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ARTICLE
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Recovery of metabolites via subnivean photosynthesis in Arctic tundra plants: Implications for climate change

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Abstract

Plants have evolved numerous strategies for surviving the harsh conditions of the Arctic. One strategy for Arctic evergreen and semi-evergreen species is to photosynthesize beneath the snow during spring. However, the prevalence of this photosynthesis and how recent photosynthates are used is still unknown. Here we ask, *how is newly acquired carbon beneath the snow allocated?* To answer this question, we delivered isotopically labeled $^{13}CO_2$ to tussock tundra plants before snowmelt. Soluble sugars and starches were preferentially enriched with ^{13}C in all five species tested, with lipids having comparatively low ^{13}C enrichment. These results provide evidence of the recovery of metabolites used over the long winter. Additionally, these new soluble sugars may function in photoprotection and cold tolerance as plants release from snow cover. Climate change, by reducing the duration of subnivean photosynthesis of these species, will limit metabolite production before snowmelt, which may lead to a reduction in the ability of these species to compete effectively during the growing season, potentially leading to changes in community structure.

KEYWORDS
carbon allocation, climate change, metabolic activity, stable isotope labeling, subnivean photosynthesis, tussock tundra

INTRODUCTION

The survival of plants and, by extension, ecosystems in Arctic tundra necessitates maintaining photosynthesis across an array of extreme conditions. A variety of adaptations facilitate this ability (Iversen et al., 2015; Mølmann et al., 2021; Natali et al., 2012; Niittynen et al., 2020), but an in situ study of such adaptations in the remote and harsh Arctic environment has proved challenging. As a result, many gaps still exist in our knowledge of Arctic plant ecophysiology.

One area particularly understudied is subnivean photophysiology. With sufficient available light, tundra evergreen and semi-evergreen species photosynthesize under snow, where severe cold is ameliorated, and respiratory CO_2 is trapped and may be recycled by plants (Lundell et al., 2010; Starr & Oberbauer, 2003). Despite knowledge of its existence, little research has investigated the underlying physiology of subnivean photosynthesis or its role in tundra evergreen and semi-evergreen survival. This study provides critical insight into both topics, which could improve the prediction of tundra community dynamics in the face of climate change.

Subnivean photosynthesis could potentially fulfill multiple adaptive functions. Photosynthesis beneath the snow is hypothesized to allow evergreen and semi-evergreen species to compete with more productive growth forms, such as deciduous shrubs, by providing a head start in CO_2 uptake in spring, allowing for the synthesis of structural and/or nonstructural carbohydrates before releasing from snow cover. Subnivean photosynthesis could also help evergreen vascular and nonvascular plants recover depleted energetic metabolites used for the maintenance of homeostasis over the long winter. This second hypothesis is reinforced by Patankar et al. (2013), who observed that many vascular tundra species, including evergreens, experience a significant decline in leaf starch content in fall, presumably to synthesize protective compounds for winter cold resistance, and that evergreen species show the lowest concentrations of simple sugars directly after snowmelt.

The critical need to establish a "function" for subnivean photosynthesis should not be understated. Knowledge of the primary role of this process will directly inform our understanding of the fate of tundra plant communities under climate change. Changes in atmospheric temperature have already led to reduced snow cover in the tundra (Box et al., 2019), mostly due to earlier spring melt, with the annual duration of snow cover across the Arctic decreasing by around 4 days per decade (Box et al., 2019; Callaghan et al., 2011; Hinzman et al., 2005). This reduction of snow cover, an integral abiotic component of Arctic systems, may have profound implications for ecosystem structure and function, especially for species that may be dependent on subnivean photosynthesis. Early melt-out may limit the premelt accumulation of depleted metabolites. Compounding this, the loss of snow will expose already active photosystems to both high light and extreme cold, a potentially disastrous combination (Pardee et al., 2019; Saarinen et al., 2016; Taulavuori et al., 2011). As a result of evolved capacity for photosynthetic activity beneath the snow, evergreen and semi-evergreen—and potentially moss—species may be substantially more vulnerable to climate change than deciduous growth forms. Therefore, this comparatively understudied process may be the basis for a dramatic shift in the relative abundance of tundra plants. These profound changes in plant community structure will likely have circumpolar energetic and climatic implications and a variety of bottom-up effects (Mekonnen et al., 2018; Pearson et al., 2013; Vowles & Björk, 2019).

Knowledge of the physiological importance of subnivean photosynthesis is critical for the development of a holistic understanding of how climate change will impact tundra plant communities and, by extension, the ecosystem. Through two years of early spring in situ $^{13}CO_2$ isotope labeling under snow, we address the questions: *how widespread is subnivean photosynthesis, and how is newly acquired carbon allocated?* Our study answers these questions in terms of both energy-storing metabolites and tissue types while quantifying differences in allocation among different growth forms of Arctic tundra.

METHODS

This study was conducted for two consecutive years (2010, 2011) near Toolik Field Station, in the foothills of the Brooks Range, Alaska, USA (68°37.082′ N, 149°36.209′ W, ~760 m above sea level) on the northeastern side of a 10° slope. The study site is a moist acidic tussock-dwarf-shrub tundra (Bliss & Matveyeva, 1992), typical for the Alaskan Arctic, and dominated by graminoids (mainly *Eriophorum vaginatum* L. and *Carex bigelowii* Torr. ex Schwein.), deciduous shrubs (mainly *Betula nana* L. with *Vaccinium uliginosum* L. and *Salix pulchra* Cham.), evergreen shrubs (mainly *Rhododendron tomentosum* Harmaja. and *Vaccinium vitis-idaea* L.), and mosses (mainly *Hylocomium splendens* (Hedw.) Schimp., *Aulacomnium turgidum* (Wahlenb.) Schwägr., *Dicranum* spp., and *Sphagnum* spp.) (Bret-Harte et al., 2004; Shaver & Chapin, 1991). The climate is continental arctic type and, as of December 31, 2010, the site had a mean hourly temperature of −8.5°C, mean hourly photosynthetically active radiation (PAR) of 230.6 $\mu mol\ m^{-2}\ s^{-1}$, and mean annual precipitation of 254.4 mm (Environmental Data Center Team, 2021). The soil is a Pergelic Cryaquept developed on Itkillik glacial till (Walker & Barry, 1991), with a maximum active layer depth of 53 cm in 2010 and 47 cm in 2011, the years of this study (Streletskiy, 2019).

