design

This study was conducted at Mer Bleue bog near Ottawa, Ontario, Canada (45°40′N, 75°50′W), which has a cool

Table 1 Experimental set-up with NPK fertilization levels equal to 5 and 20 times the ambient growing season wet N deposition

Treatment	N (g m^{-2} a^{-1})	P (g m^{-2} a^{-1})	K (g m^{-2} a^{-1})
Control	0	0	0
5N	1.6	0	0
5NPK	1.6	6.3	5
20N	6.4	0	0
20NPK	6.4	6.3	5

continental climate and a mean annual temperature of 6.0°C (monthly range −11 to 21°C), as well as a mean annual precipitation of 944 mm (Canadian Climate Normals 1971–2000). The experimental site is located in the ombrotrophic part of the peatland, where vegetation is dominated by dwarf ericaceous shrubs along with a ground layer of the mosses *Sphagnum magellanicum* Brid., *Sphagnum capillifolium* (Ehrl.) Hedw., and *Polytricum strictum* Brid. Background inorganic wet N deposition in this region is ~0.8 g N m^{-2} a^{-1} (Turunen et al. 2004). We chose N amendments of 1.6 and 6.4 g m^{-2} a^{-1} because they represent the range of probable increases in N deposition in peatland regions of North America and Europe in the twenty-first century (e.g., Reay et al. 2008). Thus, we experimentally increased the ambient growing season wet N deposition by factors of 5 and 20.

We began treatments (control, 5N, 5NPK and 20NPK) in 2000–2001; an additional treatment (20N) was initiated in 2005 (Table 1). Each treatment had three replicate plots 3 × 3 m in size. Fertilizer was given in soluble form, N as NH_4NO_3 and PK as KH_2PO_4, dissolved in 18 L distilled water (equivalent to the application of 2 mm of water), at 3 week intervals from early May to late August. Control plots were treated with distilled water.

Aboveground growth and leaf morphology

The response of whole plant growth to fertilization was examined by measuring the abundance of vascular plant species in a 60 × 60 cm quadrat in each treatment plot at the beginning of the experiment in 2000 and again in 2008. Stem number and stem height of each species were recorded in 2000. The number of hits to a metal rod (radius 4 mm) in 61 grid points of a 60 × 60 cm frame was recorded in 2008. Owing to the different methods of estimating abundance, differences between control and nutrient treatments were examined for each year separately.

In 2008, we measured the length, width, and thickness of 8–9 leaves per treatment for each of the three shrub species from different evenly spaced plants in each plot. These leaves were first measured for CO_2 exchange. We only used leaves from the top canopy, and performed the measurements within 3 weeks during the growing season from mid-July to mid-August. Leaves were frozen after removal from the field, weighed, then oven-dried for 48 h at 50°C and re-weighed. We determined leaf moisture content for each species and treatment and calculated specific leaf area (SLA, cm^2 g^{-1} leaf). We compared these leaf measurements with a larger set of randomly selected leaves and found that treatment had a similar effect on leaf area and mass in both datasets.

CO_2 exchange measurements and parameter estimation

We measured the CO_2 exchange of intact leaves (same leaves were measured for morphology) using a portable photosynthesis system LI-6400 (Li-Cor, Lincoln, NE, USA), including an infrared gas analyzer and a leaf cuvette equipped with temperature, light, CO_2 and humidity controls. The response of net photosynthesis (*A*) to intercellular CO_2 concentration (*Ci*) was measured in 13 set points of external CO_2 concentration ranging from 50 to 2,100 ppm. Chamber conditions other than CO_2 were kept constant: temperature, 25°C; flow, 150 µmol s^{-1}; humidity, ~50%; and photosynthetic photon flux density (PPFD) 1,300 µmol photons m^{-2} s^{-1}. We found that CO_2 uptake was light saturated at 1,300 µmol photons m^{-2} s^{-1}.

We used an application (Sharkey et al. 2007) based on equations in Farquhar et al. (1980) to fit *A*/*Ci* curves and estimate parameters for maximum carboxylation capacity (V_{cmax}), maximum RuBP regeneration (J_{max}) and triose phosphate utilization (TPU). We estimated parameters for each leaf sample individually, and light saturated net photosynthesis (A_{max}) was measured at the ambient CO_2 concentration. Parameters are expressed mainly per unit leaf area (µmol CO_2 m^{-2} s^{-1}), but were also calculated per unit leaf mass (µmol CO_2 g^{-1} s^{-1}). We determined photosynthetic N use efficiency (PNUE), expressing A_{max} per unit leaf N. Leaf areas inside the cuvette, as well as whole leaf areas, were determined from digital images of the leaves.

Biochemical analyses

Leaves measured for CO_2 exchange were oven dried and analyzed for total C and N concentrations using a Carlo Erba (Milan, Italy) NC2500 elemental analyzer. A separate set of leaves was collected for biochemical analyses conducted at the US Forest Service, Durham, New Hampshire: leaves from five evenly spaced plants from each of two replicate plots (ten plants/treatment/species). The only exception to this was *V. myrtilloides*, as there were not enough plants to sample equally in both plots: (1) a 20NPK treatment, where eight plants were sampled from one plot and two from a second plot; (2) a 20N treatment, where all ten plants were sampled from one plot. Freshly excised leaves

were cut into approximately 3 mm squares using sharp scissors to create a pool of foliage sample for every plant. This pool was divided into two subsamples: one (approximately 200 mg fresh weight) was placed in a preweighed microfuge tube with 1 mL of 5% perchloric acid ($HClO_4$); the other was placed in a separate tube without anything. All samples were transported to the laboratory on ice and stored at −20°C until further analysis. The samples in $HClO_4$ were weighed, frozen and thawed three times, and centrifuged at 13,000×*g* for 10 min. The supernatants were used for the analyses of $HClO_4$-extractable free PAs, free amino acids, and soluble inorganic ions (Minocha et al. 1994). The other set of subsamples was used for soluble protein and total chlorophyll analyses. Leaf extracts from each of ten plants/treatment/species were analyzed individually without pooling for all biochemical analyses.

Soluble inorganic ions were quantified using a simultaneous axial inductively coupled plasma emission spectrophotometer (Vista CCD, Varian, Palo Alto, CA, USA) and Vista Pro software (Version 4.0), following appropriate dilutions with deionized water (Minocha et al. 1994). For analysis of common amino acids and PAs (Putrescine, Spermidine, Spermine), the supernatants from $HClO_4$-extracted samples were subjected to dansylation and quantification by HPLC according to Minocha and Long (2004). The reaction was terminated by using 50 µL of L-asparagine (20 mg mL^{-1} in water) instead of alanine. The protocol did not always separate glycine, arginine and threonine; therefore, the peak areas for these three amino acids were pooled for each standard to derive a combined calibration curve for their quantification.

