



Presence of N-fixing neighbors increases leaf N and $\delta^{13}\text{C}$ in *Castilleja applegatei*, a root hemiparasite

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Abstract Parasitic plants are known for their high transpiration rates and low water use efficiency (WUE), which the N-parasitism hypothesis posits is driven by N limitation. Thus, availability of N-fixing hosts may affect parasite's WUE and in turn impact the surrounding plant community. Here, I investigate how the availability of an N-fixing host affects the root hemiparasite, *Castilleja applegatei*, and examines host-mediated effects on community structure and soil moisture. I surveyed plant diversity and percent cover and measured soil moisture in 120 1×1 m plots within Sagehen Experimental Forest, CA. Fifty percent of the plots included *C. applegatei*. In a subset of plots, I measured leaf N, C/N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in *C. applegatei* and in one N-fixer (*Ceanothus prostratus*) and two non-N-fixing plants (*Artemisia tridentata* and *Wyethia mollis*). In *C. applegatei* availability of N-fixing hosts corresponded to a significant increase in leaf %N, a distinct $\delta^{15}\text{N}$ signature, and an increase in $\delta^{13}\text{C}$ (which typically signifies an increased WUE). The presence of parasites was associated with a marginally significant decrease in WUE in N-fixing neighbors, but had no effect on the two non-N-fixing species. The presence of parasites did not impact

diversity, percent cover, or soil moisture. These results broadly support the N-parasitism hypothesis and indicate that host type can affect parasite's physiology and therefore have the potential to mediate parasite's effects in the community; however, community-level impacts were not found here.

Keywords Host-mediated effects · Leaf $\delta^{13}\text{C}$ · Leaf $\delta^{15}\text{N}$ · Orobanchaceae · Parasitic plants

Introduction

Parasitic plants can play important roles in nutrient cycling and plant community structure (Quasted 2008; Fisher et al. 2013). The decomposition of parasitic plant leaf litter, typically rich in nitrogen (N), may increase overall N mineralization, available soil N, and ecosystem productivity and drive shifts in plant community assemblages (Press 1998; Spasojevic and Suding 2011; Fisher et al. 2013). Parasitism may also suppress dominant plant species in a community, indirectly increasing community diversity via competitive release (Marvier 1998a; Joshi et al. 2000; Pennings and Callaway 2002). Because their influence is often disproportionately high compared to their abundance or biomass, parasitic plants can in some instances be considered keystone species or resources (Press and Phoenix 2005; Watson and Herring 2012).

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Parasitism has evolved independently multiple times across the angiosperms (Barkman et al. 2007; Naumann et al. 2013). As parasitic plants are not monophyletic, they are defined functionally by the parasitic uptake of resources from other plants via specialized organs called haustoria (Těšitel 2016). Within this larger umbrella, however, parasites exhibit a wide range of forms and physiology (Teixeira-Costa and Davis 2021). Understanding their unique eco-physiology is critical to illuminating their ecological roles and unique interactions in plant associations across the globe (Phoenix and Press 2005).

Parasitic plants are also known to generally have high transpiration rates and low water use efficiency (WUE) (Schulze et al. 1984; Press et al. 1987, 1988; Scalon and Wright 2017), defined as the ratio of carbon assimilation (A) to transpirational water loss (E) (denoted as A/E) (Farquhar et al. 1982). Parasites which primarily feed from the host xylem (i.e., xylem-tapping or xylem-feeding parasites) must maintain a favorable water potential gradient, achieved through high stomatal conductance, in order to redirect water and solutes from host xylem stream to the parasitic body (Press et al. 1987; Stewart and Press 1990). The resulting low WUE can potentially reduce soil moisture which has implications for broader community structure and function (Lauenroth et al. 1978; Stephenson 1990; Sala et al. 2001). Similarly, parasitic plants can decrease drought tolerance in hosts and associated plants (Press et al. 1987; Stewart and Press 1990; Sala et al. 2001).