Study design

We used in situ $^{13}CO_2$ labeling of vegetation to measure carbon dioxide uptake by vascular and nonvascular species in the subnivean environment in spring 2010 and 2011. A pulse labeling experiment was not achievable because installing chambers would disturb the snow cover and the microenvironment around the vegetation. Therefore, a continuous-flow system was used to deliver $^{13}CO_2$ to the vegetation under the snow (Appendix S1: Figure S1).

Tissue collection and isotopic analysis

Leaf and stem samples were collected in the center of the experimental plots 10 cm from the labeling tube, along the 50 cm containing the dispensing holes. Data collection was performed 24 h after the labeling period. In the control plots, tissue samples were collected randomly in the 20 × 50 cm area (Appendix S1: Figure S2). Leaves were collected from the evergreen dwarf shrubs *Rhodedendron tomentosum* (RT) and *Vaccinium vitis-idaea* (VVI), and the semi-evergreen sedge *Eriophorum vaginatum* (EV). Samples of two groups of mosses—the fronds of the feather moss *Hylocomium splendens* (HS) and the capitula of mosses in the genus *Sphagnum* (SPH)—were collected as well. Stem tissue of *R. tomentosum* and *V. vitis-idaea*, were also collected in the first year of labeling.

Tissues were collected from four individuals per species per plot in the first year. However, green leaves of EV were found only in one experimental plot and the two control plots, HS was only found in one experimental plot, and *Sphagnum* mosses were not found in experimental Plot 2. In the second year, three individuals from each species were sampled in the control plot while sample numbers per species in the labeled plots varied from 0 to 6 in each plot. At least one individual per species was found in each plot, excluding EV, which was not found in two of the four plots. After collection, all tissue samples were freeze-dried and ground to fine powder.

Extraction and isolation of carbohydrates and lipids

A sub-sample of 100 mg powdered dry plant material was suspended in 1 mL methanol/chloroform/water (MCW, 12:5:3, v/v/v) for 30 min at 70°C (water bath). On cooling, samples were centrifuged at 10,000*g* for 2 min. An aliquot of 0.5 mL of the supernatant was transferred into a new vial. The phases were then separated by adding 0.4 mL de-ionized water and 0.15 mL chloroform. After being shaken vigorously, samples were centrifuged again at 10,000*g* for 2 min. Next, an aliquot (0.125 mL) of the lower phase containing chloroform and the lipids was collected in a centrifuge tube. The aliquot (0.75 mL) of the upper phase containing the methanol and the soluble sugars was collected and 0.3 mL chloroform was added to this extract. Thereafter, the extract and chloroform were mixed thoroughly and centrifuged again at 10,000*g* for 2 min. An aliquot (0.25 mL) of the lower phase containing chloroform and the lipids was collected and added to the first aliquot of chloroform + lipids. An aliquot of the supernatant (0.7 mL) containing the methanol + water + soluble sugars was freeze-dried and re-dissolved in 1 mL de-ionized water to prevent interference of traces of chloroform with the ion-exchange resin. The lipids + chloroform fraction was dried at 60°C until 100 μL remained in the tube. The aliquot was then transferred into tin capsules and dried at 60°C until complete evaporation prior to measurement.

The neutral fraction of soluble sugars was obtained via an ion-exchange technique. First, soluble carbohydrates were isolated by applying 1 mL of the chloroform-free, re-dissolved extract to columns (13 mm inner diameter) filled with a mixture of anion-exchange resin (1.2 g, DOWEX 1 × 8, 50–100 mesh, prepared in formate form) and cation-exchange resin (0.7 g, DOWEX 50W × 8, 50–100 mesh, prepared in tT form) separated with Frits discs of 20 μm PE. The ion-exchange resins were prepared immediately before they were used. The re-dissolved sugar extract was then applied on top of the column. Following this, the column was rinsed three times with a total of 11 mL of de-ionized water. The effluent was freeze-dried and re-dissolved in 0.5 mL of pure ethanol to ensure only the dissolution of low molecular weight sugars. It was then dried at 60°C until 100 μL remained. Finally, the aliquot was transferred into tin capsules and dried at 60°C prior to measurement.

Sub-samples of ground tissues were analyzed for carbon content and ^{13}C enrichment. Enrichment was evaluated using the isotopic ratio of ^{13}C:^{12}C, which is expressed in terms of the conventional δ^{13}C (in per-mille) notation according to the relationship:

$$\delta^{13}\text{C} = [(R_\text{S} - R_\text{PDB})/R_\text{PDB}] \times 1000 \quad (1)$$

where R_s and R_PDB are molar abundance ratios of the carbon isotopes, ^{13}C: ^{12}C, of the sample and the Pee Dee Belemnite standard (PDB), respectively (Farquhar et al., 1989). All isotopic analyses for bulk tissues and metabolites were conducted at the Washington State University Isotope Lab using an elemental analyzer coupled with a mass spectrometer.

Data analysis

This experiment followed a randomized complete block design; however, the distribution of data rendered several blocks incomplete for some species. In total, there were 375 measurements of δ^{13}C in the labeled individuals: 95 measurements each of bulk tissues, soluble sugars, and lipids and 90 of starch.

All analyses were performed using R statistical software (R Core Team, 2019). Because initial general linear mixed models (GLMM) estimated via nlme (Pinheiro et al., 2019) using δ^{13}C as a function of metabolite revealed significant departures from assumptions of normality and homoscedasticity, we formulated preliminary nonparametric tests to first evaluate whether uptake had occurred. As the logical organ of uptake would be the leaves/fronds/capitula, we concentrated this analysis on these tissues only. Using the Wilcoxon rank sum test (Mann & Whitney, 1947), we tested whether labeled leaf/fronds/capitula tissues had significantly greater δ^{13}C values than those of controls for each metabolite in each species in each year. Following this analysis, we performed similar tests with all species together to evaluate differences in the effect of labeling between years. While nonparametric tests are robust to nonnormality and heteroscedasticity, these preliminary analyses are, however, relatively insensitive compared to parametric tests. Thus, we used these preliminary analyses as a verification step prior to parametric analyses.