For soluble protein analysis, 50 mg of leaf pieces were placed in 0.5 mL of 100 mM Tris–HCl buffer (containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, and 10% (v/v) glycerol; pH 8.0), frozen and thawed three times, and the supernatant was used for protein analysis according to Bradford (1976). For chlorophyll analysis, 10 mg of leaf tissue was placed in 1.0 mL of 95% ethanol in the dark at 65°C for 16 h, and centrifuged (13,000×*g* for 5 min). The supernatant was scanned from λ_{350} to λ_{710} (U-2010, Hitachi Ltd., Tokyo, Japan) and chlorophyll was quantified as per Minocha et al. (2009). Results for leaf chemistry are expressed per dry weight. We used the average percent moisture for each species/treatment to calculate the dry weight (DW) from fresh weight (FW).

Statistical analyses

We studied the effect of treatments on the aboveground abundance of *C. calyculata* and *L. groenlandicum* using one-way ANOVA, analyzing years 2000 and 2008 separately. Differences in *V. myrtilloides* abundance were not analyzed, because it was not present in all survey plots, and treatment 20N was excluded due to its different experimental duration. Test variables were stem # × height in 2000, and number of point intercept hits in 2008; data were rank transformed.

Leaf level variables were analyzed using multivariate analysis of variance (MANOVA). The MANOVAs resulted in highly significant treatment, species, and species × treatment effects (see Resource 1 of the Electronic supplementary material, ESM). We used one-way ANOVA to assess the treatment effects on variables with significant between-subject treatment or treatment × species effects. The set of leaves measured for CO_2 exchange, morphology and N concentration was analyzed separately from the leaf samples used for biochemical analyses. All data were first tested for normality and equality of variances using Levene's test. Response variables with unequal variances were rank transformed. Bonferroni adjustment was used to evaluate statistical significance (adjusted *P* values were 0.003 for photosynthesis variables, and 0.001 for biochemical variables). We examined differences between treatments and the control with Dunnett's post-hoc test. Relationships among photosynthetic parameters, leaf morphological and biochemistry variables were quantified using Pearson's correlation and regression analyses. Statistical analyses were performed using the SPSS statistical package 11.0 for MS Windows (Lead Technologies, Inc. 2001).

Results

Aboveground growth and leaf morphology

Chamaedaphne calyculata was the most abundant vascular plant species in all plots (Fig. 1a, b). Analysis of variance did not show any differences between treatments and control in the abundance of either *C. calyculata* or *L. groenlandicum* in the first year of the experiment (2000). After 8–9 treatment years, we found no significant differences in the abundance of these species, but there were trends for increased growth. For example, the abundance of *C. calyculata* nearly doubled in the treatment 20NPK (Fig. 1).

We expected an increase in SLA with fertilizer treatment for the plants to maximize light interception. We found no changes, except for *L. groenlandicum*, which increased from ~60 $cm^2\ g^{-1}$ SLA in the control plots to ~100 $cm^2\ g^{-1}$ with 5NPK treatment (Fig. 2a), perhaps due to a decrease in leaf mass (Table 2), but these changes were not significant. Overall, the deciduous *V. myrtilloides* had a higher SLA (~100–115 $cm^2\ g^{-1}$) than the two evergreen species (~65–100 $cm^2\ g^{-1}$), and a higher moisture content (~50% DW) than the evergreens (~10–30% DW) in control plots. Compared with the control, percent moisture was lower in

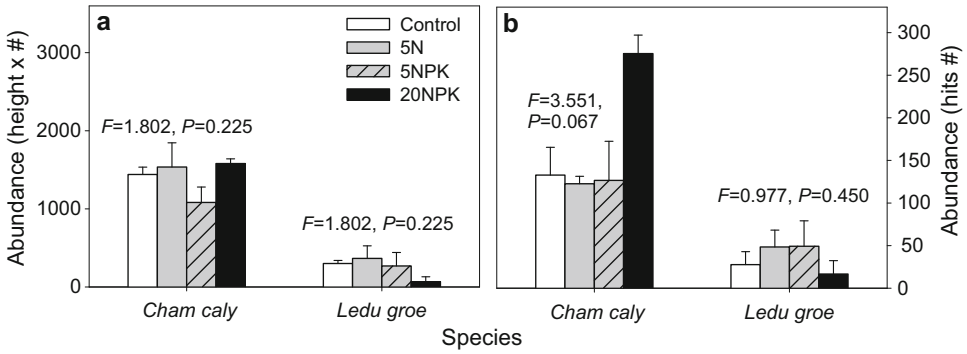

Fig. 1 Abundance of *Chamaedaphne calyculata* and *Ledum groenlandicum* in survey plots (mean ± SE, $n = 3$) in **a** 2000, at the beginning of the experiment, and **b** 2008, after 9 years of fertilization. ANOVA results for differences among the treatments are indicated in the panels. Test variables were height × number of stems in 2000 and number of point intercept hits in 2008

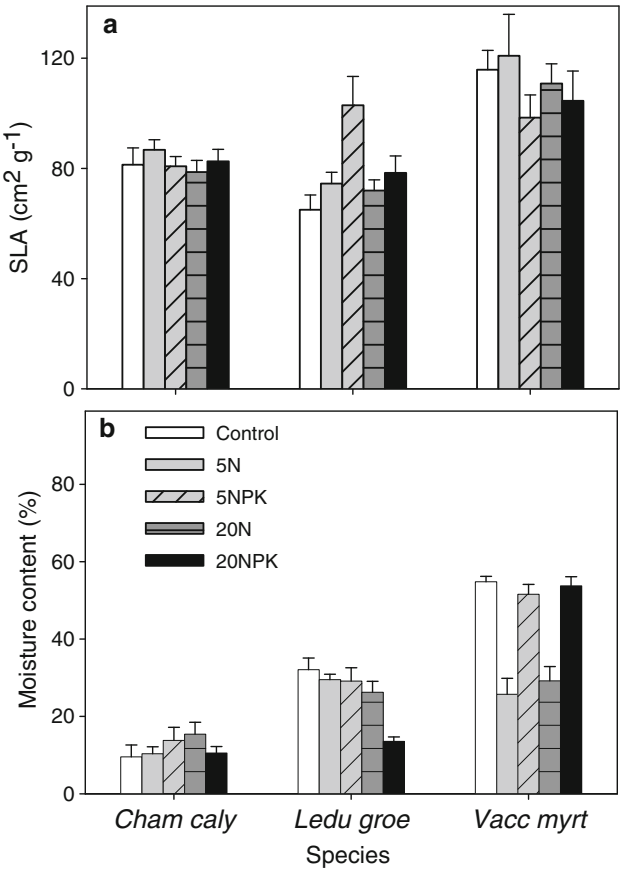

Fig. 2 **a** Specific leaf area (SLA) and **b** moisture content in fresh leaves (mean ± SE, $n = 9$)

V. myrtilloides leaves in the 5N and 20N treatments (declining from ~50–30%) and in *L. groenlandicum* leaves in the 20NPK treatment (declining from ~30–15%). *C. calyculata* had the lowest moisture content of all three species (~10–15%), and did not change with treatment (Fig. 2b). A similar trend was observed in soluble proteins (Fig. 3f).