The physiology and subsequent impact of parasitic plants are likely often host-mediated. For example, host type has been shown to affect parasites' palatability to herbivores (Schädler et al. 2005). In particular, the N-parasitism hypothesis describes an important role for N-fixing hosts, positing that parasites are N limited and N acquisition then drives the observed high transpiration rates (Schulze et al. 1984). Although xylem-tapping parasites do not have access to the host phloem, they acquire dilute C, N, and other solutes from the host xylem stream (Bollard 1960) but the low concentrations require profligate transpiration. The N-parasitism hypothesis predicts that higher N availability would in turn lead to decreased transpiration by the parasite.

Research into the N-parasitism hypothesis has yielded mixed results. Supporting evidence includes observations that N-fixing hosts cause increased

growth rates in parasites and a decrease in WUE difference between host and parasite (Schulze et al. 1984; Schulze and Ehleringer 1984; Ehleringer et al. 1985; Seel and Press 1993, 1994). The fertilization of host plants has also been shown to increase WUE in mistletoes (aerial stem parasites) through increased photosynthesis and stable stomatal conductance (Marshall et al. 1994). It is not clear, generally, how much observed increases in WUE are due to a downregulation in transpiration versus an increase in photosynthesis, that is, whether the numerator or denominator in A/E is shifting (Seel and Press 1994). Evidence against the hypothesis includes a more recent survey on WUE in host–mistletoe pairs, which found no effect of differences in host and parasite foliar N on WUE, nor any effect of N-fixing hosts on host–mistletoe differences in WUE (Scalon and Wright 2015). In a follow-up study, the authors also measured nutrient resorption prior to leaf senescence (Scalon et al. 2017). If N were a limiting nutrient, one would expect N resorption, which was not observed. The authors did, however, find P resorption suggesting a possible alternative: that P limitation drives high transpiration. In both the above studies, there was a strong effect of site.

The N-parasitism hypothesis has largely been investigated in stem parasites, such as the mistletoes above. Functionally distinct, root hemiparasites are relatively common and widespread but host–root hemiparasite interactions have more often been the subject of greenhouse studies, which while valuable are not sufficient because controlled greenhouses cannot fully replicate field dynamics (Marvier 1996; Matthies 1997; Joshi et al. 2000; Schädler et al. 2005; Matthies 2017; Sandner and Matthies 2018, but see Tennakoon et al. 1997; Marvier 1998a; Adler 2002). This paucity of research is understandable: in situ investigation is difficult when the host–parasite connection is hidden from view. Furthermore, root hemiparasites commonly attach to several hosts at once and the connections are small, fragile, and difficult to detect in nature. Plant physiology methods, in particular stable isotopes, are a powerful tool to elucidate interactions like this, which would be intractable with traditional ecological methods (Dawson et al. 2002).

Castilleja, a genus of root hemiparasitic plants, is an ideal group to investigate in this context. Part of the Orobanchaceae family, the second largest family of

parasites (Westwood et al. 2010), *Castilleja* spp., commonly known as paintbrushes, are widespread across North America, occurring in a wide range of habitats. Like other parasites, they typically have high transpiration rates, and at least some species benefit from N-fixing hosts (Seel and Press 1993; Matthies 1997). In addition, work in other systems suggests that *Castilleja* substantially affects ecosystem structure and function through depositing N-rich litter, decreases in host biomass, and host-mediated effects on herbivores (Marvier 1996; Spasojevic and Suding 2011). As root hemiparasites, *Castilleja* can theoretically survive without a host and can photosynthesize, but still obtain nutrients, carbon, and water from a wide variety of hosts via haustorial root attachments (Heckard 1962; Stewart and Press 1990). *Castilleja* are generalists, capable of parasitizing a wide variety of hosts, and host identity and quantity may significantly alter *Castilleja* individuals' physiology and their interactions with neighbors and other trophic levels, such as herbivores and pollinators (Matthies 1997; Marvier 1998b; Adler 2000).