Because δ^{13}C values can be negative, we transformed δ^{13}C with the function $\ln(\delta^{13}\mathrm{C} + c)$ as a function of metabolite, testing the sensitivity of results to the value of the constant, c, and evaluating how changes in c affected p values by metabolite, as well as homoscedasticity and normality of the residuals. For all models, a c value 1‰ greater than the absolute value of the lowest ^{13}C value did not eliminate heteroscedasticity or non-normality; however, it substantially reduced both from the nontransformed models. After transforming, we estimated several multi-factor GLMMs, with plot as a random effect. To elucidate differences among δ^{13}C values by species, the first model included fixed effects for species, metabolite, and their interaction. To compare allocation by tissue type, we estimated a second model that included these effects, as well as tissue type and its interactions with species and metabolite. However, this analysis could only be conducted using data from the two dwarf evergreen shrubs, RT and VVI, in Year 1. Finally, we estimated a third model to elucidate differences among ^{13}C values by growth form. This model included fixed effects for growth form, metabolite, and its interaction. All models used the Kenward–Roger adjustment to the denominator degrees of freedom (Kenward & Roger, 1997). For each model, we estimated marginal means and conducted post hoc Tukey's honestly significant difference tests (package emmeans; Lenth, 2020) to elucidate significant effects ($p < 0.05$).

Preliminary analyses revealed substantial differences in the variance of values between Year 1 (2010) and Year 2 (2011). Therefore, we analyzed the two years separately in all analyses. In addition, to account for physiological differences between vascular and nonvascular species, we analyzed bryophytes independently in all analyses, except in the comparative analysis among growth forms. Tissue-based allocation in dwarf evergreen shrubs in Year 1 and overall growth form allocation was also evaluated using logarithmic transformation and GLMM. Unless otherwise stated, all analyses were applied only to leaf, frond, or capitula tissue, as stem tissue was collected only from the dwarf evergreen shrubs in Year 1 of the study.

RESULTS

Climatic conditions

In Year 1, labeling commenced around 1 month before summer solstice (May 14, 2010), when snow cover was 25–29 cm deep in our experimental plots, 8 days before daily mean temperature exceeded the freezing point (May 22, 2010) and 13 days before 80% of snow cover at the site had melted (May 27, 2010). In 2010, the daily mean air temperature above the snow during labeling was −0.4°C, and air temperature ranged from −3.4 to 1.6°C.

In Year 2 (2011), deep snow persisted much later than in 2010 until a warm period triggered rapid snowmelt. The average snow depth was 49.5 cm when labeling began (May 21) but dropped to 23.8 cm over the 2-day sampling period. This sudden decrease in snow depth over the labeling period was coupled to approximately 10°C increase in air temperature, from 5.8°C on May 22 to 15.3°C on May 23. This limited the physiological window for plants under the snow, which is expected as climate warming advances in the region.

Allocation of ^{13}C among metabolites in leaves and capitula, Years 1 and 2

Nonparametric analyses confirmed that there was significant ^{13}C enrichment in our experimental plots (see Appendix S1: Supplemental Results) ($n = 94$ experimental and $n = 27$ control individuals). Across all species and years, we found consistent differences in δ^{13}C among metabolites and bulk tissue across species ($n = 94$). Soluble sugars and starch had the highest δ^{13}C, while lipids consistently had the lowest and bulk tissue was midrange (Figure 1). We also found differences in δ^{13}C of these metabolites among species and growth forms. In Year 1, C enrichment was consistently much higher than in Year 2 with much higher variation (Figure 1).

δ^{13}C in leaves of vascular species, Year 1

The GLMM comparing vascular species in Year 1 ($n = 34$) indicated that there were significant differences

