

## Effects of elevation and nitrogen and phosphorus fertilization on plant defence compounds in subarctic tundra heath vegetation

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### Summary

1. Plant chemical and structural defence compounds are well known to impact upon herbivory of fresh leaves and influence decomposition rates after leaf senescence. A number of theories predict that alleviating nutrient limitation and reducing other environmental stressors will result in decreased production of plant chemical defences.

2. In this study, we measured plant defence properties [total polyphenols (TP), condensed tannins (CT) and lignin concentrations, and protein complexation capacity (PCC)] in both fresh and senesced plant leaves in a fully factorial N and P fertilization experiment set-up at each of three elevations along an elevational gradient in Swedish subarctic tundra heath vegetation. Further, we performed a decomposition of variance analysis on community-weighted averages (CWAs) of plant defence properties to determine the relative contributions of interspecific and intraspecific variation to the total variation observed in response to elevation and nutrient addition.

3. We hypothesized that N fertilization would reduce plant defence properties and that this reduction would be greater at higher elevations, while the effects of P fertilization would have no effect at any elevation.

4. At the community level, N addition reduced CT and PCC in both fresh and senesced leaves and TP in senesced leaves, while P addition had few effects, broadly in line with our hypothesis. The effects of N addition frequently varied with elevation, but in contrast to our hypothesis, the said effects were strongest at the lowest elevations. The effects of N addition and the interactive effect of N with elevation were primarily driven by intraspecific, rather than interspecific, variation.

5. Our findings suggest that as temperatures warm and N availability increases due to global climate change, secondary metabolites in subarctic heath vegetation will decline particularly within species. Our results highlight the need to consider the effects of both nutrient availability and temperature, and their interaction, in driving subarctic plant defence.

**Key-words:** condensed tannins, decomposition, global climate change, litter feedback, nutrient addition, plant defence theory, protein complexation capacity, subarctic tundra

### Introduction

Plants have evolved numerous chemical and structural mechanisms to protect themselves from damage. Poly-

phenols are a broad class of secondary carbon-based compounds found in nearly all higher plants (Lattanzio, Lattanzio & Cardinali 2006), where they convey such benefits as protection against UV radiation (Close & McArthur 2002) and microbial pathogens (Scalbert 1991). In living foliage, polyphenols also defend against herbivory

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(Coley, Bryant & Chapin 1985) and plants may increase the production of these compounds in response to herbivore damage (Gatehouse 2002). After leaf senescence, polyphenols, specifically tannins, can regulate nutrient cycling through protein complexation capacity (PCC), which is the formation of protein-stable tannin compounds. This process may reduce soil nitrogen (N) mineralization rates by inhibiting microbial activity and thereby reduce plant-available N (Fierer *et al.* 2001). The legacy effects of polyphenols in senesced plant material can influence soil nutrient dynamics in ecosystems by initiating plant–soil feedbacks that may maintain conditions favourable to the dominant plants species (Wurzburger & Hendrick 2009; Gundale *et al.* 2010). In addition to chemical defence compounds, plants allocate carbon (C) to structural defences such as lignin, which can regulate palatability and digestibility (Cornelissen *et al.* 2004) in living foliage, and decomposition rates in leaf litter (Taylor, Parkinson & Parsons 1989). Due to their different roles in fresh and senesced leaves, quantifying polyphenols and structural defences in both leaf types is important for understanding the role of plant secondary chemistry in driving ecological processes.

Many theories exist concerning the differences observed in plant defences between species and across ecosystems due to abiotic or biotic factors. The ‘carbon nutrient balance’ hypothesis (Bryant, Chapin & Klein 1983) proposes that C is fixed in surplus when N is limited, which causes increases in C-based defence compounds, the ‘resource availability’ hypothesis (Coley, Bryant & Chapin 1985) postulates that slower-growing plants in nutrient-limited environments will invest in greater defences to minimize tissue loss due to herbivory, and the ‘protein competition’ hypothesis (Jones & Hartley 1999) suggests trade-offs in the metabolic pathways of plants for the production of photosynthetic vs. defence compounds. Despite myriad studies on the topic, there is considerable debate surrounding the predictive power of these theories (Koricheva 2002; Endara & Coley 2011; Johnson 2011). Consistent with these theories, higher N availability is typically associated with lower polyphenol production (Bryant, Chapin & Klein 1983; Koricheva *et al.* 1998; Kraus, Zasoski & Dahlgren 2004), but the effect of N availability on polyphenols has been found to vary, particularly in subarctic/alpine plant species (Nybakken, Klanderud & Totland 2008; Sundqvist *et al.* 2012). In contrast, it has been proposed that phosphorus (P) availability is unimportant in polyphenol production, because it does not directly affect the phenylalanine pathway responsible for synthesizing proteins and many polyphenols (Jones & Hartley 1999). However, while some results provide evidence for this (Koricheva *et al.* 1998), others have found polyphenol concentrations in plants to increase (Feller 1995), or decrease (Zhang *et al.* 2012) with increasing P availability. Further, structural defences such as lignin have been shown to decrease with increasing N (Haukioja *et al.* 1998) and P (Santiago, Schuur & Silvera 2005) availability.

Ratios of N and P to lignin have also been shown to control litter decomposition (Zhang *et al.* 2008), highlighting the importance of considering lignin in tandem with polyphenols for understanding responses of ecosystem processes to changes in environmental conditions.

Elevational gradients are powerful tools for determining how nutrient cycling and plant communities respond to changes in temperature when other extrinsic factors such as precipitation remain relatively constant (Körner 2007; Sundqvist, Sanders & Wardle 2013). Decreasing temperature associated with increasing elevation has been shown to have positive (Bernal *et al.* 2013), negative (Wallis, Huber & Lewis 2011) and neutral (Rasmann *et al.* 2014) effects on plant chemical defences. Furthermore, in subarctic systems, invertebrate herbivory tends to be highest near the tree line (Hagen *et al.* 2007), while vertebrate herbivory often increases with elevation (Vistnes *et al.* 2008; Hoset *et al.* 2014), which may also lead to increases in plant defence at both the highest and lowest elevations. In the present study, we utilized a subarctic elevational gradient near Abisko in northern Sweden (Sundqvist, Giesler & Wardle 2011b; Sundqvist *et al.* 2011a). Along this gradient, Sundqvist *et al.* (2012) found contrasting species responses in phenols to elevation, with different species increasing, decreasing or showing no response. We focused on heath vegetation along this gradient (Fig. 1); this vegetation is dominated by ericaceous dwarf shrubs, which tend to be high in polyphenols (Gundale *et al.* 2010). For heath vegetation along this gradient, previous studies have shown increasing elevation to be associated with decreases in plant-available P (Vincent *et al.* 2014) and N, increasing fungal to bacterial ratios and low species turnover (Sundqvist *et al.* 2011a). Additionally, foliar N:P ratios increase with increasing elevation, while decomposition rates are relatively constant; these shifts occur at both the intra- and interspecific levels (Sundqvist, Giesler & Wardle 2011b). We utilized a N and P fertilization experiment on