In the present study I investigate the interaction between *Castilleja applegatei* and available hosts, paying particular attention to the N-fixer *Ceanothus prostratus* (Rhamnaceae). In each species, I measured the leaf traits: C/N, %N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$. Carbon isotopes are commonly used as a proxy for WUE. Carbon isotope composition of plant tissues ($\delta^{13}\text{C}$) can be an integrator of the time-averaged c_i/c_a (ratio of internal to ambient [CO_2]) of an individual plant. C_i/c_a is in turn a reflection of the rates of carbon assimilation (demand for CO_2) and stomatal conductance (loss of water). As such, $\delta^{13}\text{C}$ has been used to infer the WUE of a particular plant species (Farquhar et al. 1982). Under the N-parasitism hypothesis, I expected that when *Castilleja* individuals associate with N-fixing hosts, they will exhibit higher N content and higher WUE evidenced by $\delta^{13}\text{C}$ and a distinct $\delta^{15}\text{N}$ signature, closer to the $\delta^{15}\text{N}$ of an N-fixing plant. Because of this higher WUE on the part of the parasite, I also expected that WUE of N-fixer hosts would be less impacted by parasitism than non-N-fixers. In addition, I investigate the effect of parasites on community diversity and productivity. Because the study site has relatively low plant cover and low soil nutrient content (Johnson et al. 1997), I anticipated that the deposit of N-rich litter would lead to higher percent cover and higher diversity in plots with the parasite and/or the N-fixer.

Materials and methods

Study sites

I conducted fieldwork at Sagehen Creek Experimental Forest (N39°25.981', -W120°14.758'), located in the Central Sierra Nevada north of Truckee, CA, USA. Vegetation types include mixed-conifer forest, meadow, shrub, and conifer plantations. The shrub type occurs on poor and/or shallow soils unable to support conifer forests and on more productive soils after disturbance (fire, logging) (USFS 2008). The site has a broadly Mediterranean-type climate, characterized by warm, dry summers and cool, wet winters. Sagehen Creek's receives significant snowfall, which accounts for 80% of the annual precipitation in a typical year (USFS 2008). California was in the midst of a historic drought for part of this project (CADWR 2015). The period between fall 2011 and fall 2015 was the driest since record keeping began, while 2014 and 2015 were the hottest years on record in California at the time. In 2016, average precipitation in Northern California, in combination with above average temperatures, reduced but did not eliminate drought across the state (Griffin and Anchukaitis 2014; Hanak et al. 2016).

Study species

I focused on a root hemiparasite, *Castilleja applegatei* ssp. *pintorum* (Fernald) T.I. Chuang & Heckard (Orobanchaceae), one N-fixer, *Ceanothus prostratus* Benth. (Rhamnaceae), and two non-N-fixers, *Artemisia tridentata* ssp. *vaseyana* (Rydb.) Beetleand (Asteraceae) and *Wyethia mollis* A. Gray (Asteraceae). These species were chosen because they are common in the shrub ecosystem at Sagehen, as demonstrated by the surveying (below). *C. prostratus* is a mat-forming shrub found in dry sites in pine forests in the Sierra Nevada and Cascade ranges (Conard et al. 1985). *C. prostratus* is actinorhizal, meaning individuals fix N through a symbiotic relationship with a soil actinomycete (Busse 1996). *A. tridentata* is an important, often dominant, woody shrub found from the arid lands of the Great Basin to the cooler climes of Western mountains (Barker and McKell 1983). There is considerable intraspecific variation within *A. tridentata* (Winward 1980). The subspecies studied here, mountain sagebrush, is found in dry sites in the upper foothills and mountain regions (Barker and McKell

1983). *W. mollis* or mule's ears is an herbaceous perennial that forms rosettes of large leaves, which die back to ground level each winter. It commonly co-occurs with *A. tridentata* in shallow, dry soils, and conifer understories in the Sierra Nevada (Parker and Yoder-Williams 1989; Karban 2007). *Castilleja applegatei* is a highly variable species of perennial root hemiparasites, characterized by wavy leaf margins. The subspecies here, *C. applegatei* ssp. *pinetorum*, is typically found in open conifer forest and sagebrush scrub environments throughout the Sierras, the Southern Cascades, the High North Coast Ranges, and the Modoc plateau (Baldwin and Goldman 2012). At Sagehen, *C. applegatei* ssp. *pinetorum* can be found within the shrub type and on more productive soils after disturbance.