Foliar chemistry

The total N concentration in the leaves of all three species increased from ~1% in the control to ~1.5% in the highest fertilizer treatments (Fig. 3a). Consequently, the C:N ratios decreased from ~52 in the control to ~34 in 20NPK treatments (Fig. 3b). Total chlorophyll ranged from approximately 0.9 to 1.8 mg g^{-1} and followed a trend similar to that of foliar N for *C. calyculata*, resulting in a twofold increase in 20N compared to the control (Fig. 3c). Trends for the other two species were not so clear. For example, chlorophyll in *L. groenlandicum* leaves showed a small (but insignificant) increase with 20N, but there was a significant decrease in the 20NPK treatment.

Species differed in the partitioning of N into amino acids, PAs, and soluble proteins. While none of the species showed significant differences between treatments and control in total levels of amino acids and levels of either total or individual PAs, *L. groenlandicum* had higher total amounts of amino acids and PAs than the other two species in all treatments (Fig. 3d, e, Resources 2 and 3 of the ESM). *V. myrtilloides* had the highest total amounts of soluble proteins among the three species in the control plots (Fig. 3f), but showed declines between the control and the two N-only treatments (5N and 20N). Supplying P along with N prevented this decline in soluble proteins. *L. groenlandicum* had a small but insignificant decline in soluble proteins between the control and 20NPK.

Individual amino acids showed species-specific patterns. In the two evergreens, the combined concentrations of four amino acids [GABA, alanine, glutamic acid, and arginine (+ glycine and threonine)] constituted more than 50% of the total amino acid pool under normal growth conditions, histidine and tryptophan dominated the amino acid pool in the deciduous *V. myrtilloides*. Since histidine and tryptophan share a common pathway, an increase in histidine with 5N significantly reduced tryptophan (Resource 2 of the ESM). More important than alterations in total amino acids were the effects of treatments on the relative ratios of different amino acids. Leaves of both evergreen species had higher concentrations of glutamic acid, alanine, arginine (plus glycine and threonine) and GABA in the 20N and/or 20NPK treatments compared with the control, although some of these increases were not significant. *V. myrtilloides* had higher concentrations of GABA accompanied by a decrease

Table 2 Specific leaf area SLA ($cm^2\ g^{-1}$ leaf), individual leaf area (cm^2), leaf mass (g), and thickness (mm) (mean ± SE) with test statistics from one-way ANOVA

Species	Treatment	SLA	Area	Mass	Thickness
C. calyculata	Control	81.4 (6.1)	1.68 (0.16)	21.4 (2.5)	0.3 (0.03)
	5N	86.8 (3.7)	2.00 (0.1)	22.5 (1.5)	0.36 (0.03)
	5NPK	80.8 (3.5)	1.67 (0.12)	21.0 (1.7)	0.41 (0.04)
	20N	78.7 (4.2)	2.13 (0.22)	27.3 (2.7)	0.39 (0.02)
	20NPK	86.6 (4.3)	1.94 (0.14)	24.1 (2.2)	0.38 (0.02)
	F	0.45	1.57	1.67	1.15
	P	0.77	0.20	0.18	0.35
L. groenlandicum	Control	65 (5.4)	1.78 (0.11)	28.3 (1.9)	0.76 (0.06)
	5N	74.5 (4.1)	1.74 (0.22)	23.5 (2.7)	0.59 (0.03)
	5NPK	103 (10.5)	1.64 (0.12)	16.8 (1.7)	0.63 (0.04)
	20 N	72 (3.8)	1.78 (0.11)	27.6 (0.7)	0.66 (0.06)
	20NPK	78.4 (6.2)	1.9 (0.11)	18.9 (2.4)	0.61 (0.06)
	F	5.23	0.73	6.36	1.82
	P	<0.001	0.58	1.67	0.15
V. myrtilloides	Control	115.9 (7.0)	3.8 (0.51)	22.3 (4.9)	0.41 (0.04)
	5N	120.9 (15.0)	3.1 (0.28)	27.1 (3.1)	0.36 (0.02)
	5NPK	98.4 (8.3)	4.4 (0.49)	47.7 (7.5)	0.50 (0.04)
	20N	110.8 (7.1)	3.8 (0.26)	35.1 (2.1)	0.48 (0.05)
	20NPK	104.5 (10.8)	5.7 (0.58)	59.6 (8.9)	0.46 (0.03)
	F	0.96	4.59	6.36	1.69
	P	0.44	<0.001	1.67	0.18

Differences are considered significant at a Bonferroni adjusted *P* level of 0.003. Sample size was eight leaves per species per treatment. Either three or two leaves were sampled from each plot

in tryptophan in 5NPK and 20NPK treatments compared with the control (Fig. 4, Resource 2 of the ESM). These amino acids are some of the most N-rich of the major amino acids, in particular arginine and histidine with N:C ratios of >0.5. All of these amino acids are derived from glutamate. Consequently, there were significant correlations between foliar N and glutamic acid for *L. groenlandicum*, alanine for *C. calyculata*, and GABA for *C. calyculata* and *V. myrtilloides* (Fig. 4).

Fertilizer treatment had a significant effect on the accumulation of several soluble ions in the foliage of all three species (Fig. 5). Ca was lowered significantly in all species by almost all treatments; Mg concentration was lowered for *C. calyculata* and *V. myrtilloides* in the N-only treatments. *V. myrtilloides* contained higher amounts of Ca and Mg than the other two species in control plots (Fig. 5a, b). Phosphorus (P) increased significantly in response to the NPK treatments, as expected, and the increases were similar in the 5NPK and 20NPK treatments, as these plots received the same amount of P. P concentration was significantly lower in N-only treated plants as compared with controls in *L. groenlandicum* and *V. myrtilloides* (Fig. 5c). Relative to P, changes in potassium (K) concentration were smaller, but K increased in *C. calyculata* in NPK treatments. *V. myrtilloides* leaves had only about half the concentration of K in N-alone treatments compared to the control (Fig. 5d). Manganese (Mn) and aluminum (Al) concentrations in the foliage of the evergreen species were generally smaller in treatment plots than in the controls. Manganese declined from ~6 to ~2 μmol g^{-1} DW in *C. calyculata* and from ~4 to ~0.5 μmol g^{-1} in *L. groenlandicum* (Fig. 5e, f). In contrast, *V. myrtilloides* had higher Mn in the 5NPK treatment than in the control.

Photosynthetic parameters

Nutrient addition had few significant effects on photosynthetic parameters. Rates of carboxylation (V_{cmax} per unit mass) in *L. groenlandicum* in 5N and 5NPK treatments were higher than in the control (Table 3). However, there were no significant differences between the treatments and control for rates of light-saturated photosynthesis (A_{max}) or for other photosynthetic parameters (Table 3). A_{max} ranged from ~8 to 13 μmol CO_2 m^{-2} s^{-1} and V_{cmax} ranged from ~67 to 137 μmol CO_2 m^{-2} s^{-1} among the three species and treatments, with *C. calyculata* having slightly higher rates than the other species.