**Fig. 1.** An example of heath vegetation used for collection of fresh and senesced leaves for plant defence measurements in this study located on Mount Suorooaivi near Abisko, Sweden. Plots pictured are located at 800 m. Photograph credit: Tyler Logan.

heath-dominated vegetation along this gradient from 500 to 1000 m elevation (Sundqvist *et al.* 2014), to explore how polyphenols in leaves and litter responded to nutrient addition and decreasing temperature and nutrient availability associated with increasing elevation. To our knowledge, no study to date has explicitly examined the interactive effects of nutrient availability and temperature on plant defence compounds on both live and senesced leaves in a natural ecosystem. This experiment allows for the opportunity to address questions of how nutrient limitation affects plant defence compounds under contrasting climatic conditions.

We collected both live and senesced leaves from each of the major vascular plant species in heath vegetation from N- and P-fertilized plots across this elevational gradient, and measured chemical and structural defences as well as nutrient concentrations in these leaves to test the following two hypotheses: (i) total polyphenol (TP), condensed tannins (CT) and lignin concentrations, as well as PCC, will decrease with N fertilization because plants will prioritize resource allocation to growth vs. secondary metabolite production (Bryant, Chapin & Klein 1983; Endara & Coley 2011), but will be unaffected by P fertilization because P does not directly affect the phenylalanine pathway (Jones & Hartley 1999); (ii) nitrogen fertilization will reduce polyphenol and lignin concentrations more at higher elevations, due to the observed decline in soil N availability with increasing elevation (Sundqvist *et al.* 2011a), while there will be no effects of P fertilization at any elevation despite P availability varying across the gradient (Vincent *et al.* 2014), because P is not involved in polyphenol production (Jones & Hartley 1999). To further investigate the link between nutrient availability and plant defence compounds among fertilizer treatments, we also measured foliar C:N, C:P and N:P ratios in both fresh and senesced leaves. We address these hypotheses using fresh leaves, where plant defences can mitigate UV damage, pathogenesis and herbivory, as well as senesced leaves, where plant defences can constrain litter decomposition dynamics and nutrient cycling. We explored the responses of phenolic and lignin concentrations both for individual species and at the whole community level to fertilization and elevation, and further assessed the relative contributions of inter- vs. intraspecific variation in driving community-level responses. Thus, investigating how plant defence compounds are affected by contrasting temperatures and nutrient addition in this manner will allow us to better predict responses of subarctic plant communities to global climate change, and the consequences of these responses for ecosystem functioning.

## Materials and methods

### STUDY SYSTEM

The experiment was performed along an elevational gradient on the north-east facing slope of Mount Suorooaivi (1193 m), located approximately 20 km south-east of Abisko, Sweden (68°21'N,

18°49'E) (Sundqvist, Giesler & Wardle 2011b; Sundqvist *et al.* 2011a). Air temperature along this gradient declines by around 2.5 °C from 500 to 1000 m elevation (Sundqvist *et al.* 2011a), which is on par with projected increases in average mean subarctic surface temperatures over the next century (IPCC 2013). Between 1913 and 2000, the mean annual precipitation at the nearby Abisko Scientific Research Station was 310 mm, with highest and lowest values occurring in July (51 mm) and April (12 mm), respectively (Kohler *et al.* 2006). Along this elevational gradient, precipitation ranges from 230 to 290 mm from June to October and shows no change with elevation (Sundqvist *et al.* 2014), in line with other comparable elevational gradients in the region (Karlsson, Jonsson & Jansson 2005). However, decreasing temperature associated with increasing elevation may indirectly enhance moisture availability via decreasing evapotranspiration when precipitation remains constant. Parent soil material consists of salic igneous rocks and quartic and phyllitic hard schists. The tree line is located at 500–600 m and is formed by *Betula pubescens* ssp. *czerepanovii* Ehrh. Heath vegetation is widespread throughout the region at all elevations and is composed primarily of ericaceous dwarf shrubs and *Betula nana* ssp. *nana* L. (hereafter *B. nana*) with interspersed graminoids.

### EXPERIMENTAL SET-UP

For this study, we utilized plots within a fertilization experiment described by Sundqvist *et al.* (2014). During July 2008, a total of 48 plots measuring 1 × 1 m (with an outer 10 cm buffer) were established in heath vegetation at each of the three elevations along the gradient, that is 500, 800 and 1000 m. The mean minimum and maximum distance between plots within an elevation was approximately 10 and 100 m, respectively. Considering the high level of spatial variability observed in this system (Björk *et al.* 2007), it is expected that this distance is sufficient to ensure independence between plots (Sundqvist *et al.* 2011a, 2012). At the 500 m elevation, plots were located in open *Betula pubescens* ssp. *czerepanovii* forest, while plots at 800 and 1000 m were above the tree line. Plots all had a similar slope and a north-east facing aspect.

At each elevation, 16 replicate plots were established with random assignment to one of four fertilization treatments in one of four blocks: control, N addition, P addition and N+P addition. Fertilizer was applied to the treatment plots annually in the amount of 10 g N m<sup>-2</sup> year<sup>-1</sup> as NH<sub>4</sub>NO<sub>3</sub> and 5 g P m<sup>-2</sup> year<sup>-1</sup> as superphosphate (Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>·H<sub>2</sub>O), with the first application on 26 July 2008. For the next 2 years, half of the annual amount of N and P fertilizers was added when all plots were snow free (25 June 2009, 8 June 2010), with the second half added 3 weeks thereafter (16 July 2009, 29 June 2010). In 2011 and 2012, fertilizer was added in one application (27 June 2011; 26–29 June 2012). The amounts of N and P fertilization used in this experiment are consistent with previous studies that have sought to study N and P limitation in arctic ecosystems (Jonasson 1992; Chapin *et al.* 1995; Mack *et al.* 2004; Rinnan *et al.* 2007).