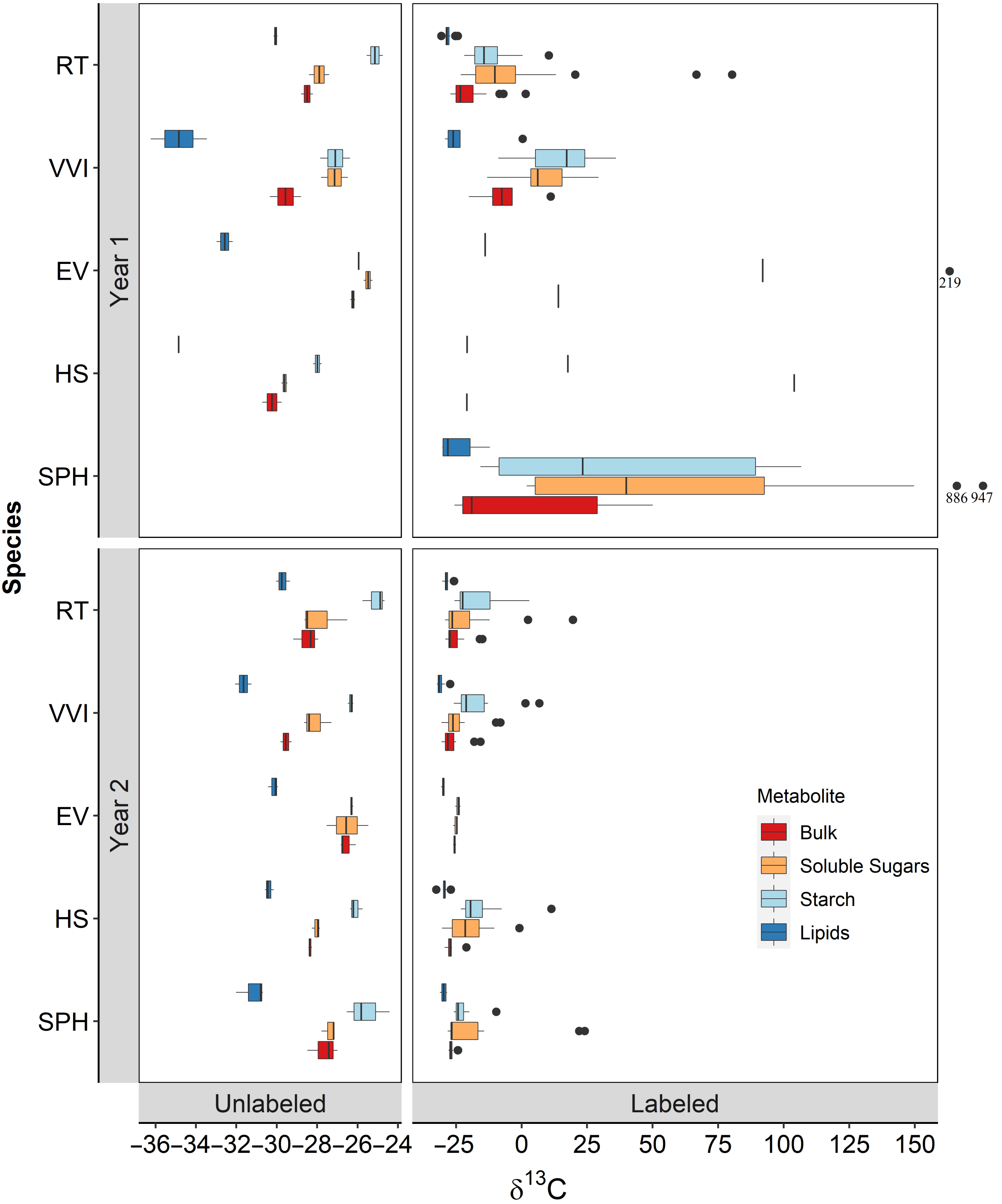

FIGURE 1 Boxplot of control (unlabeled) and experimental (labeled) isotopic ratio (δ^{13}C) values for the photosynthetic tissue by species over both Year 1 and Year 2 of the study. Upper and lower bounds of boxes represent first and third quartiles. Whiskers extend to the highest/lowest values unless such values are outside 1.5× the interquartile range from the box. The three labeled dots represent points removed from the graph for clarity (219‰ for soluble sugars in EV, 886‰ and 947‰ for soluble sugars in SPH); all three points are from labeled individuals in Year 1. EV, *Eriophorum vaginatum*; HS, *Hylocomium splendens*; RT, *Rhododendron tomentosum*; SPH, *Sphagnum* mosses; VVI, *Vaccinium vitis-idaea*.

in δ^{13}C among metabolites and among species ($p < 0.036$; Table 1). Estimated marginal means suggested that soluble sugars and starch had the highest δ^{13}C (30.4‰ and 16.3‰, respectively) while lipids had the lowest (−24.2‰; Figure 2). This result was confirmed by pairwise post hoc tests of the marginal means; soluble sugars and starch had significantly higher δ^{13}C than bulk tissue or lipids ($p < 0.05$) and were not significantly different from one another in labeled plots. The species effect proved to be the result of significantly greater δ^{13}C in EV than in RT ($p = 0.0399$), while VVI was not significantly different from EV or from RT.

δ^{13}C in fronds and capitula of *Hylocomium* and *Sphagnum* species, Year 1

In Year 1, δ^{13}C in HS and SPH were similar ($n = 7$; $p > 0.08$; Table 1), but, as in the vascular species, δ^{13}C was significantly different among metabolites. Soluble sugars (estimated marginal mean: 119‰) showed significantly greater δ^{13}C than starch (estimated marginal mean: 13‰; $p = 0.004$) and both metabolites were significantly enriched with ^{13}C than bulk tissues and lipids ($p < 0.01$). Both moss species proved highly variable in δ^{13}C, with SPH having a maximum δ^{13}C of 947.2‰ (728‰ higher than the maximum value of the next highest species, EV) and HS having a maximum δ^{13}C of 103.9‰.

δ^{13}C in leaves of vascular species, Year 2

Analyses of Year 2 vascular plant data ($n = 33$ experimental, $n = 9$ control) showed a significant effect of metabolite on ^{13}C enrichment, but this effect was species-dependent ($p = 0.0019$; Table 1). Post hoc marginal means tests revealed a mix of significant differences in δ^{13}C across species by metabolite combinations. Generally, starch in all species had significantly higher δ^{13}C than the lipids of any other species, except for EV starch compared to RT lipids. In addition, RT soluble sugars had significantly higher δ^{13}C than VVI lipids. The difference between soluble sugars of one species and lipids of another was not significant in all other contexts. Comparing across metabolites, starch showed significantly higher δ^{13}C than bulk tissue ($p = 0.0061$) while soluble sugars did not ($p = 0.5870$). Both soluble sugars and starches had significantly higher δ^{13}C ($p \leq 0.0001$) than lipids but were not significantly different from one another ($p = 0.0764$). Estimated marginal means across species ranged from −31.3‰ for lipids in VVI to −19.4‰ for starch in VVI. Starch, the metabolite with the highest δ^{13}C, had an estimated marginal mean δ^{13}C of −22.2‰ for

TABLE 1 Type 3 tests of fixed effects for general linear mixed models of δ^{13}C in leaves, fronds, or capitula of tundra plants by year.

Year	Model	Fixed effect	df	*F*	*p*
1	Vascular	Species	2, 4.43	7.72	0.036
		Metabolite	3, 54.29	25.1	<0.0001
		Metabolite × Species	6, 109.46	0.62	0.7158
	Bryophyte	Species	1, 2.01	0.01	0.9283
		Metabolite	3, 9.8	57.56	<0.0001
		Metabolite × Species	3, 9.8	3	0.083
	All	Growth form	2, 3.65	3.07	0.1649
		Metabolite	3, 6.62	29.31	<0.0001
		Metabolite × Growth form	6, 135.5	4.54	0.0003
2	Vascular	Species	2, 4.47	0.85	0.4858
		Metabolite	3, 27.57	27.56	<0.0001
		Metabolite × Species	6, 110.19	3.77	0.0019
	Bryophyte	Species	1, 2.71	0.12	0.7544
		Metabolite	3, 8.46	13.02	0.0016
		Metabolite × Species	3, 70.37	1.62	0.1929
	All	Growth form	2, 5.12	0.14	0.8695
		Metabolite	3, 24.07	21.32	<0.0001
		Metabolite × Growth form	6, 197.808	1.33	0.2437

all species, while lipids had an estimated marginal mean δ^{13}C of −30.3‰ (Figure 3).