Surveys

To address the impacts of parasitic plants on community diversity and productivity, I established 12 50 m transects in the summer of 2015. Along each transect 10 1 × 1 m quadrats were established (120 plots total). I established quadrats every 5 m along each transect, alternating sides until I reached five plots with *C. applegatei* and five plots without. In order to reach ten total plots, transects were sometimes extended beyond 50 m (but none exceeded 75 m). I surveyed each quadrat for all species presence, count, and percent canopy cover using visual estimation (Meese and Tomich 1992). Volumetric water content (VWC%) was recorded at mid-day in each plot using a FieldScout TDR 150 Soil Moisture Meter (Spectrum Technologies, Aurora, Illinois) with 12 cm probes at three regular locations, forming an equilateral triangle, in the quadrat or where the soil was sufficiently soft and rock-free to allow measurement.

Leaf sample collection

To address questions about N-parasitism, WUE, and host detection (via $\delta^{15}\text{N}$), I collected leaf samples for elemental and stable isotope analysis (specifically %N, %C, C/N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$) of the parasite, common N-fixers, and non-N-fixers. *C. prostratus* emerged from these surveys as the most common N-fixer available to *C. applegatei*. In a subset of transects, where *C. prostratus* was well represented (Table 1), I collected leaf samples from *C. applegatei*,

C. prostratus, and the two most common non-N-fixing available hosts: *A. tridentata* and *W. mollis*. For the host species, I collected leaf samples from individuals in quadrats with and without the parasite, *C. applegatei* ($n = 2 \times 10$ each $\times 3$ species = 60 individuals). For the parasite I collected samples from individuals in quadrats with and without the N-fixer (*C. prostratus*) ($n = 20$ each, 40 total individuals). Mature, sun-exposed leaves were collected from each individual.

Leaf sample preparation

I dried the leaf samples in a 45–55 °C oven for at least 48 h, and then I manually removed the midveins with a razor blade under a dissecting scope. I ground all leaves from an individual plant together into a fine powder, from which I packed 5–7 mg per sample into a tin capsule for elemental and isotope analyses. Samples were submitted to the Center for Stable Isotope Geochemistry at UC Berkeley for %C, %N, C/N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ analysis using a CHNOS Elemental Analyzer interfaced to an IsoPrime100 mass spectrometer. The Center for Stable Isotope Geochemistry corrected raw instrument data for drift over time and linearity and normalized data to the international stable isotope reference scale. Long-term external precision is $\pm 0.1\%$ and $\pm 0.2\%$, respectively, for C and N isotope analyses. For the four runs of samples for this work, the analytical standard deviations were $\pm 0.02\%$, $\pm 0.07\%$, $\pm 0.06\%$, and $\pm 0.15\%$ for ^{13}C and $\pm 0.17\%$, $\pm 0.06\%$, $\pm 0.12\%$, and $\pm 0.28\%$ for ^{15}N . All isotope values are expressed in delta notation.

Data analysis

Unless otherwise noted I used the native R *stats* package for all statistics (R Core Team 2020). For all models below I used visual inspection of the residuals, Q–Q plots, and Cook's distance to confirm that the model met all assumptions and no data points had undue leverage. Where appropriate I also ran Shapiro–Wilk normality tests, *F* tests, and/or Levene's test to check for normality and heterogeneity of variance.