### LEAF ANALYSES

To determine the effects of elevation and fertilization on leaf chemical and structural traits, approximately 3 grams dry weight per species of both fresh and senesced fully expanded undamaged leaves of all major vascular plant species was collected from all plots during 11–27 July and 3–20 September 2012, respectively. Leaves were sampled from each individual of each species in each plot, which is considered sufficient to capture total intraspecific variability (incorporating variability both among and within individuals) at the plot scale (Albert *et al.* 2011; Lepš *et al.* 2011; Violle *et al.* 2012). The species selected represent over 80% of the



vegetation cover at each elevation (Sundqvist *et al.* 2014; Table S1 in Supporting Information), as determined by point quadrat analysis (Goodall 1952). This resulted in a total of eight species collected from the gradient: *B. nana* (800, 1000 m), *Calamagrostis lapponica* Wahlenb. (800, 1000 m), *Cassiope tetragona* D. Don (1000 m), *Deschampsia flexuosa* L. 500 m, *Empetrum hermaphroditum* L. (500, 800, 1000 m), *Vaccinium myrtillus* L. (500 m), *V. uliginosum* L. (800, 1000 m) and *V. vitis-idaea* L. (500, 800, 1000 m). Please see Appendix S1 for details on the natural history of herbivores and the role of defences in deterring herbivores in the study species.

Subsamples of both fresh and senesced leaves from each species from each plot were dried (40 °C) and sorted in the laboratory prior to being ground in a Wiley Mill (Model #: 3383-L40; Thomas Scientific, Swedesboro, NJ, USA). A subsample of each dried leaf sample was analysed for foliar C and N with a LECO TruSpec CN analyzer (St. Joseph, MI, USA), for P using nitric-perchloric acid digestion analysed by inductively coupled plasmography (ICP) and for lignin using the H<sub>2</sub>SO<sub>4</sub> acid digestion method (Chemists 1990). An additional subsample of 0.05 g leaf litter was extracted in 20 mL 50% analysis-grade methanol and shaken for 1 h. Two subaliquots of this subsample were each analysed for TP and CT using the Prussian blue technique with a catechin standard (Stern *et al.* 1996) and the acid butanol method with procyanidin standard B2 (Sigma-Aldrich, St Louis, MO, USA) (Porter, Hrstich & Chan 1986), respectively. Another subsample of 0.25 g leaf litter was extracted in 25 ml de-ionized water and shaken for 24 h. This subsample was used to determine PCC using bovine serum albumin (BSA) to measure the amount of protein precipitated by each leaf extract (Gundale *et al.* 2010). All of the above extractions were filtered through 0.2-µm filter paper (Munktell Filter AB, Falun, Sweden) under vacuum filtration, stored at -18 °C until further analysis and removed from the freezer 24 h prior to analysis to eliminate temperature-induced interference with reaction kinetics.

## SOIL ANALYSES

We measured soil abiotic properties for all plots at all elevations to aid in the interpretation of how soil conditions influence leaf traits. On 23 July 2012, a total of 3–6 soil cores were taken from each plot with a 45-mm-diameter PVC corer to a depth of 10 cm, the humus layer depth measured, and then samples were bulked to yield a minimum of 0.2 L. Soil was kept at 4 °C overnight and then passed through a 4-mm sieve to remove plant matter and stones. Soil pH was determined on a subsample of fresh soil (2.5 g dry weight equivalent) after shaking for 12 h in 40 ml deionized water. Soil organic matter content was determined on a subsample of soil after drying (105 °C, 24 h) and combustion in a muffle furnace (550 °C, 4 h). A subsample of fresh soil (5 g dry weight equivalent) was extracted with 80 ml 1 M KCl after shaking for 2 h; extracts were frozen until analysed for NO<sub>3</sub>-N, NH<sub>4</sub>-N and PO<sub>4</sub>-P by colorimetry on an AutoAnalyser III (SEAL Analytical; Kontram OmniProcess AB, Solna, Sweden). A subsample of soil was dried (60 °C, 72 h), ground with a ball mill (Retsch, Haan, Germany) and analysed for total C and N with a CHN furnace and for P with nitric-perchloric acid digestion, as described above.

## STATISTICAL ANALYSES

To test for the effects of elevation, N addition, P addition and all possible interactions on soil abiotic properties, and on the measured leaf trait values of all species that occurred at two or more elevations, we used a three-way ANOVA. Whenever ANOVA yielded significant effects for species present at all three elevations or

between the four fertilizer treatments, data were further subjected to Tukey's honestly significant difference (hereafter h.s.d.) *post hoc* tests to assess differences between means. Elevation, N addition and P addition were considered fixed factors, with block as a random factor. Block was rarely found to be significant and, although always included in the analyses, was omitted from the results shown. To further examine the link between plant secondary metabolites and foliar nutrient ratios, we utilized Pearson's correlation coefficients. This analysis is justified on the basis that increasing foliar nutrient concentrations are usually associated with declines in secondary metabolites (Zhang *et al.* 2012).

In order to explore changes in the fresh and senesced leaf trait values measured at the whole community level across the elevational gradient and in response to N and P addition, community-weighted averages (CWAs) were calculated as described by Garnier *et al.* (2007). The use of CWAs is based upon the idea that the contribution of each species to ecological processes at the whole community level is related to the proportion of total community biomass that it represents (Grime 1998; Garnier *et al.* 2004). For each leaf trait in each plot, CWAs were calculated as:

$$\text{Weighted plot-average} = \sum_{i=1}^n (p_i \times \text{trait}_i),$$

where  $p_i$  is the relative abundance of species  $i$  in a plot (as a proportion of the total abundance), and  $\text{trait}_i$  is the measured leaf trait of species  $i$ . A three-way ANOVA was then used on the CWA values for TP, CT, PCC, lignin and foliar nutrient ratios, and whenever the ANOVA revealed significant effects, the data were further subjected to Tukey's h.s.d. *post hoc* tests to assess differences between means ( $n = 48$ ).

Using the method developed by Lepš *et al.* (2011), we determined the relative contributions of intra- and interspecific trait variation on the response of weighted average trait values across elevation and nutrient treatments. A detailed description of the procedure used to do this is presented in Appendix S2.