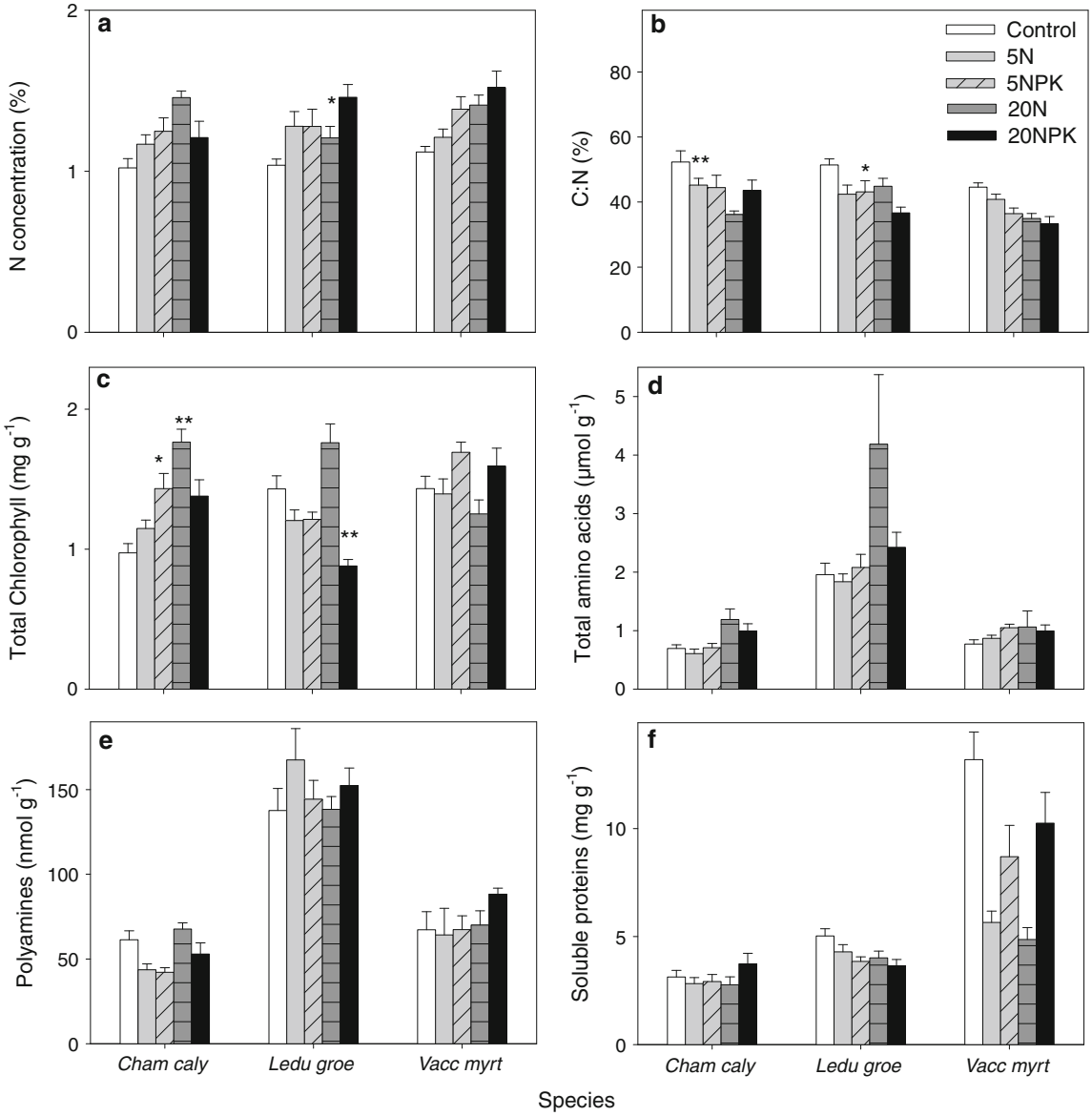

Fig. 3 Concentrations (mean ± SE) of foliar: **a** nitrogen, **c** chlorophyll, **d** amino acids, **e** polyamines (Putrescine, Spermidine, Spermine), **f** soluble proteins, and **b** the C:N ratios for *Chamaedaphne calyculata*, *Ledum groenlandicum* and *Vaccinium myrilloides*. * and ** denote significant differences between the treatment and control conditions ($P < 0.05$ and 0.01, respectively)

The highest rates of A_{max} or V_{cmax} seemed to co-occur with intermediate leaf N concentrations (g m^{-2} leaf) (Fig. 6a, b, e, f, i, j). Only *C. calyculata* had a weakly positive linear relationship between foliar N and A_{max}. PNUE had a significantly negative relationship with foliar N for *V. myrtilloides* and a weakly negative relationship (although not significant) with foliar N for the evergreens (Fig. 6c, g, k). The V_{cmax} : foliar N ratio had a significant negative correlation with foliar N for all three species (Fig. 6d, h, l). Foliar N tended to be higher in leaves with low SLA for all three species combined, which likely is a result of more N per unit leaf area in thicker leaves. Chlorophyll had a positive relationship with A_{max} (area) and a negative relationship with V_{cmax} (mass), J_{max} (area), and TPU (area) (Table 4).

Discussion

N deposition at Mer Bleue was ~0.2 g m^{-2} a^{-1} in pre-Industrial times, but has since increased to its current level of ~0.8 g m^{-2} a^{-1}. Contrary to our expectations, additions of 1.6 and 6.4 g N m^{-2} a^{-1} alone or in combination with P resulted in few significant responses in shrub biomass or leaf biochemistry. *C. calyculata* was clearly the dominant vascular species and had the largest growth increase after