δ^{13}C in fronds and capitula of bryophyte species, Year 2

The bryophytes showed the same pattern as the vascular species whereby the magnitude of ^{13}C enrichment in Year 2 was dramatically reduced from that of Year 1. Just as in Year 1, there was no significant difference in δ^{13}C between HS and SPH in Year 2, while there was a significant effect of metabolite ($p = 0.0016$; Table 1; $n = 21$). Soluble sugars and starch again had a significantly higher δ^{13}C than lipids (p values < 0.01; Figure 1). Unlike in Year 1, however, neither had a significantly higher δ^{13}C than bulk tissue ($p = 0.1546$ for soluble sugars and 0.0518 for starch), and there was no significant difference in δ^{13}C between soluble sugars and starch (estimated marginal means of −22.6‰ and −20.6‰,

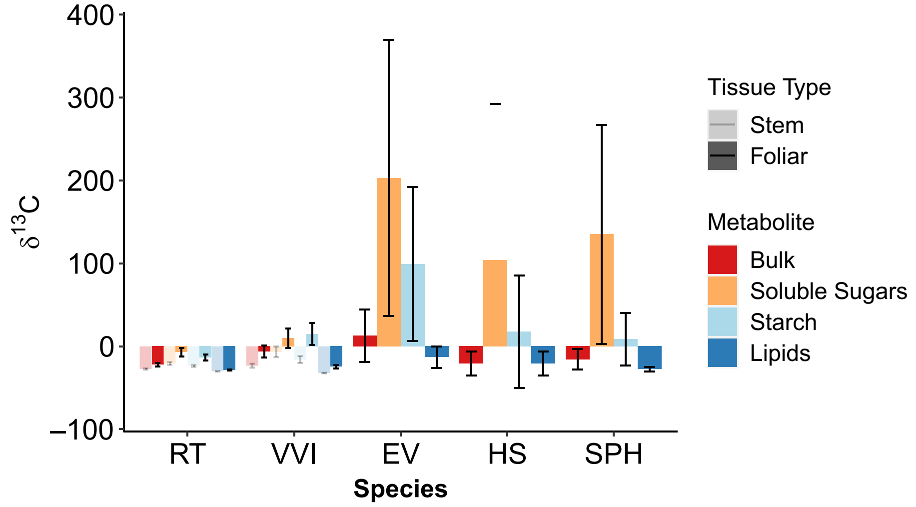

FIGURE 2 δ^{13}C estimated marginal means for foliar and stem tissue by species in Year 1 in experimental plots. Less opaque bars for RT and VVI represent stem tissue. Error bars represent ±1 SE. $n = 5$ for all metabolites in VVI stems and leaves, while $n = 28$ for all metabolites in RT stems and leaves, except starch in leaves ($n = 24$). For stem tissue, $n = 1$ for each in EV. $n = 1$ for all metabolites in HS. $n = 5$ for bulk and starch and $n = 6$ for soluble sugars and lipids in SPH. EV, *Eriophorum vaginatum*; HS, *Hylocomium splendens*; RT, *Rhododendron tomentosum*; SPH, *Sphagnum* mosses; VVI, *Vaccinium vitis-idaea*.

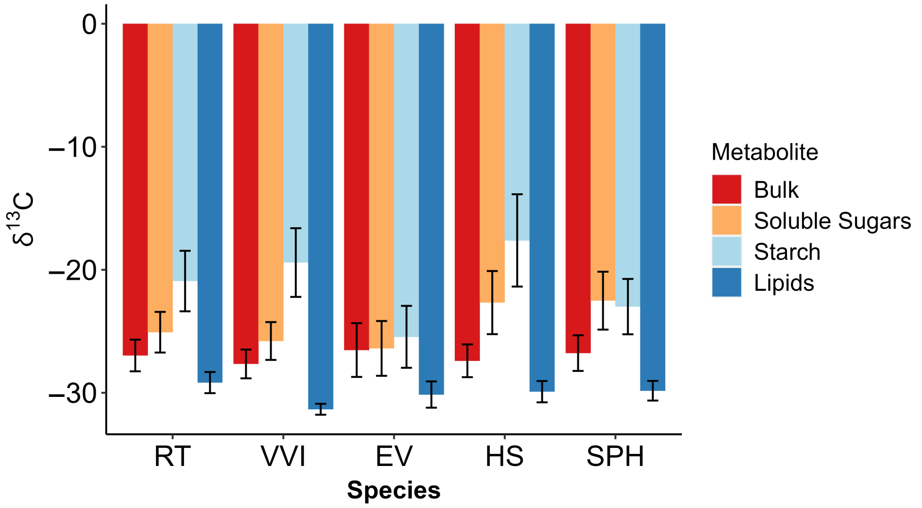

FIGURE 3 δ^{13}C estimated marginal means for foliar tissue by species in Year 2 in experimental plots. Error bars represent ±1 SE. $n = 16$ for all metabolites in RT, $n = 14$ for all in VVI, and $n = 3$ for all in EV. $n = 10$ for all metabolites in HS and $n = 11$ for all in SPH. EV, *Eriophorum vaginatum*; HS, *Hylocomium splendens*; RT, *Rhododendron tomentosum*; SPH, *Sphagnum* mosses; VVI, *Vaccinium vitis-idaea*.

respectively; $p = 0.8716$; Figure 1). As in the vascular species, values of $\delta^{13}C$ in mosses proved much less variable in Year 2 versus Year 1.

Tissue allocation

Analyses of the effect of tissue type on allocation for the two dwarf evergreen species, RT and VVI, in Year 1 ($n = 33$) revealed a significant three-way interactive effect of metabolite, tissue, and species ($p = 0.008$; Table 2), implying that species had a significant effect on the tissue-dependent effect of metabolite on $\delta^{13}C$. In other words, the interactive effect of metabolite with species varied across tissue types. Post hoc tests revealed that $\delta^{13}C$ was higher for all metabolites in VVI than in RT except for the lipids in the branch tissue, where RT showed significantly higher $\delta^{13}C$ ($p = 0.0042$). We also found that all metabolites had significantly higher $\delta^{13}C$ in leaves than in stems for both species, the only exception being soluble sugars in VVI ($p = 0.9939$), likely due to high variability. In tissues of both species, lipids always had lower $\delta^{13}C$ than the other metabolites.