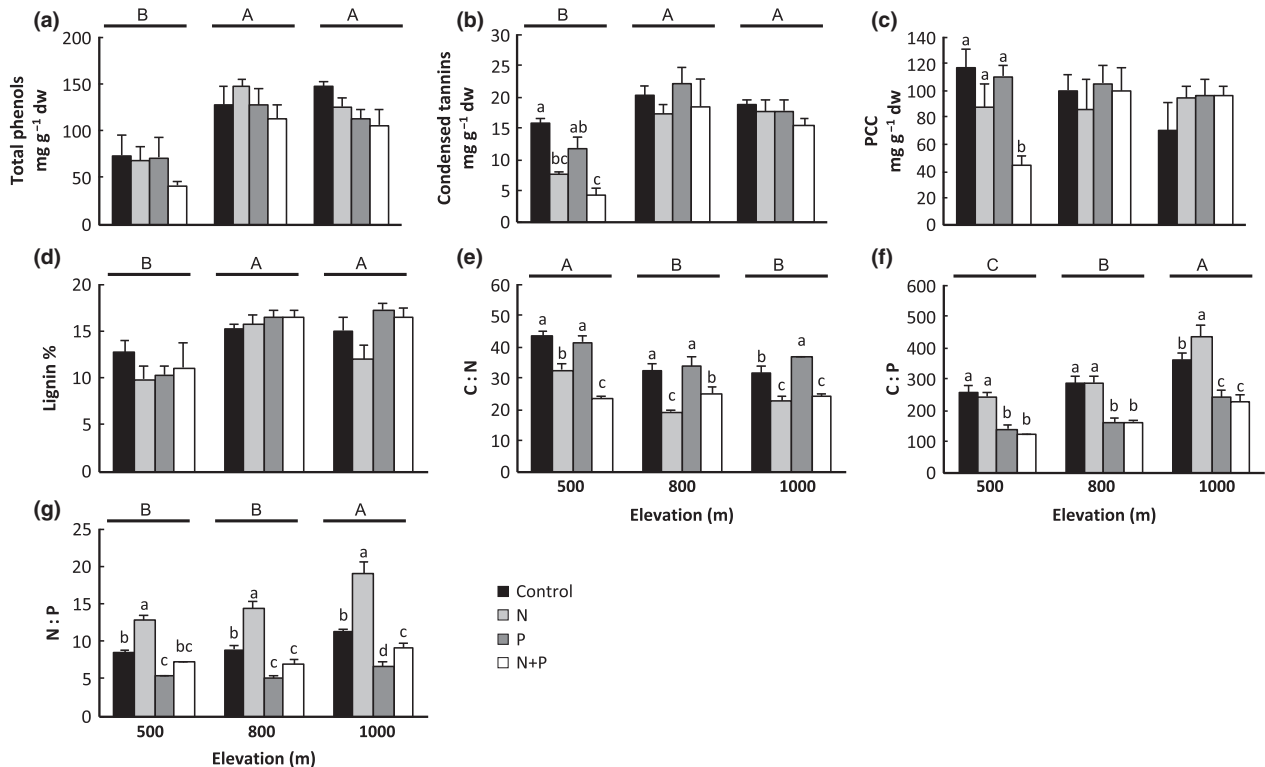
Data were transformed when necessary to meet assumptions of parametric testing. All statistical analyses were performed in SPSS (PASW statistics 21.0; IBM Corporation, Armonk, NY, USA).

## Results

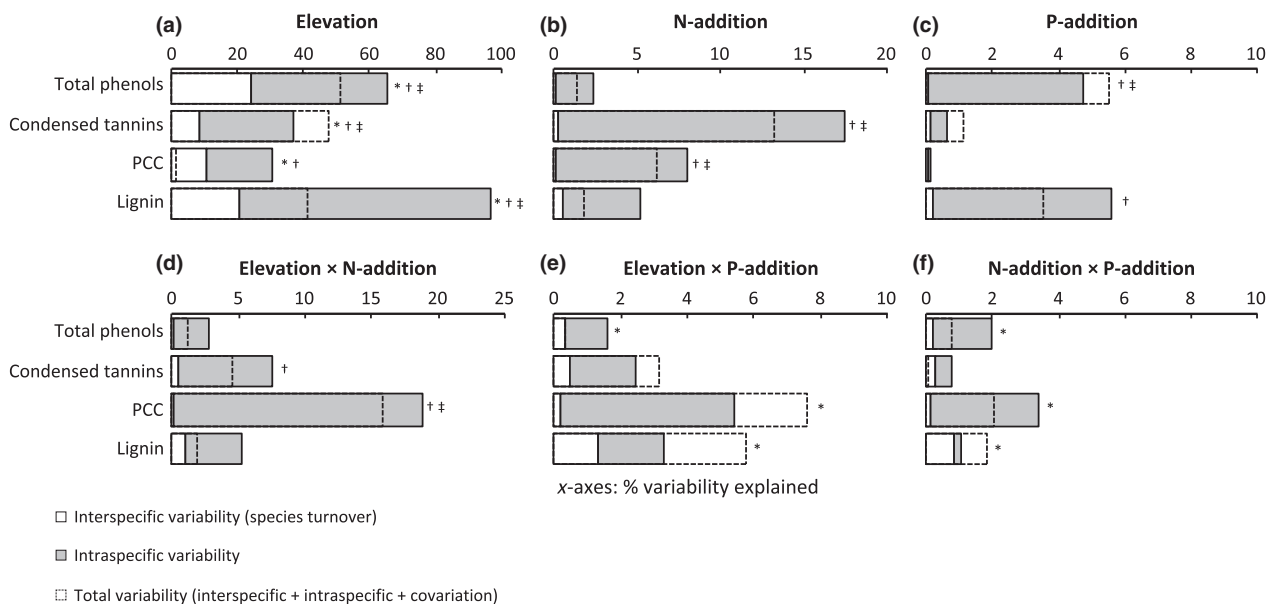
### COMMUNITY-WEIGHTED RESPONSES TO ELEVATION AND FERTILIZATION

The CWAs of defence traits in fresh leaves often responded to elevation and nutrient addition (Fig. 2, Table S2). Total phenols, CT and lignin were lowest at the 500 m elevation. Overall, N addition decreased CT and PCC, and P addition decreased TP. When interactive effects were considered, N addition reduced PCC most at the 500 m elevation.

Total variability, as well as inter- and intraspecific contributions to this variability, was responsive to elevation for all fresh leaf defence traits, except total variability for PCC (Fig. 3). Concerning elevation, all fresh leaf defence traits showed negative covariation between inter- and intraspecific variability (hereafter 'covariation'), except CT, which showed positive covariation. For N addition, intraspecific variability drove the response of total variability for CT and PCC, both showing negative covariation. Addition of P affected intraspecific and total variability



**Fig. 2.** Community-weighted average measures for total phenols (a), condensed tannins (b), protein complexation capacity (PCC)(c), lignin (d), C to N (e), C to P (f) and N to P (g) foliar ratios measured on fresh leaves of dominant plant species in plots without fertilizer (control) or amended with N, P or N+P from subarctic heath vegetation along an elevational gradient (500, 800, 1000 m). Error bars = standard errors ( $n = 4$ ). Within each panel, groups of four bars topped with the same capital letters do not differ significantly at  $P = 0.05$  (Tukey's h.s.d.). Within each group of four bars, bars topped with the same lower case letters do not differ significantly at  $P = 0.05$  (Tukey's h.s.d.). When no letters are used, there is no significant difference among treatments. ANOVA results given in Table S2.



**Fig. 3.** Decomposition of total variation of community-weighted averages of dominant plant species into interspecific, intraspecific and covariation components for fresh leaf traits for total phenols, condensed tannins, protein complexation capacity (PCC) and lignin explained by elevation, (a), N addition (b), P addition (c) and their interactions (d–f) along an elevational gradient (500, 800, 1000 m) in subarctic heath vegetation. The symbols \*, † and ‡ indicate significance ( $P \leq 0.05$ ) of interspecific, intraspecific and total variability effects, respectively. Total variability (denoted by the dashed line) greater than the sum of inter- and intraspecific variability indicates positive covariation, while total variability less than the sum of inter- and intraspecific variability indicates negative covariation. Note the different scales on axes denoting % variability explained.