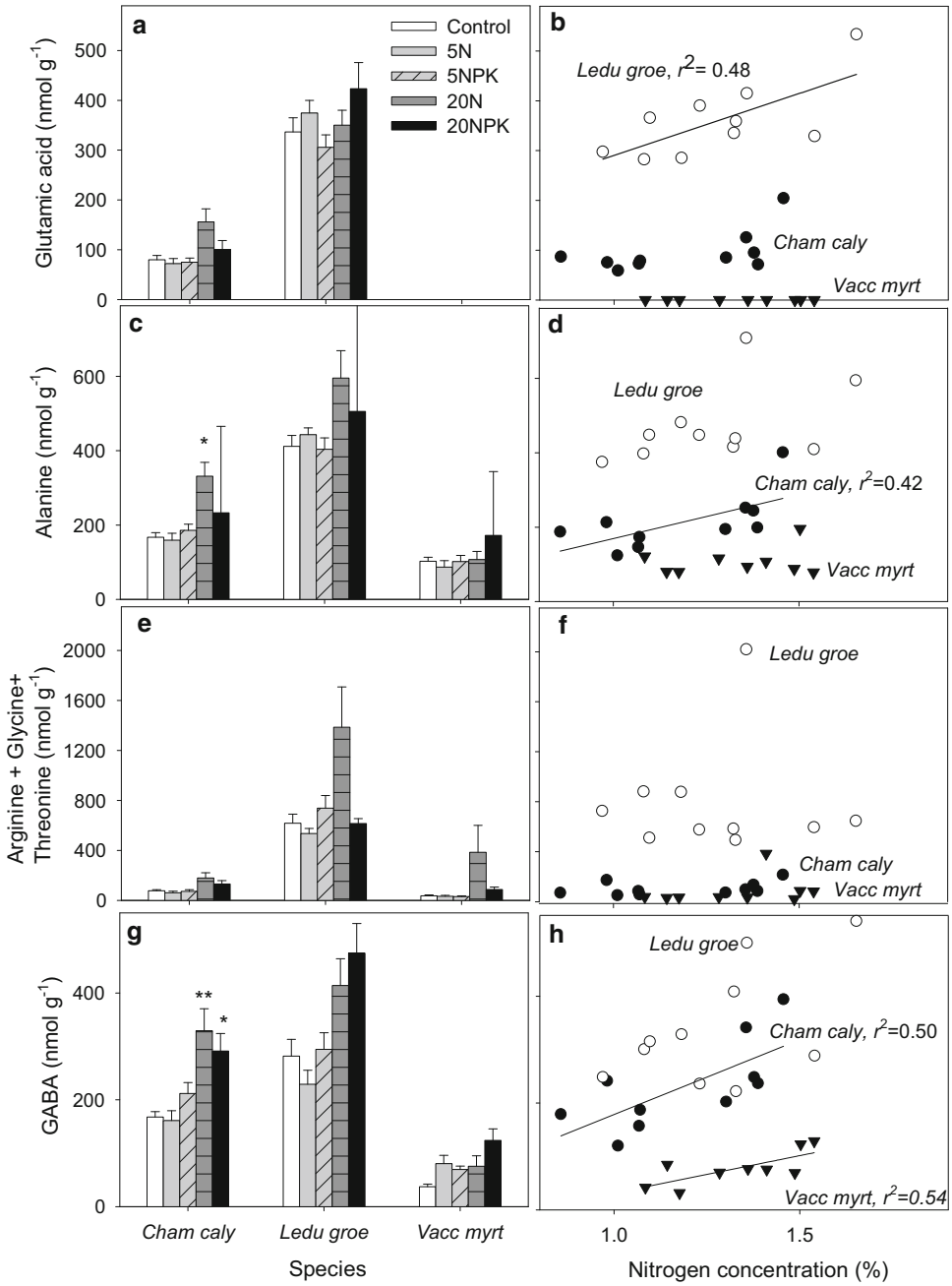

Fig. 4 Amino acid concentrations (means ± SE, $n = 10$) for **a** glutamic acid, **c** alanine, **e** arginine + glycine + threonine, and **g** GABA. Significant differences between fertilizer and control treatments are denoted by * ($P < 0.05$) or ** ($P < 0.01$). **b**, **d**, **e**, and **h** show plot means ($n = 5$) of individual amino acid concentrations versus plot means of foliar nitrogen concentration. Fitted regression lines and r^2 values denote linear regressions with slopes that are significantly ($P < 0.05$) different from zero

8 years of fertilization, but only in the 20NPK treatment (Fig. 1). However, growth led to a larger investment into stems and woody biomass than in leaves (Juutinen et al. 2010). *Ledum groenlandicum* was less abundant, but there was a trend for increasing aboveground biomass of this species with N treatment, particularly 5NPK. Nitrogen concentrations per unit leaf mass increased with nutrient addition, and increases in chlorophyll were the largest in *C. calyculata* leaves compared with other species (Fig. 3).

Relative to the control, we did not observe consistent changes in chemistry, morphology and photosynthetic capacity with fertilizer treatment. However, levels of Ca in leaves were lowered significantly in all three species, and a declining trend was seen in Mg (Fig. 5). Loss of these cations from soil is a known consequence of excess N and acidification, and has been implicated as a primary cause of forest decline in the US and in Europe (Schulze 1989; Magill et al. 2004). Similar losses in Ca and Mg occurred concomitant with a decline in *Sphagnum* productivity with increased N deposition in European peatlands (Bragazza et al. 2004). A decline in moss cover and biomass at Mer Bleue bog upon high N additions has also been reported earlier by Bubier et al. (2007) and Juutinen et al. (2010). In the present study, we observed a species-specific increase