Table 1 Sampling locations at Sagehen Experimental Forest, 2015. All transects were surveyed and a subsection was sampled for leaf $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, %N, and C/N

Transect	Location (DM)	Altitude (m)	Leaf samples
1	39°25.981', – 120°14.758'	1940	N
2	39°26.050', – 120°14.790'	1975	N
3	39°26.050', – 120°14.791'	1975	N
4	39°26.147', – 120°15.623'	1988	Y
5	39°26.201', – 120°15.755'	2006	Y
6	39°25.913', – 120°16.927'	2092	N
7	39°25.913', – 120°16.928'	2092	N
8	39°26.488', – 120°14.766'	2061	Y
9	39°26.512', – 120°14.781'	2064	Y
10	39°26.763', – 120°16.066'	2091	N
11	39°26.337', – 120°15.806'	2022	Y
12	39°26.182', – 120°15.937'	1999	Y

Diversity, productivity, and soil moisture

Using the *vegan* package in R (Oksanen et al. 2019), I calculated rarefied species richness (using the minimum number of individuals [3] within a plot as the subsample size) and two diversity indices: Shannon index and inverse Simpson index for each plot. The Shannon and inverse Simpson indices were calculated using both percent cover and counts, resulting in five diversity metrics. In addition, I analyzed percent cover and soil moisture (an average of the three measurements in each plot). To examine the effect of *C. applegatei* on each of these factors, I ran type III ANOVAs on a linear mixed-effects model (nlme package) (Pinheiro et al. 2017). For each model, the metric was the response variable, binary presence/absence of *C. applegatei* was the parameter, and transect (i.e., location) was a random effect. I ran a type III ANOVA for each of the five metrics. For these models *C. applegatei* was excluded from the metrics calculation, including percent cover. To look for the effect of *C. prostratus* on diversity I repeated this process, using binary presence/absence of *C. prostratus* as the parameter and metrics from which *C. prostratus* was excluded. Finally, I ran a type III ANOVA with both presence of *C. prostratus* and *C. applegatei* and their interaction as parameters. Both species were excluded from metric calculations for these ANOVAs (Spasojevic and Suding 2011). For soil moisture I only ran one type III ANOVA with both presence of *C. prostratus* and *C. applegatei* and their interaction as parameters. Due to the multiple comparisons (19), a Bonferroni correction was applied,

resulting in an adjusted α of 0.002 (Quinn and Keough 2002).

Leaf traits

For the leaf samples, I looked at the effect of the presence of the parasite (*C. applegatei*) on the N-fixer (*C. prostratus*) and vice versa for four traits: $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, %N, and C/N. When I found heterogeneity and/or non-normality I initially transformed the data; however, no transformations improved the violations. Two sets of measurements showed significant heterogeneity of variance: %N in *C. applegatei* (F test: $P < 0.0001$, ratio of variances = 0.056) and $\delta^{15}\text{N}$ in *C. prostratus* (F test: $P = 0.045$, ratio of variances = 0.24). For this reason, I subsequently ran Welch's two sample t tests, which allow for unequal variance. Only $\delta^{15}\text{N}$ in *C. prostratus* showed significant violation of normality in one of the groups (Shapiro–Wilk test: group = w/o *C. applegatei*, $W = 0.84$, $P = 0.044$). For this measurement I ran the non-parametric Asymptotic Wilcoxon–Mann–Whitney test. For the two non-parasite, non-N-fixer species (*W. mollis*, *A. tridentata*) I ran type III ANOVAs on a linear model fit on $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, %N, and C/N where both presence of *C. prostratus* and *C. applegatei* and their interaction were parameters.

I was also interested in the relationship between leaf traits and how that was affected by species and the presence of the parasite and/or N-fixer. To address these questions I ran ANCOVAs for each species looking at $\delta^{13}\text{C}$ as a function of leaf %N and $\delta^{15}\text{N}$ as a function of $\delta^{13}\text{C}$. For the parasite (*C. applegatei*) the