(positive covariation) for TP and intraspecific variability for lignin. Elevation  $\times$  N interaction influenced intraspecific variability for CT and intraspecific and total variability for PCC; both showed negative covariation. Elevation  $\times$  P and N  $\times$  P influenced interspecific variability for TP, PCC and lignin.

The CWAs of foliar nutrient ratios for fresh leaves responded to both elevation and nutrient addition (Fig. 2, Table S2). With regard to elevation, overall C:N was greatest at 500 m, while both C:P and N:P were greatest at 1000 m. Addition of N decreased C:N ratios and increased N:P ratios, while P addition decreased C:P and N:P ratios. Overall, P addition reduced N:P ratios and increased C:N more at the 1000 m elevation. Generally, N:P ratios were elevated by N when P was not also added.

The CWAs of defence traits in senesced leaves often responded to elevation and nutrient addition (Fig. 4, Table S3). Values of all defence traits (except lignin) were highest at the 500 m elevation and showed overall decreases with N addition. When interactive effects were considered, TP and CT were reduced most by N addition at 500 m.

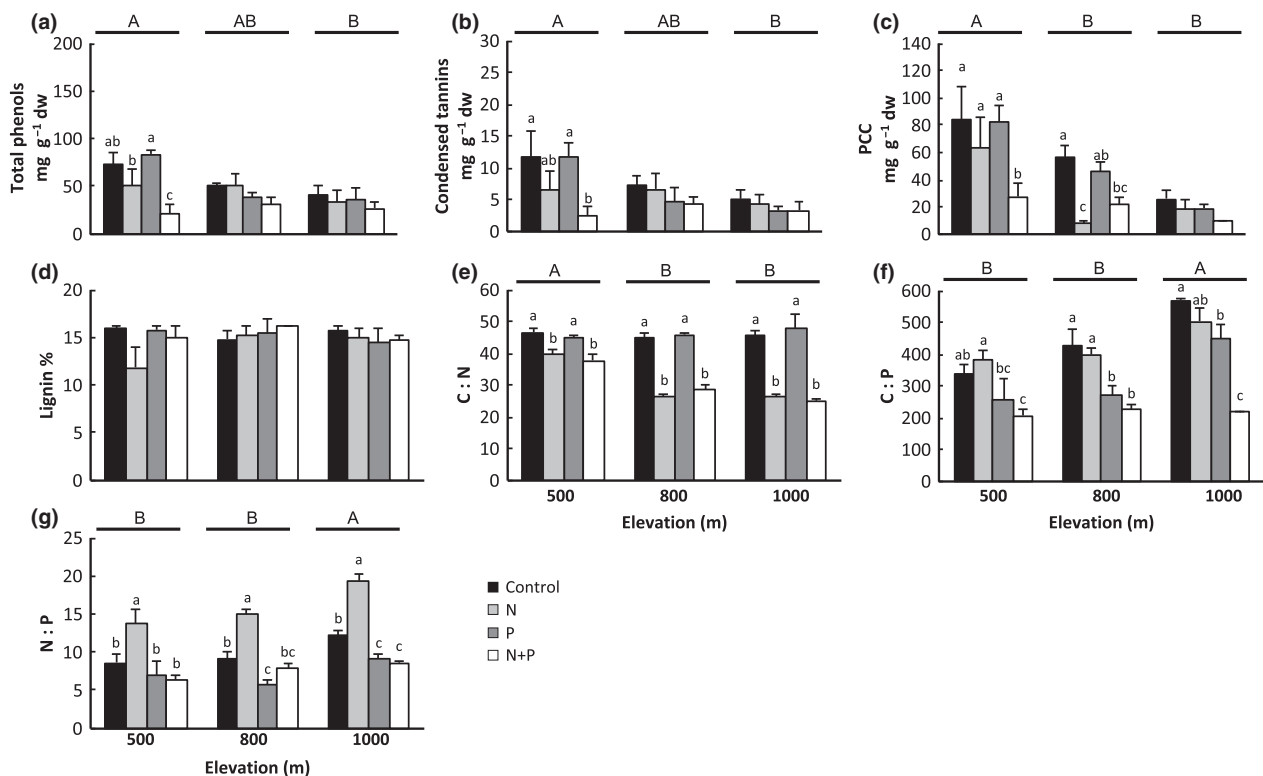
Concerning senesced leaves, elevation drove interspecific variability for all defence traits, while also driving intraspecific variability for CT and lignin and total variability for

TP, CT and PCC; all traits showed negative covariation (Fig. 5). For N addition, intraspecific variability explained the response of total variability for all defence traits except lignin; all showed negative covariation. In response to the elevation  $\times$  N interaction, intraspecific variation explained the total variability of TP and CT, while intraspecific variability was significant for lignin; all showed negative covariation. Interspecific variability explained the response of lignin to the elevation  $\times$  P and the N  $\times$  P interactions, with negative and positive covariation, respectively.

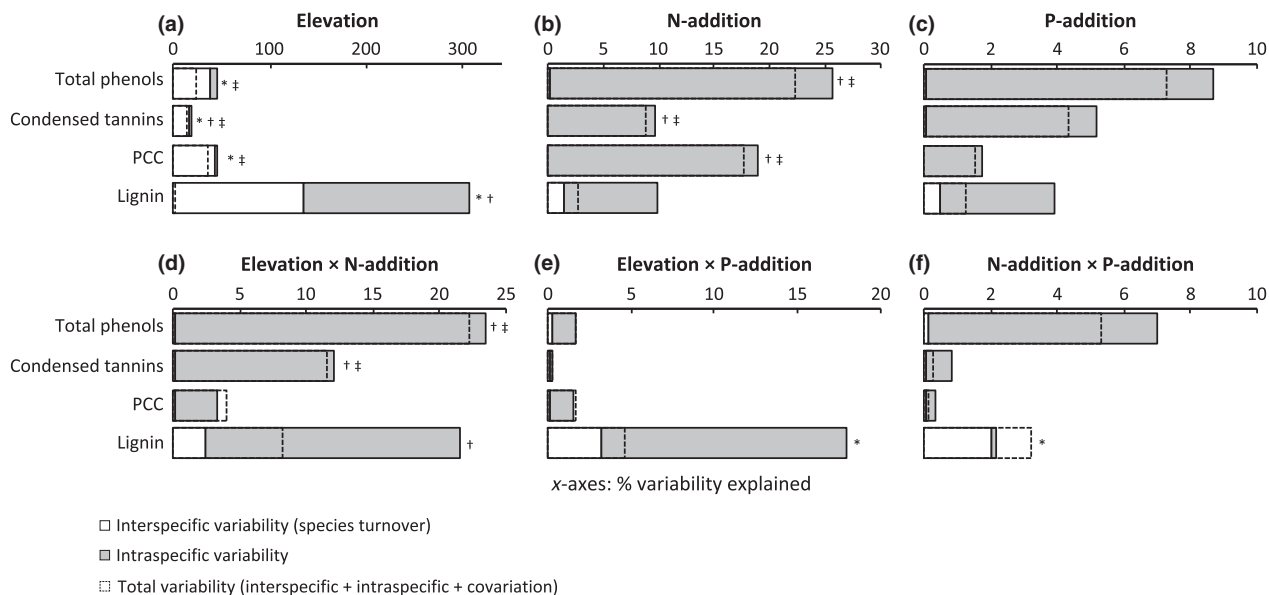
The CWAs of foliar nutrient ratios in senesced leaves often responded to elevation and nutrient addition (Fig. 4, Table S3). Higher elevations had lower C:N, but higher C:P and N:P ratios. Generally, N addition decreased C:N and C:P ratios and increased N:P ratios. Both C:P and N:P ratios declined with P addition. Considering interactive effects, N addition decreased both C:N and C:P ratios more at higher elevations. Adding N+P decreased C:P more than when nutrients were added singly, while N:P ratios increased only when N was added alone.