Growth form allocation

For growth form analysis ($n = 96$), RT and VVI were classed as "evergreen;" EV was classed as a "sedge;" and HS and SPH were classed as "moss." As with all other models, metabolite was once again a significant predictor of $\delta^{13}C$; however, it interacted with growth form in Year 1 ($n = 41$; $p = 0.0003$; Table 1). Post hoc tests revealed that this was partially due to soluble sugars having higher $\delta^{13}C$ than starches in mosses but not in the other growth forms (Figure 4). In addition, while starches had significantly higher $\delta^{13}C$ than lipids in evergreens and mosses ($p < 0.0001$), the two were not significantly different in the sedge group ($p = 0.0759$). Of all six pairwise contrasts of metabolites, only starch and soluble sugars were not significantly different from one another. Generally, the two in tandem both had higher $\delta^{13}C$ than bulk or lipids.

TABLE 2 Type 3 tests of fixed effects for general linear mixed model of ^{13}C tissue allocation in dwarf evergreen shrubs, *Rhodedendron tomentosum* and *Vaccinium vitis-idaea* (Year 1).

Fixed effect	df	F	p
Tissue	1, 6.48	47.17	0.0003
Metabolite	3, 24.46	64.68	<0.0001
Species	1, 3.82	30.19	0.0061
Metabolite × Tissue	3, 284.62	3.70	0.0122
Metabolite × Species	3, 285.82	7.90	<0.0001
Tissue × Species	1, 285.64	14.67	0.0002
Metabolite × Tissue × Species	3, 284.59	4.01	0.0080

In Year 2, as in all other models, post hoc tests revealed that bulk tissues, soluble sugars, and starch all had higher $\delta^{13}C$ than lipids ($n = 54$; $p < 0.005$). There was no significant difference in $\delta^{13}C$ between soluble sugars and starch ($p = 0.4718$), and only starch had higher $\delta^{13}C$ than bulk tissue ($p = 0.0133$). Soluble sugars, on the other hand, showed no difference from bulk tissue in $\delta^{13}C$ ($p = 0.2628$).

DISCUSSION

This study provides the first evidence of the allocation of carbon from subnivean photosynthesis into metabolites in both nonvascular and vascular growth forms in the Arctic tundra. It suggests an energetic function, aligning well with the hypothesis that subnivean photosynthesis provides evergreen, semi-evergreen, and bryophyte species a head start in productivity (Starr & Oberbauer, 2003) and allows for recovery of depleted energy stores (Patankar et al., 2013). Such conclusions have important implications for the future community structure and carbon cycling of Arctic tundra.

We show the prioritization of soluble sugar and starch over that of lipids across all species. In the first year of our study, the $\delta^{13}C$ in vascular plants was species dependent—with semi-evergreen EV showing significantly higher and more variable ^{13}C than evergreen RT, and VVI being found between the two. This finding aligns with Starr and Oberbauer (2003), who found that EV, VVI, and RT had photosynthetic activity near their compensation points beneath the snow, that VVI had the highest ambient rate (A_{amb}), and that EV had the highest saturated rate (A_{max}). However, there was no difference among vascular species in Year 2 of the study or between nonvascular species in either year.

In Year 1, our model also revealed an interactive effect of growth form with metabolite on $\delta^{13}C$, suggesting that different growth forms prioritize metabolite production differently. We found that mosses exhibited higher allocation to soluble sugars than to starch while sedges and evergreens did not. We also did not find that $\delta^{13}C$ of any specific metabolite was different among growth forms, nor that bulk tissue concentrations of $\delta^{13}C$ differed between growth forms in Year 2. These results are surprising as tundra graminoids are known to have significantly higher A_{max} than evergreen (Johnson & Tieszen, 1976; Oberbauer & Oechel, 1989), and we would expect the photosynthetic rate to affect the magnitude of increases in

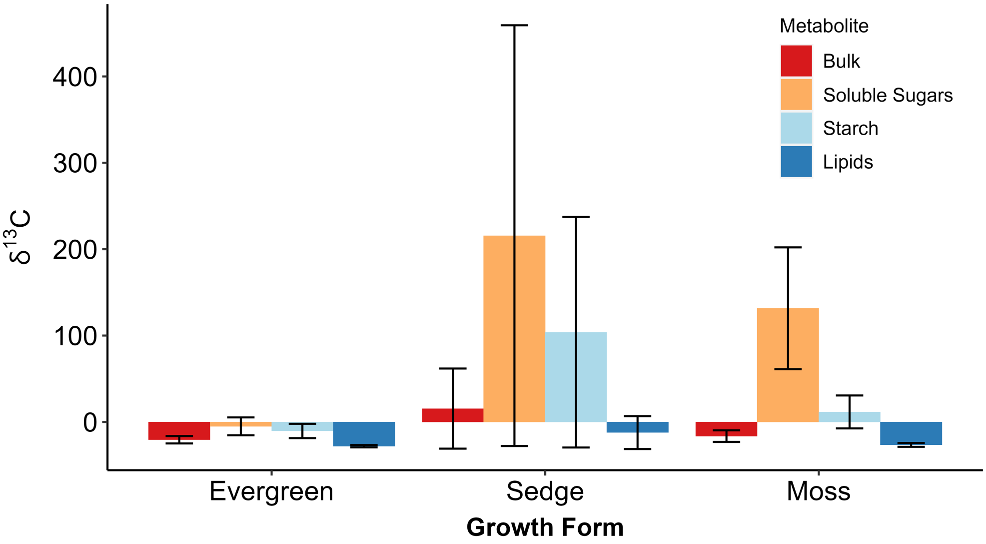

FIGURE 4 $\delta^{13}C$ estimated marginal means by growth form in Year 1 in experimental plots. Error bars represent ±1 SE. $n = 33$ for all metabolites in the evergreens except starch ($n = 29$), $n = 1$ for all in the sedge, $n = 6$ for bulk and starch, and $n = 7$ for soluble sugars and lipids in the mosses.

$\delta^{13}C$. While growth forms might not photosynthesize at substantially different rates under snow with elevated CO_2, our low sample size likely renders our analyses insensitive to differences in photosynthetic physiology. Concrete conclusions about such growth form-based differences will require larger sample sizes.