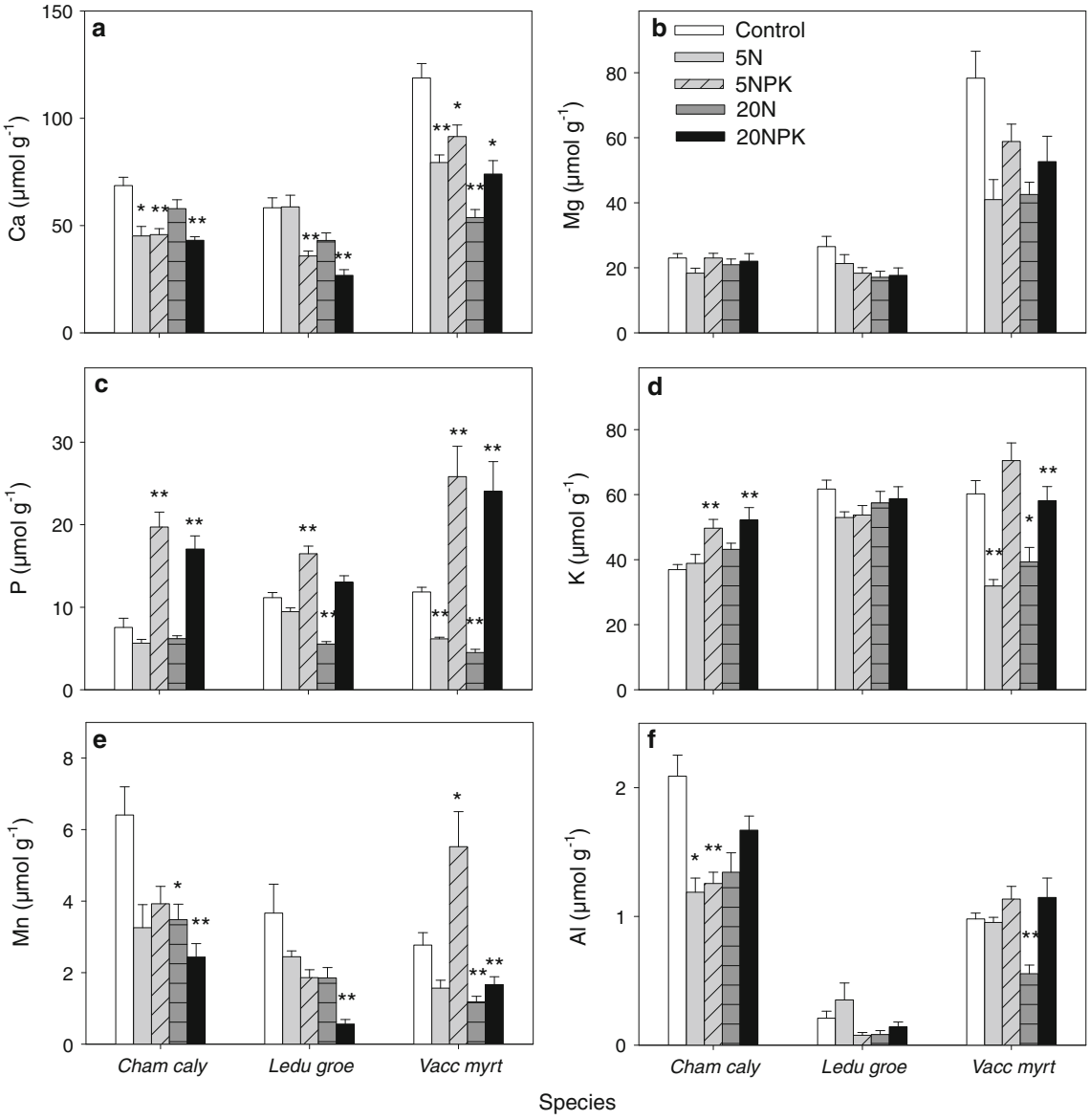

Fig. 5 Foliar concentrations (mean ± SE, $n = 10$) of soluble **a** Ca, **b** Mg, **c** P, **d** K, **e** Mn, and **f** Al. * and ** denote significant differences between the treatment and control conditions ($P < 0.05$ and 0.01, respectively)

in a few N-rich amino acids with treatments, which is perhaps a strategy to avoid NH_3 toxicity at the cellular level. However, in the absence of significant changes in photosynthetic parameters, PAs and most other amino acids, it is not evident at this time if the changes observed in the few amino acids are large enough to indicate significant physiological stress in these shrubs.

Photosynthetic capacity and foliar chemistry

In the present study, the lack of strong relationships between foliar N and either A_{max} or V_{cmax}, coupled with negative relationships between foliar N, SLA and PNUE (Table 4; Fig. 6), are opposite to our hypotheses. The results also disagree with earlier meta-analyses of natural ecosystems predicting that increased foliar N should lead to a corresponding increase in photosynthetic capacity (Reich et al. 1998). We found no increase in photosynthetic parameters (A_{max} or V_{cmax}) to accompany the increases in foliar N, with the exception of an increase in V_{cmax} in *L. groenlandicum* at moderate N addition of 1.6 g N m^{-2} a^{-1} (+ background deposition ~0.8 g m^{-2} a^{-1}) (Table 3). We also found a weakly positive correlation between $A_{max,}$ foliar N, and chlorophyll (Fig. 6; Table 4) and increased chlorophyll in *C. calyculata* leaves (Fig. 3c), indicating that this species is using some of the excess N to invest in light harvesting and photosynthetic capacity. This was also the only species to increase in biomass with fertilization, but only in the

Table 3 V_{cmax} and A_{max} per unit area (μmol CO_2 m^{-2} s^{-1}) and per unit mass (μmol CO_2 g^{-1} s^{-1}), J_{max}, and TPU (μmol CO_2 m^{-2} s^{-1}) (mean ± SE, n = 7–8), along with test statistics of one-way ANOVA

Species	Treat.	V_{cmax} (area)	A_{max} (area)	V_{cmax} (mass)	A_{max} (mass)	J_{max} (area)	TPU (area)
Cham caly	Control	132.1 (31.2)	10 (4.9)	1.1 (0.4)	0.08 (0.04)	93.4 (4.2)	7.2 (0.3)
	5N	129.7 (30.6)	9.1 (4.3)	1.1 (0.4)	0.08 (0.03)	94.7 (7.7)	7.6 (0.6)
	5NPK	122.9 (14.7)	11.8 (6.2)	1.0 (0.2)	0.09 (0.05)	122.7 (15.4)	9.1 (1.2)
	20N	117.3 (23.1)	12.9 (3.2)	0.9 (0.1)	0.1 (0.03)	120.4 (9.7)	8.6 (0.7)
	20NPK	129.7 (27.5)	8.6 (5.0)	1.1 (0.3)	0.09 (0.04)	117.2 (8.5)	8.7 (0.7)
	F	0.44	0.75	0.36	1.12	0.21	0.34
	P	0.78	0.57	0.84	0.37	0.93	0.85
Ledu groe	Control	78.1 (13.4)	11.0 (2.6)	0.5 (0.2)	0.07 (0.02)	119.4 (12.1)	10.1 (1.0)
	5N	137.4 (21.9)	9.6 (3.3)	**1.0 (0.3)**	0.07 (0.02)	177.8 (17.0)	14.4 (1.3)
	5NPK	123.5 (49.1)	7.6 (3.0)	**1.2 (0.4)**	0.07 (0.02)	170.0 (18.5)	13.3 (1.3)
	20N	118.4 (38.5)	9.5 (2.1)	0.8 (0.2)	0.07 (0.02)	141.5 (15.6)	11.5 (1.2)
	20NPK	103.1 (34.2)	10.3 (4.7)	0.8 (0.4)	0.09 (0.03)	170.0 (16.6)	13.7 (1.3)
	F	3.57	2.06	6.80	0.91	2.04	1.76
	P	0.02	0.11	<0.001	0.47	0.11	0.16
Vacc myrt	Control	84.6 (13.5)	9.8 (1.9)	0.8 (0.2)	0.11 (0.02)	164 (12.1)	13.4 (1.0)
	5N	66.7 (14.3)	10.9 (4.3)	0.8 (0.2)	0.12 (0.04)	152.9 (6.3)	12.6 (0.5)
	5NPK	96.6 (32.1)	10.8 (2.5)	1.0 (0.5)	0.11 (0.03)	159.2 (9.6)	12.7 (0.7)
	20N	90.7 (25.6)	11.0 (4.3)	1.0 (0.4)	0.12 (0.06)	156.9 (11.8)	12.5 (1.0)
	20NPK	92.4 (27.8)	10.1 (3.6)	1.0 (0.4)	0.1 (0.02)	152.1 (12.7)	12.0 (1.0)
	F	3.0	0.17	0.68	0.49	1.69	1.02
	P	0.03	0.95	0.61	0.74	0.17	0.41

Differences are considered significant at a Bonferroni-adjusted P level of 0.003. Significant ($P < 0.05$) differences from the control conditions were assessed with Dunnet's test and are highlighted in *boldface*

20NPK treatment (Fig. 1). In the other two species, we observed either no change or a decline in chlorophyll concentrations, and no change in biomass. While it might be expected that N addition would have an even stronger positive influence on photosynthetic capacity in nutrient-limited ecosystems than in other ecosystems, results have been mixed. For example, studies have found increases (St. Clair et al. 2009), no change (Bowman et al. 1995; Starr et al. 2008), and decreases (Bigger and Oechel 1982; Bauer et al. 2004) in A_{max} in response to nutrient addition. The differences in the species composition and their threshold for N tolerance, initial N status of the site, and the duration of N addition probably contribute to these varied results (Aber 1992).