presence of the N-fixer (*C. prostratus*) was an additional factor and conversely for the N-fixer the presence of the parasite was an additional factor. For the two non-parasitic, non-N-fixing species (*A. tridentata* and *W. mollis*) both presence of the parasite and N-fixer were included as factors. I used a backward model selection, initially including all factors and their interaction terms and dropping factors one at a time, using AIC and BIC to compare models (Zuur et al. 2007). If no factors were significant results are shown for the linear model that just includes the leaf traits (i.e., $\delta^{13}\text{C}$ as a function of leaf N (%) and $\delta^{15}\text{N}$ as a function of $\delta^{13}\text{C}$). One model showed heterogeneity (F test: $P = 0.0075$, ratio of variances = 8.17): [$\delta^{15}\text{N} \sim \delta^{13}\text{C} * \text{with } C. \text{ applegatei}$] within *C. prostratus*. Various data transformations did not improve the result. In this case I applied a generalized least squares fit by restricted maximum likelihood with a variance structure that allows for different standard deviations in each stratum (*varIdent* variance structure from the *nlme* package) (Zuur et al. 2009).

Results

Diversity, productivity, and soil moisture

There was no effect of the presence of parasites or N-fixers on plot-level diversity as measured by inverse Simpson index, Shannon index, rarefied richness, or plot-level soil moisture (Tables 2, 3, 4). There was significant negative effect of *C. prostratus* on percent

cover (51% decrease when cover is calculated without *C. prostratus*) (Table 3).

Leaf traits

The N-fixer and parasite each significantly affected leaf traits in the other. In the parasite, the presence of an N-fixer corresponded to a 125.31% increase in leaf %N ($P < 0.0001$), a significant decrease in leaf C/N ($P < 0.0001$), a significant decrease in $\delta^{15}\text{N}$ values ($P < 0.0001$), and significant increase in $\delta^{13}\text{C}$ values ($P < 0.0001$) (Table 5, Fig. 1). Conversely in the N-fixer, *C. prostratus*, the presence of the parasite corresponded to no significant change in leaf %N and leaf C/N but a significant decrease in $\delta^{15}\text{N}$ values ($P = 0.0019$) and decrease in $\delta^{13}\text{C}$ values ($P = 0.012$), signifying a decrease in WUE (Table 5, Fig. 2).

The presence of the parasite was not associated with significant changes in leaf traits within the two non-N-fixers, *W. mollis* and *A. tridentata*. The presence of the N-fixer, however, did correspond to a 15.79% decrease in leaf %N ($P = 0.025$) and a significant increase in leaf C/N ($P = 0.016$) in *A. tridentata* (Table 6). In *W. mollis*, the presence of the N-fixer corresponded to a significant decrease in $\delta^{15}\text{N}$ values ($P = 0.020$) (Figs. 2, 3, Table 6).

When controlling for the presence of the parasite and/or N-fixer, $\delta^{13}\text{C}$ was significantly correlated with leaf %N in *C. applegatei* ($P = 0.060$, $R^2 = 0.610$), *C. prostratus* ($P = 0.044$, $R^2 = 0.402$), and *A. tridentata* ($P = 0.007$) (Figs. 3, 4, Table 7). *W. mollis* was the only species where $\delta^{15}\text{N}$ was correlated with $\delta^{13}\text{C}$ ($P = 0.016$, $R^2 = 0.465$) (Fig. 4, Table 7).

Table 2 Diversity and productivity in 120 plots surveyed in Sagehen Experimental Forest, 2015

Metric	<i>Castilleja applegatei</i> present		<i>Castilleja applegatei</i> absent		<i>Ceanothus prostratus</i> present		<i>Ceanothus prostratus</i> absent	
	Value	SE	Value	SE	Value	SE	Value	SE
Inverse Simpson (counts)	3.39	0.17	3.17	0.16	3.24	0.18	3.28	0.15
Shannon (counts)	1.34	0.05	1.27	0.05	1.27	0.05	1.33	0.05
Rarefied richness	2.28	0.05	2.22	0.05	2.20	0.05	2.25	0.04
Inverse Simpson (percent cover)	2.36	0.13	2.48	0.15	2.88	0.16	2.74	0.17
Shannon (percent cover)	1.02	0.05	1.03	0.06	1.17	0.05	1.09	0.07
Percent cover (actual)	52.50	3.22	39.58	3.06	56.15	3.06	35.59	2.85
Percent cover (w/o species of interest)	44.38	3.11	39.58	3.06	18.41	2.13	35.59	2.85