The higher $\delta^{13}C$ of starch and soluble sugars in all analyses is consistent with plant photosynthetic pathways (Smith & Stitt, 2007). Soluble sugars, specifically sucrose, are vital for transporting carbon throughout the plant, while starch is the fundamental energy storage molecule (MacNeill et al., 2017). The extensive interconversion between the two which occurs along with the diurnal cycle (Weise et al., 2011) helps to explain why both were prioritized in tandem. When adequate PAR is available, carbon dioxide is converted to triose phosphates via the Calvin–Benson cycle (Dusenge et al., 2019). These molecules can then be converted into starch in the chloroplast or sucrose in the cytosol. Alternatively, when PAR is too low, such as during the long winters, starch stores can be broken down into monosaccharides which are then used to synthesize sucrose dimers (MacNeill et al., 2017; Patankar et al., 2013).

In vascular plants, the elevated $\delta^{13}C$ of soluble sugars, the predominant molecule of which is sucrose, could also suggest that fixed carbon is being funneled into sugar transport to other parts of the plant (Brüggemann et al., 2011) or into short-term energy usage. In addition, due to sucrose's role in phloem movement through phloem loading (Brüggemann et al., 2011; Eom et al., 2015; López-Salmerón et al., 2019), elevated $\delta^{13}C$ of soluble sugars may imply that the plant is increasing the movement of other key nutrients. Both conclusions suggest prioritization of growth. This would support the idea that evergreen plants that photosynthesize beneath the snow have a growth advantage directly after snowmelt. However, this idea does not entirely align with our prior observations that the evergreens initiate growth after deciduous species during the growing season. We have observed, however, the growth of *E. vaginatum* beneath the snow (Starr, personal observation). This qualitative observation is supported by the observed higher $\delta^{13}C$ of EV (Figure 2).

The lack of evidence for subnivean growth in evergreen dwarf shrubs necessitates an alternative interpretation of elevated soluble sugar $\delta^{13}C$. A compelling explanation lies in the role of sucrose in the production of secondary compounds. Sucrose has been shown to act as a signaling molecule for the synthesis of anthocyanins (Yoon et al., 2021). This aligns with Oberbauer and Starr (2002), who showed that anthocyanin concentrations are highest in tundra evergreens directly after snowmelt. It has been suggested that anthocyanins reduce light stress by simultaneously dissipating excess solar radiation and acting as antioxidants (Gould et al., 2018; Steyn et al., 2002). In addition, anthocyanins appear to be more effective at lower temperatures (Gould et al., 2018) and synthesis of anthocyanins appears to be correlated with cooler conditions (Ahmed et al., 2015; Chalker-Scott, 2002; Li et al., 2015). Thus, anthocyanin accumulation before snowmelt may be advantageous for evergreens and semi-evergreens in the tundra, facilitating photoprotection and, potentially, cold tolerance. Both factors are critical as snow both shades and insulates plants (Saarinen et al., 2016).

Starch, on the other hand, is especially important for maintaining homeostasis at night when there is no sunlight to promote sucrose synthesis. Without sun, starch must be broken down into its constituent sugars to form new sucrose and maintain phloem translocation to the rest of the plant (Graf & Smith, 2011). During the Arctic winter, plants are not just exposed to low temperatures, but also receive little or no PAR (Gerland et al., 1999). Without sufficient PAR, the light reactions of photosynthesis cannot occur, and starch must thus be catabolized. Enriched $\delta^{13}C$ of starch in subnivean species likely implies that subnivean photosynthesis functions to aid in replenishing depleted starch stores after the long winter. This aligns with Patankar et al. (2013) in that RT, VVI, and EV all had significantly more starch upon snowmelt than in the fall season before snowfall.

Just as tandem ^{13}C enrichment of starch and sucrose is expected, the comparative lack of lipid enrichment is unsurprising. We would predict relatively higher $\delta^{13}C$ in lipids if reproductive structures had been present and sampled. Seeds especially would be expected to be high in fat content and would likely have high lipid $\delta^{13}C$. Lipids do not, however, play the same role in day-to-day energy provision in plants that they do in animals. Most lipid synthesis for energy storage occurs in the seed (Xu & Shanklin, 2016). Despite this purpose, the significantly lower $\delta^{13}C$ of lipids in evergreens could also, at least partially, be the result of naturally occurring ^{13}C discrimination. As observed in our control data (Figure 1), lipids in plants are naturally more ^{13}C depleted due to fractionation in the conversion of pyruvate to acetyl-CoA, the primary building block of fatty acid chains, during cellular respiration (Ghashghaie et al., 2003; Zhou et al., 2015). In addition, ^{13}C depletion in the control plants may have been higher than is generally seen in C_3 plants due to the photosynthetic uptake of recycled respiratory CO_2 beneath the snow.

Our analysis of dwarf evergreen shrubs also provided evidence of prioritization of leaf allocation over stems. Nearly all metabolites had higher $\delta^{13}C$ in leaves than stems in both evergreen species, in alignment with previous studies (Johnson & Tieszen, 1976; Shaver & Chapin, 1991). Few studies provide empirical information as to why more photosynthate is allocated to leaves rather than stems in Arctic tundra plants; however, through ^{14}C labeling of *V. vitis-idaea*, Karlsson (1985) found that photosynthate from the previous year's leaves, those which had overwintered, was the primary driver for growing season shoot allocation. It could be that the evergreen shrubs can enhance shoot growth through initial subnivean allocation to leaves when the growing season begins. This could also be a natural product of the route taken by carbon after photosynthesis. Fundamentally, new photosynthate must travel through the leaves to reach the stem, where they would be mixed with existing unenriched metabolites. Thus, it stands to reason that $\delta^{13}C$ would be comparatively elevated. Both explanations are compelling, and both may be true in tandem.