#### LEAF PROPERTIES AT THE SPECIES LEVEL

Several fresh leaf defence traits of individual species were significantly affected by elevation and nutrient addition



**Fig. 4.** Community-weighted average measures for total phenols (a), condensed tannins (b), protein complexation capacity (PCC)(c), lignin (d), C to N (e), C to P (f) and N to P (g) foliar ratios measured on senesced leaves of dominant plant species in plots without fertilizer (control) or amended with N, P or N+P from subarctic heath vegetation along an elevational gradient (500, 800, 1000 m). Error bars = standard errors ( $n = 4$ ). Within each panel, groups of four bars topped with the same capital letters do not differ significantly at  $P = 0.05$  (Tukey's h.s.d.). Within each group of four bars, bars topped with the same lower case letters do not differ significantly at  $P = 0.05$  (Tukey's h.s.d.). When no letters are used, there is no significant difference among treatments. ANOVA results given in Table S3.



**Fig. 5.** Decomposition of total variation of community-weighted averages of dominant plant species into interspecific, intraspecific and covariation components for senesced leaf traits for total phenols, condensed tannins, protein complexation capacity (PCC) and lignin explained by elevation, (a), N addition (b), P addition (c) and their interactions (d–f) along an elevational gradient (500, 800, 1000 m) in subarctic heath vegetation. The symbols \*, † and ‡ indicate significance ( $P \leq 0.05$ ) of interspecific, intraspecific and total variability effects, respectively. Total variability (denoted by the dashed line) greater than the sum of inter- and intraspecific variability indicates positive covariation, while total variability less than the sum of inter- and intraspecific variability indicates negative covariation. Note the different scales on axes denoting % variability explained.

(Table S4, Fig. S1). Total phenols in *B. nana* and *V. vitis-idaea* were lowest at the 1000 and 500 m elevations, respectively, while PCC for *V. vitis-idaea* was lowest at 1000 m. Overall, N addition increased TP in *V. vitis-idaea* and PCC in *E. hermaphroditum* and *V. vitis-idaea*, but decreased PCC in *B. nana*. Addition of P increased PCC in *B. nana* and lignin in *E. hermaphroditum* (latter not shown). For *V. vitis-idaea*, P addition decreased lignin at the 500 m elevation, but increased it at the highest elevations (data not shown), while N addition increased TP more when P was not also added and generally increased PCC more when P was also added. For *E. hermaphroditum*, TP tended to increase with N+P addition at the 500 m elevation but to decrease at higher elevations.

Nutrient properties of fresh leaves were frequently affected by elevation and nutrient addition (Fig. S1, Table S5). For all species, C:N ratios were overall lowest at the highest elevations, while C:P and N:P ratios were highest at 1000 m for *E. hermaphroditum* and *V. vitis-idaea*. For all species, C:N decreased, and N:P generally increased with N addition, and C:P tended to increase for *V. vitis-idaea*. Addition of P had mixed effects on C:N ratios for all species except *B. nana*, and decreased C:P and N:P ratios for all species. With increasing elevation, N addition reduced C:N ratios less for *B. nana*, but reduced them more for *E. hermaphroditum* and *V. vitis-idaea*. For *V. vitis-idaea*, P addition reduced, while N addition increased N:P ratios more at higher elevations. Further, C:N ratios were lowest with N+P addition for *C. lapponica*, while they were overall lower when N was added alone for

*E. hermaphroditum* and *V. vitis-idaea*. For *E. hermaphroditum*, N:P ratios were lowest when P was added alone. There was an elevation  $\times$  N  $\times$  P addition interaction on C:P and N:P for *B. nana* because N+P addition increased these ratios relative to P addition more at 800 m than at 1000 m.

Defence traits (except TP) in senesced leaves showed some responses to elevation and nutrient addition (Table S6, Fig. S2). Condensed tannins were greatest at 1000 m for *E. hermaphroditum* and *V. vitis-idaea* (data not shown), and PCC was lowest at 500 m for *E. hermaphroditum*. Further, N addition decreased lignin in *C. lapponica* (data not shown) and PCC in all species except *C. lapponica*, while P addition had mixed effects on PCC in *B. nana* and decreased it in *V. vitis-idaea*. Concerning interactive effects, N addition reduced PCC in *E. hermaphroditum* less at the lowest elevation. Generally, N+P addition reduced PCC less in *B. nana* than when N was added alone and more in *V. vitis-idaea* than when P was added alone.

Senesced leaf nutrients often responded to elevation and nutrient addition (Fig. S2, Table S7). Overall, C:N ratios decreased with increasing elevation for all species except *V. vitis-idaea*, where they tended to increase. The C:P and N:P ratios were highest at 1000 m for all species, except C:P ratios for *C. lapponica*. Nitrogen addition decreased C:N ratios for all species, increased C:P ratios in *B. nana* and increased N:P ratios in all species except *C. lapponica*, but decreased C:P in *C. lapponica* and *E. hermaphroditum*. Overall, P addition increased C:N

ratios in *E. hermaphroditum*, but decreased C:P and N:P ratios for all species. With regard to interactive effects, N addition decreased C:N ratios of *E. hermaphroditum* and *V. vitis-idaea* least and increased N:P ratios of *V. vitis-idaea* least at the 500 m elevation. Further, the negative effect of P addition on C:N ratios of *V. vitis-idaea* was least at the 1000 m elevation, while it was generally greatest at higher elevations for C:P and N:P ratios of *E. hermaphroditum* and *V. vitis-idaea*. For *V. vitis-idaea*, C:N ratios were generally lowest when N was added alone. For *C. lapponica*, C:P ratios were lowest with N+P addition, while for *B. nana*, C:P ratios were only increased by N when it was added alone. Generally, N:P ratios increased in all species except *E. hermaphroditum* when N was added alone.