Our results indicate that most of the additional N was stored in simple organic compounds rather than invested in photosynthetic machinery (i.e., Rubisco and chlorophyll). Some of these metabolites, such as polyamines and amino acids, N-rich compounds essential for growth and development, in combination with chlorophyll and total soluble proteins have been used as indicators of environmental stress before the morphological symptoms of stress are visible (Näsholm et al. 1994; Bauer et al. 2004). PAs (specifically putrescine) and certain amino acids are indicative of the physiological response of forest trees to an array of environmental stress conditions, including a shortage of soil-available Ca, excess Al, and chronic N accumulation (Minocha et al. 1997, 2000; Wargo et al. 2002). It has been suggested that, under conditions of stress, PAs impart stress tolerance by lowering NH_3 toxicity and scavenging free radicals. Polyamines also act as signal molecules to regulate gene activity related to cellular N metabolism and the metabolism of several amino acids: proline, arginine, γ-aminobutyric acid (GABA) and glutamic acid, all of which play important roles in plant responses to higher N exposure (Näsholm et al. 1994; Bauer et al. 2004; Bouché and Fromm 2004). Storing excess N in organic forms is more favorable to plant health (reduces NH_3 toxicity) and growth, as the plant can access the stored N when supplies diminish (Rabe 1990; Näsholm et al. 1994; Limpens and Berendse 2003; Bauer et al. 2004).

We found increases in alanine and GABA with the highest N treatment (Fig. 4), which, in boreal understory species, are thought to indicate N saturation from the shoot to the root, and therefore have the potential to inhibit further root uptake of nutrients (Näsholm et al. 1994; Limpens and Berendse 2003; Tomassen et al. 2003). Significant changes in most amino acids and all PAs were observed in a red pine stand at Harvard

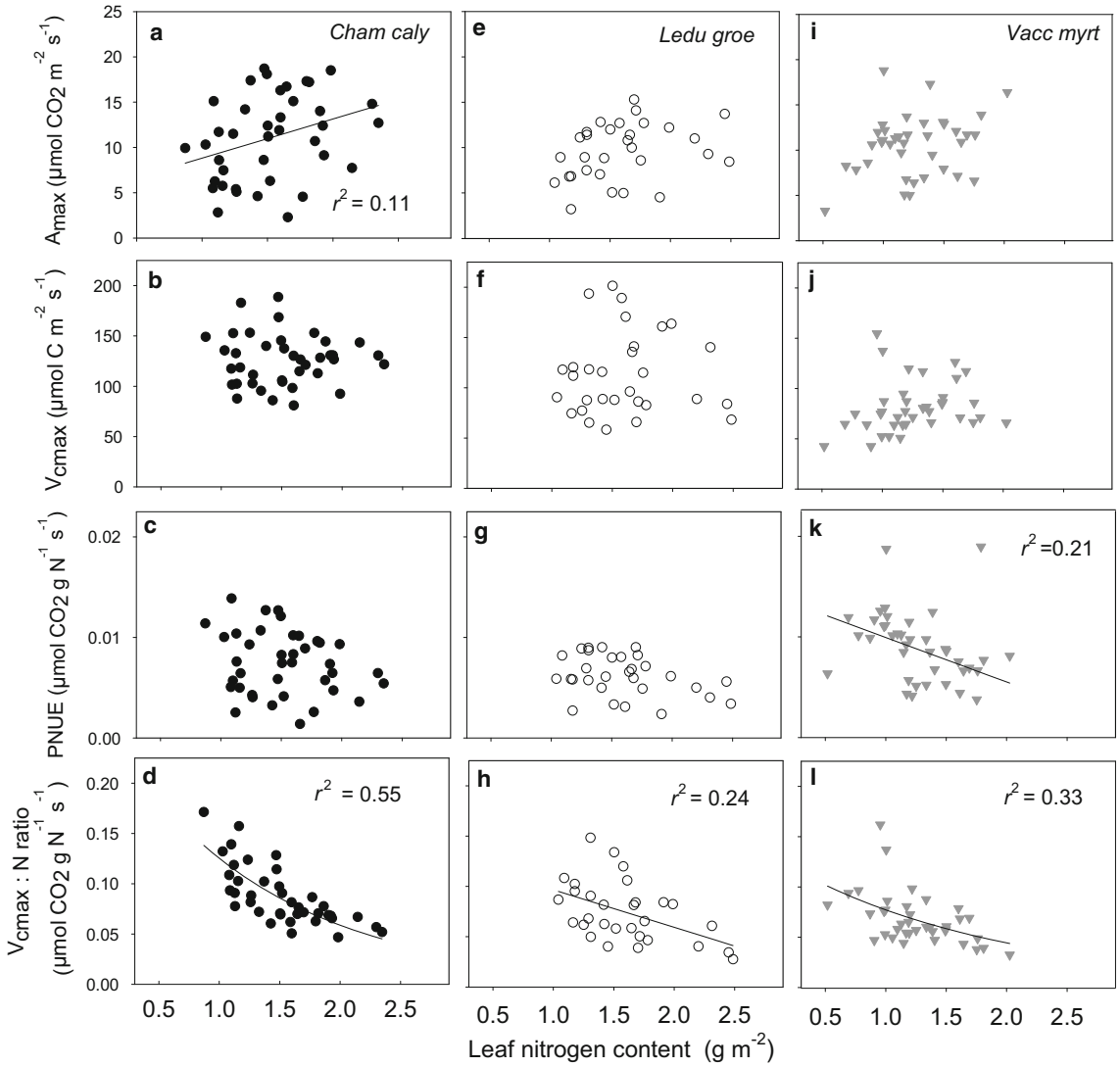

Fig. 6 A_{max} (area), V_{cmax} (area), PNUE, and V_{cmax} : N ratio in relation to leaf N content for *Chamaedaphne calyculata* (**a–d**), *Ledum groenlandicum* (**e–h**), and *Vaccinium myrtilloides* (**i–l**). Fitted regressions and coefficients of determination are indicated only for significant relationships ($P < 0.05$). Each point represents one leaf

Forest, MA, when it was subjected to long-term chronic N additions (Bauer et al. 2004). The metabolic costs of maintaining high levels of free amino acids so as to avoid the toxic effects of free ammonia in our study would also considerably increase maintenance respiration of the cells, thus removing energy from growth processes (De Vries 1975).

Compared to the other species, total levels of PAs were the highest in *L. groenlandicum* (Fig. 3d). However, we did not find significant changes in the two major PAs (putrescine and spermidine) between the treatments and control in this study (Resource 3 of the ESM). The data collected so far indicate that these shrub species may be in the earlier phases of N sufficiency/saturation with 20N and/or 20NPK additions. While all three species showed significant changes in some metabolites, we did not observe reductions in photosynthetic capacity.