Owing to substantial differences in physiology and life history, our results may have different implications for bryophytes. To our knowledge, no previous study has demonstrated subnivean photosynthesis in bryophytes, though Bubier et al. (2002) reported some gross primary production in a temperate peatland system beneath the snow. The lack of record of subnivean photosynthesis in tundra bryophytes is surprising as they account for a substantial portion of tundra productivity (Olivas et al., 2011; Street et al., 2012), and *Sphagnum* are critical for the maintenance of net ecosystem exchange during drought in peatlands (Kuiper et al., 2014). Studies on mosses, especially *Sphagnum*, suggest a normal $\delta^{13}C$ range in the dominant gametophyte tissue between roughly −33‰ and −23‰ (Griffin-Nolan et al., 2018; Rice & Giles, 1996). Our estimated marginal means in Year 1 showed much higher $\delta^{13}C$ values, suggesting uptake, and by extension photosynthesis (Figures 3 and 4). Our study suggests, as with the vascular plants, that *Sphagnum* and *H. splendens* prioritize soluble sugars and starch. The lack of vasculature in the bryophytes rules out the attribution of elevated soluble sugar $\delta^{13}C$ to phloem movement. Though sucrose signaling appears to be less well-studied in Bryophyta, increases in the stress-tolerance hormone abscisic acid (ABA) in some mosses are associated with increases in sucrose (Takezawa et al., 2011). In turn, such increases in soluble sugar content are linked to moss cold tolerance (Bhyan et al., 2012; Nagao et al., 2005). This would align with our finding that soluble sugars had a significantly higher $\delta^{13}C$ enrichment than starch in Year 1 but not Year 2. It is likely that with comparatively minimal subnivean photosynthesis, soluble sugar synthesis cannot occur. Thus, sucrose may be prioritized in moss photosynthesis to provide freezing tolerance as snow melts (Saarinen et al., 2016). Despite the evidence that starch concentration decreases with increases in soluble sugar content, starch $\delta^{13}C$ was not significantly different from that of soluble sugars in Year 2. Starch may thus also be a critical sink in mosses. A study of *Sphagnum* in alpine Japan (Shiraishi et al., 1996) shows a similar trend in starch storage to that observed in vascular plants by Patankar et al. (2013), with stores dropping substantially late in the growing season, before snowfall. Therefore, an increase in $\delta^{13}C$ in starch may imply that mosses are recovering starch stores lost over the winter.

Differences in weather conditions, specifically rapid temperature increases in Year 2, help to explain the substantial shift in our results and serve as a case study for how subnivean photosynthesis may be affected by climate change. Values of $\delta^{13}C$, though still significantly greater than the control plots, were lower in the labeled individuals in Year 1 of the study. This result is likely the product of deeper snow at the start of labeling in Year 2 compared to Year 1. Snow depth at the beginning of labeling in Year 2 was greater than the snow depth of <30 cm thought to be necessary for subnivean photosynthesis (Starr & Oberbauer, 2003), thus the lower $\delta^{13}C$ values are likely due to insufficient PAR. This also may explain why preliminary nonparametric analyses showed fewer differences from control plants in Year 2 and may clarify why there was no significant effect of species. The conditions in Year 2 align well with what is predicted with climate change. While snowmelt is predicted to occur earlier, snowfall events in many locations in the Arctic have proven to be more severe, resulting in more snow on the ground for a shorter period (Box et al., 2019; Callaghan et al., 2011; Hinzman et al., 2005). These potential future conditions were demonstrated in Year 2, in which we waited for the snow cover to reach the optimum depth to drive photosynthesis but were forced to quickly begin the experiment when the temperature increased dramatically. Under these conditions, snow melted rapidly, which limited the optimal window for physiological activity in the subnivean environment. Thus, little subnivean photosynthesis occurred under these conditions. Under these conditions, soluble sugar and starch synthesis are limited which could exacerbate cold stress and photoinhibition after snowmelt (Pardee et al., 2019; Saarinen et al., 2016; Taulavuori et al., 2011). Evidence for photoinhibition has already been recorded by Lundell et al. (2010) in *V. vitis-idaea* and it may prove to be a significant issue for the other species studied. It is thus possible that any conferred advantage of this process will be lost with climate change.

Temperature changes may prove to be influential in determining how changes in snow patterns affect the long-term fitness and abundance of plants that photosynthesize beneath the snow. The Arctic is currently warming almost four times faster than the global average (Rantanen et al., 2022). Thus, any potential frost effects that come from an earlier loss of insulating snow cover could be offset by a correlated increase in temperature. If spring temperature increases at a similar rate to that of snowmelt advancement, no major change in relative abundance may occur. In this scenario, the only significant abiotic shift from current conditions would be a decrease in sunlight intensity before and after snowmelt. This could still decrease the potential yield of subnivean photosynthesis and could reduce the effectiveness of the process for giving these growth forms the ability to compete with deciduous species.

There are other important limitations to our study that provide guidance for future research. While our sample size was higher for the two dwarf evergreen shrubs, we found comparatively few individuals of the sedge *E. vaginatum* in the site and were unable to find samples in more than two plots in either year. Thus, our sample size for the semi-evergreen was far smaller, reducing statistical power and rendering conclusions about the sedge or growth form-based differences less robust. This renders our study less representative of the tussock tundra, which is characterized by tussocks of *E. vaginatum*. In addition, measured concentrations of subnivean CO_2 in the labeled plots in 2011 were lower (511–931 ppm) than those in 2010 (1377 ppm). With lower concentrations, ^{13}C enrichment may have been less efficient and any differences between species or growth forms may have been less clear. Finally, from a statistical perspective, it is important to note the limitations of performing a logarithmic transformation when there is no clear basis for its usage (Feng et al., 2014). The transformation renders our results more difficult to interpret but was preferable to violation of the assumptions of GLMM.

With accelerating climate change, studies on the physiological factors which may affect plant performance and community composition in the tundra are critical. Our results elucidate some of the allocation patterns underlying subnivean photosynthesis and point to the preferential prioritization of starch and sucrose in nearly all evergreen, semi-evergreen, and moss species. Further research on subnivean photosynthesis is needed to fully determine its metabolic role; however, this study has shown evidence that aligns with the hypothesis that it provides a variety of tundra species a head start in the accumulation of energetic metabolites and, in addition, may point to the recovery of starch after the long winter. Furthermore, it may be critical for photoprotection and cold tolerance.

Further research on this unique physiological process is urgently needed so that better predictions can be made about how climate change will affect long-term community dynamics and, by extension, tundra feedbacks to climate change.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Starr, 2022) are available from Figshare: https://doi.org/10.6084/m9.figshare.21065893.v1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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