#### RELATIONSHIPS OF SECONDARY METABOLITES WITH NUTRIENT RATIOS

Defence traits were sometimes significantly correlated with nutrient ratios in fresh leaves (Table S8). Specifically, C:N ratios were negatively correlated with TP for *V. vitis-idaea* and positively correlated with PCC for the CWA values and *B. nana* and with lignin for *E. hermaphroditum*. The C:P ratios were positively correlated with TP and CT for the CWA values and negatively correlated with lignin in *E. hermaphroditum*. Ratios of N to P were negatively correlated with CT for *B. nana* and lignin for *E. hermaphroditum*.

Similarly, defence traits were sometimes significantly correlated with nutrient ratios in senesced leaves (Table S8). Specifically, C:N ratios were positively correlated with TP, CT and PCC for the CWA values, CT and PCC for *V. vitis-idaea*, and PCC for *E. hermaphroditum*. The C:P ratios were positively correlated with TP for *C. lapponica* and PCC for *V. vitis-idaea*, and negatively correlated with PCC for *B. nana* and lignin for *V. vitis-idaea*. The N:P ratios were positively correlated with TP for *C. lapponica*, and negatively correlated with PCC for the CWA and for *B. nana* and with lignin for *V. vitis-idaea*.

#### SOIL PROPERTIES

Regarding soil abiotic properties (Tables S9, S10), pH,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  were affected by elevation, with highest pH at 1000 m, lowest  $\text{NH}_4\text{-N}$  at 800 m and  $\text{PO}_4\text{-P}$  increasing with increasing elevation. Soil  $\text{NH}_4\text{-N}$ , C:P and N:P ratios overall increased with N addition, while P addition generally lowered pH and N:P ratios although never significantly so within elevations, and raised  $\text{PO}_4\text{-P}$  concentrations. With regard to interactive effects, N addition tended to increase pH, but only significantly at 800 m, and N addition alone generally increased  $\text{NH}_4\text{-N}$ ,  $\text{NO}_3\text{-N}$ , C:N and C:P; these variables tended to decrease with N+P addition relative to when N was added alone. Further,  $\text{PO}_4\text{-P}$  increased with N+P addition and this increase was greatest at the higher elevations.

## Discussion

In this study, we explored how N and P fertilization affected the secondary metabolites of fresh and senesced leaves along a subarctic elevational gradient. At the community level, secondary metabolites were often reduced by N addition while P addition had few effects. These reductions were primarily driven by intraspecific, as opposed to interspecific, responses to fertilization, with different species responding in contrasting manners to nutrient addition. At the community level, N addition reduced secondary metabolites most at the lowest elevation, while the effects of P addition were independent of elevation. Below, we discuss our findings in the context of the drivers behind plant defence and their implications for the functioning of subarctic tundra ecosystems under future global climate change.

#### EFFECTS OF N AND P ADDITION ON PLANT DEFENCE

In support of our first hypothesis, and in line with previous work (Bryant, Chapin & Klein 1983; Endara & Coley 2011), N fertilization generally decreased CT and PCC in fresh and senesced leaves, as well as TP in senesced leaves, at the whole community level. However, lignin concentrations were non-responsive to N addition, meaning that despite N addition influencing chemical defences, it does not impact on structural defences in our study system. The concomitant declines of PCC and CT in response to N addition were likely due to PCC being driven primarily by CT (Wurzburger & Hendrick 2009). The decline in community-level defences caused by N addition corresponded with a reduction in foliar C:N ratios, especially for senesced leaves (Table S8), which is in line with work showing that C-based defences decrease with increasing foliar N (Kraus, Zasoski & Dahlgren 2004). Despite this overall trend, N addition increased TP in the fresh leaves of *V. vitis-idaea* and PCC in fresh leaves of *E. hermaphroditum* and *V. vitis-idaea*, although PCC in senesced leaves of both species was reduced. This is in line with studies that have shown increased N availability to lead to greater investment by some plant species in secondary metabolites to protect against herbivory of fresh leaves (Keinänen *et al.* 1999), as well as those that point to a decreased need for PCC in senesced leaves for ericaceous species (e.g. *E. hermaphroditum* and *V. vitis-idaea*), which typically secure nutrients from recalcitrant litter that only their own mycorrhizae can access (Wurzburger & Hendrick 2009). However, other species such as graminoids may instead invest resources into growth under increasing N availability, as we observed (Table S1). Our results are relevant for understanding the effects of increased N mineralization rates expected to result from climate warming in the Arctic (Aerts 2006), because they show that higher N availability may downregulate tannins and PCC for some species which could contribute to a positive feedback between



decomposition, plant N availability and plant litter chemistry (Dorrepaal, Cornelissen & Aerts 2007).

Most secondary metabolites in both fresh and senesced leaves were unresponsive to P at the community level, which is in line with our first hypothesis. The only exception was that P addition caused a weak overall decrease in TP in fresh leaves. Moreover, the CWA values of TP in fresh leaves were positively correlated with their C:P ratios (Table S8). These findings are consistent with studies showing that increased foliar P can coincide with a reduction in polyphenols (Zhang *et al.* 2012), because of reduced plant nutrient limitation leading to greater carbon allocation to primary growth as opposed to secondary metabolite production (Bryant, Chapin & Klein 1983). In this system, N and P have been shown to co-limit plant biomass (Sundqvist *et al.* 2014) and it is therefore possible that P addition improved N availability (Güsewell 2004; Vitousek *et al.* 2010), with the resultant increase in N leading to the observed decreases in TP. Further, while TP were reduced in fresh leaves at the community level only by P addition, they were reduced in senesced leaves only by N addition. The pool of TP consists of a variety of compounds whose relative concentrations change over time during both leaf development and senescence (Gallet & Lebreton 1995), and it is possible that N and P vary in their effects on phenolic compounds that dominate in fresh vs. in senesced leaves. Further, the relative importance of N vs. P in driving C allocation may shift from peak growing season to senescence (Holeski *et al.* 2012). However, the effects of P addition on plant defence were much weaker than those of N addition, and besides the one responsive CWA measure, the TP of only one species (*V. vitis-idaea*) showed a weak response to P addition. Overall, our results suggest that variation in P availability has a minor effect on plant secondary metabolites which is likely of minimal ecological significance, and provides experimental support consistent with observational studies that plants on P-poor soils are not necessarily well defended (Wright *et al.* 2010).