N use efficiency, leaf traits, and resource allocation

Higher SLA, as observed in *L. groenlandicum* in low-N treatments, may indicate a change in nutrient allocation with regard to leaf life span and morphological adjustments (Shipley et al. 2005). Specific leaf area is usually positively correlated with light use efficiency; thinner leaves require less photosynthetic machinery per unit area (Burns 2004), while thicker or denser leaves have greater internal shading and diffusion limitations, which may restrict the potential for higher photosynthetic capacity because of the chloroplast stacking in thick leaves (Reich et al. 1998). In turn, low SLA foliage tends to be longer lived but less productive than thinner leaves (Pornon and Lamaze 2007), indicating a trade-off between photosynthetic capacity and leaf persistence (Hikosaka 2004; Shipley et al. 2006).

Table 4 Pearson's correlation coefficients between leaf dimensions, chlorophyll concentration and photosynthetic variables and N for all species and treatments

	Mass	Area	SLA	Thickness	Chlorophyll
N	0.134	**−0.198**	**−0.669**	**0.251**	0.340
V_{cmax} (area)	**−0.279**	**−0.380**	**−0.291**	−0.004	−0.298
V_{cmax} (mass)	**−0.322**	−0.128	**0.354**	−0.167	**−0.590**
A_{max} (area)	0.127	0.004	**−0.300**	−0.128	**0.372**
A_{max} (mass)	0.114	**0.284**	**0.327**	**−0.307**	0.220
J_{max} (area)	**−0.370**	**−0.498**	**−0.355**	0.095	**−0.416**
TPU (area)	**−0.425**	**−0.557**	**−0.376**	0.103	**−0.431**

Dry leaf mass (mg), specific leaf area ($cm^2\ g^{-1}$), thickness (mm), area (cm^2), total chlorophyll ($\mu g\ g^{-1}$), leaf N ($mg\ m^{-2}$ leaf), V_{cmax} (area) ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$), V_{cmax} (mass) ($\mu mol\ CO_2\ g^{-1}\ s^{-1}$), A_{max} (area) ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$), A_{max} (mass) ($\mu mol\ CO_2\ g^{-1}\ s^{-1}$). Significant correlations ($P < 0.05$) are highlighted in *boldface*

Cost–benefit models (Kikuzawa and Ackerley 1999; Wright et al. 2004; Ellison 2006) suggest that better nutrient availability and shorter leaf life spans allow the plant to reinvest nutrients in young, photosynthetically active tissues, leading to higher PNUE. We found no correlation between PNUE and SLA, and either no correlation or a negative correlation between foliar N and SLA, and foliar N and PNUE (Table 4; Fig. 6). These relationships are contrast with those predicted by some cost–benefit models (Hikosaka 2004; Poorter and Evans 1998), but are analogous to those observed by Ripullone et al. (2003) and Granath et al. (2009b) under high N deposition. The latter study found a unimodal relationship between foliar N and PNUE, with an optimum N level of approximately 9 mg N g^{-1} dry mass for *Sphagnum*, although this level would also depend on N:P:K ratios (Bragazza et al. 2004; Ellison 2006; Elser et al. 2007; Hidaka and Kityama 2009). In our study, the weak negative correlation (depending on the species) without an optimum level between PNUE and foliar N suggests that any level of N addition at Mer Bleue either has no effect or decreases the PNUE of these species (Fig. 6).

On the other hand, it can be argued that PNUE may not be the most relevant measure of lifetime nutrient use efficiency for bog species. The longer leaf life span and mean residence time of leaf N may lead to longer lifetime nutrient use efficiency in evergreens, particularly in nutrient-limited environments (Small 1972; Berendse and Aerts 1987). These species can produce two- to threefold more photosynthate using a given unit of N before it is returned to the environment than do bog deciduous species, which can produce about 60% more photosynthate per acquired unit of N than do non-bog deciduous species (Small 1972). Butler and Ellison (2007) observed that a predilection for storing nutrients, rather than using them immediately, may be one reason that photosynthetic rates of many wetland plants are lower than expected given their foliar N concentrations.

The total mass of leaves per plant can be more important than leaf photosynthetic rate in determining plant productivity, as observed in some arctic and peatland studies (Chapin and Shaver 1996; Starr et al. 2008). Bartsch (1994) found that biomass, flower production, and shoot growth in *C. calyculata* increased two to fivefold with fertilizer treatment in a Maine bog. Similarly, in our study, it appears that nutrients have been invested mainly in woody biomass, and less into new leaves; but total foliar N per unit area has increased with N addition (Fig. 1; Juutinen et al. 2010). At the leaf level, the current study shows that N has been allocated to foliar storage compounds more than photosynthetic processes (particularly in *L. groenlandicum*).

In addition to woody and foliar biomass, lifetime nutrient use efficiency includes leaf lifespan. Shaver (1983) found that *Ledum palustre* had decreased leaf longevity with nutrient addition, possibly due to failure to survive the winter. Leaves may turn over faster in order to eliminate potentially toxic levels of ammonia. In fertilized and ambient environments, older leaves serve as storage organs, but have a lower photosynthetic capacity than new leaves (Maier et al. 2008). Our species at Mer Bleue may thus be shifting their allocation patterns to a shorter leaf lifespan in the highest nutrient treatments as leaf litter accumulation has increased in these plots (Juutinen, pers. comm.). Thus far, we have not observed an invasion of deciduous and graminoid species, which have been reported to have a competitive advantage over evergreens under elevated atmospheric N and nutrient addition in Europe and North America (Bowman et al. 1995; Chapin and Shaver 1996; Van Wijk et al. 2003).

Finally, we observed lower foliar moisture content in *L. groenlandicum* in the highest nutrient treatments, and in the N-only treatments for *V. myrtillloides* (Fig. 2b). The lower moisture content may be due to changes in nutrient concentrations and consequently in root uptake mechanisms, or due to drying of the surface soil, as observed recently in the 20NPK plots with the loss of *Sphagnum* (Humphreys, pers. comm.). Bowman et al. (1995) found that photosynthetic rates in alpine tundra species were unrelated to variation in foliar N concentration, but instead correlated with variations in stomatal conductance. Starr et al. (2008) found lower stomatal conductance after drought periods, resulting in lower A_{max} values in arctic tundra. These changes in soil and plant moisture will likely have stronger effects on the physiology of these plants in the future.

Conclusions

The varied responses of plant species to N deposition globally, and at Mer Bleue, are perhaps the result of physiological

differences and evolutionary adaptations to resource use and allocation. Our biochemical data suggest that these bog shrub species tolerate and store the additional N for future use rather than invest it in increasing photosynthetic capacity. We do not know how the decreased levels of essential plant nutrients (e.g., Ca and Mg) will affect these species in the future. Moreover, in the highest nutrient treatments, the whole-plant physiology (e.g., the distributions of woody and foliar biomass and nutrients, and shifts in life history traits such as leaf longevity) and competition need to be studied to assess ecosystem changes. While we have not yet seen a change in photosynthetic capacity (either a reduction owing to nutrient stress or an increase owing to a shift in N allocation to photosynthetic processes), bog shrubs may not be able to adapt their evolutionary strategies to take advantage of elevated nutrients in the long term, resulting in replacement by species that are better adapted to a higher nutrient environment.

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