#### EFFECTS OF N AND P ADDITION ON PLANT DEFENCE WITH CHANGING ELEVATION

In contrast to our second hypothesis and despite large changes in plant-available N across this elevational gradient (Sundqvist *et al.* 2011a; Tables S9, S10), N addition did not generally reduce defences more as elevation increased. At the community level, plants generally had greater secondary metabolites at the highest elevations in fresh leaves, with this pattern being reversed in senesced leaves. This pattern may be the result of higher elevation plants producing greater concentrations of phenols that serve a photoprotective function for fresh leaves (e.g. flavonoids) (Filella & Peñuelas 1999) and which decline rapidly during senescence (Olsen *et al.* 2009), as well as lower elevation plants producing defence compounds that are more stable and less easily resorbed (Reichardt *et al.*

1991). Alternatively, in line with the resource availability hypothesis (Coley, Bryant & Chapin 1985), plants adapted to the resource-poor and more stressful higher elevations may have invested more in defence of their fresh leaves in order to deter vertebrate herbivores, such as lemmings and reindeer, which tend to increase with increasing elevation (Vistnes *et al.* 2008; Hoset *et al.* 2014). Further, shifts in the type of phenols that the plant community produces with changing elevation may explain several of the observed interactions between N addition and elevation that we observed. For instance, at high elevations, even as more N becomes available, plants may continue to allocate resources to secondary metabolites to tolerate higher levels of UV radiation (Close & McArthur 2002; Roberts & Paul 2006) and to compensate for reduced efficacy of these compounds at lower temperatures (Albert *et al.* 2009). Further, the effects of N in reducing community-level PCC in fresh leaves and TP and CT in senesced leaves were greatest at the lowest elevation. Plants at lower elevations are less temperature limited (Hoch & Körner 2012), and an increase in N may be more likely to shift C allocation from secondary metabolites to growth when conditions are warmer (Lattanzio, Cardinali & Linsalata 2012) despite higher invertebrate herbivory at the tree line in this system (Hagen *et al.* 2007). Thus, with future global warming and subsequent increases in soil N availability (Aerts 2006), it is possible that high elevation heath plant communities may exhibit reductions in polyphenol concentrations that are in line with those observed for low elevation communities in our study.

#### INTER- VS. INTRASPECIFIC DEFENCE TRAIT RESPONSES TO NUTRIENT ADDITION AND ELEVATION

Interspecific variation (i.e. species turnover) was often an important determinant of the responses of total community-level variation in defence traits to elevation, which is in line with previous work on plant functional trait variation along elevational gradients (Sundqvist *et al.* 2011a; Kichenin *et al.* 2013). However, the community-level response of these defences to N addition was often driven to a greater extent by intraspecific rather than interspecific variation among plots, suggesting that within-species plant secondary metabolites are often highly plastic and nutrient-sensitive (Bryant, Chapin & Klein 1983; Endara & Coley 2011). Changes in secondary metabolites at the species level may eventually have community-level implications because poorly defended leaves often decompose and release nutrients faster, thereby potentially leading to greater dominance of resource acquisitive species (Hobbie 1992) that are capable of compensating for herbivory with increased growth rates (Coley, Bryant & Chapin 1985). We also found that the responses of community-level secondary metabolites to the interaction of N addition and elevation were primarily explained by intraspecific variation. This indicates that while increased N availability through fertilization reduces polyphenols more at lower elevations,

the primary driver of the elevation  $\times$  N interactive effect is due to within-species plastic responses rather than species turnover. Such adaptability may serve as a short-term buffer to changing climate as higher elevations become warmer, with species capable of altering their leaf chemistry better able to adapt to temperature-induced changes in nutrient availability. Further, community-level responses to N addition and its interaction with elevation were strongly affected by covariation between inter- and intraspecific variation, pointing to both sources of variation interacting to influence the community-level responses observed. Taken together, these findings draw attention to the need to consider not just species turnover but also intraspecific variability when making predictions of how the plant community, and potentially the ecosystem processes that it drives, will respond to future changes in the environment (Violle *et al.* 2012).

## Conclusions

Our results demonstrate that N addition often reduced, while P addition only occasionally affected, plant secondary metabolites in subarctic heath vegetation. Further, N addition frequently decreased these defences more at lower elevations, meaning that a warmer climate with increased nutrient availability will likely reduce plant defences. Both intraspecific variation and interspecific variation were strong drivers of plant defence response to elevation, while intraspecific variation and its covariation with interspecific variation were the main components of defence response to N addition; these findings demonstrate the need to consider both sources of variation in explaining plant defence response to changes in environmental conditions. Subarctic heath vegetation is typically well defended against herbivory and its litter decomposition rates are slow. Responses of plant defences in subarctic heath to increasing temperatures and N availability, particularly in low elevation communities that currently have high invertebrate herbivory pressure (Hagen *et al.* 2007), will likely lead to further increased herbivory and altered decomposition rates and nutrient fluxes (Bardgett & Wardle 2003), potentially resulting in feedbacks to the plant community (Facelli & Pickett 1991). Our results pull focus on the need to consider the interactive effects of temperature and nutrients in regulating defences of subarctic heath plant communities, and the importance of taking the said effects into account when predicting ecosystem functioning under future climate change.

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## Data accessibility

Data used in this paper have been archived with Dryad Digital Repository under the DOI: 10.5061/dryad.82gq2 (De Long *et al.* 2015).

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## Supporting Information

Additional Supporting information may be found in the online version of this article:

**Fig S1.** Defence and leaf properties in fresh leaves of four dominant plant species.

**Fig S2.** Defence and leaf properties in senesced leaves of four dominant plant species.

**Table S1.** Dominant vascular plant species cover in study plots.

**Table S2.** Results ANOVA for effects of elevation, N, P on community fresh leaf properties.

**Table S3.** Results ANOVA for effects of elevation, N, P on community senesced leaf properties.

**Table S4.** Results ANOVA for effects of elevation, N, P on species fresh leaf defences.

**Table S5.** Results ANOVA for effects of elevation, N, P on species fresh leaf nutrient ratios.

**Table S6.** Results ANOVA for effects of elevation, N, P on species senesced leaf defences.

**Table S7.** Results ANOVA for effects of elevation, N, P on species senesced leaf nutrient ratios.

**Table S8.** Pearson's correlation coefficients between secondary metabolites and nutrient ratios.

**Table S9.** Results ANOVA for effects of elevation, N, P on soil abiotic properties.

**Table S10.** Soil abiotic properties.

**Appendix S1.** Natural history of herbivory and the role of secondary metabolites in deterring herbivory for dominant plant species in study area.

**Appendix S2.** Supplementary methods on decomposition of variance.