50% of plots included the parasite, *C. applegatei*

SE is short for standard error

Table 3 Results of ANOVAs on diversity, productivity, and soil moisture in 120 plots surveyed in Sagehen Experimental Forest, 2015

Parameter	Metric		Num df	Den df	F	P value
Presence of parasite (<i>C. applegatei</i>)	Inverse Simpson (counts)		1	107	0.88	0.35
	Shannon–weaver (counts)		1	107	1.17	0.28
	Rarefied richness		1	107	0.76	0.38
	Inverse Simpson (percent cover)		1	107	0.41	0.52
	Shannon (percent cover)		1	107	0.06	0.81
	Percent cover (w/o <i>C. applegatei</i>)		1	107	1.26	0.26
Presence of N-fixer (<i>C. prostratus</i>)	Inverse Simpson (counts)		1	107	0.00	0.95
	Shannon–weaver (counts)		1	107	0.30	0.59
	Rarefied richness		1	107	0.30	0.59
	Inverse Simpson (percent cover)		1	107	0.32	0.57
	Shannon (percent cover)		1	107	0.57	0.45
	Percent cover (w/o <i>C. prostratus</i>)		1	107	19.37	< 0.0001
Presence of parasite (<i>C. applegatei</i>) and N-fixer (<i>C. prostratus</i>)	Inverse Simpson (counts)	<i>C. applegatei</i>	1	105	0.33	0.57
		<i>C. applegatei</i>	1	105	0.11	0.74
		Interaction	1	105	0.02	0.88
	Shannon (counts)	<i>C. applegatei</i>	1	105	0.94	0.34
		<i>C. prostratus</i>	1	105	0.39	0.54
		Interaction	1	105	0.32	0.57
	Rarefied richness	<i>C. applegatei</i>	1	105	0.41	0.53
		<i>C. prostratus</i>	1	105	0.59	0.44
		Interaction	1	105	0.00	0.99
	Inverse Simpson (percent cover)	<i>C. applegatei</i>	1	105	0.10	0.76
		<i>C. prostratus</i>	1	105	0.22	0.64
		Interaction	1	105	0.15	0.70
	Shannon (percent cover)	<i>C. applegatei</i>	1	105	1.22	0.27
		<i>C. prostratus</i>	1	105	0.56	0.46
		Interaction	1	105	0.15	0.70
	Percent cover (w/o <i>C. prostratus</i> or <i>C. applegatei</i>)	<i>C. applegatei</i>	1	105	0.11	0.74
		<i>C. prostratus</i>	1	105	8.47	0.004
		Interaction	1	105	1.68	0.20
Soil moisture (VWC%)	<i>C. applegatei</i>	1	97	3.88	0.05	
	<i>C. prostratus</i>	1	97	1.81	0.18	
	Interaction	1	97	7.17	0.009	

Bold signifies significant result at the adjusted $\alpha = 0.002$ (Bonferroni correction)

Discussion

As predicted, the availability of an N-fixing host significantly increased leaf N in the parasite, *C. applegatei*. Parasites in plots without the N-fixer had leaf N levels in line with neighboring non-N-fixers; however, the presence of the N-fixer brought the

parasite leaf N well above that of other species, including the N-fixer itself. High leaf N is thought to be relatively common in parasitic plants and be an important contributor to indirect effects on plant communities (Pate 1995; Spasojevic and Suding 2011; Fisher et al. 2013). These data additionally

Table 4 Soil moisture (VWC%) in plots surveyed in Sagehen Experimental Forest, 2015

<i>Castilleja applegatei</i> present	<i>Ceanothus prostratus</i> present	VWC %	SE	<i>N</i>
No	No	5.86	0.49	35
No	Yes	5.61	0.49	25
Yes	No	5.46	0.66	24
Yes	Yes	5.93	0.50	36

VWC% is short for volumetric water content

Table 5 Results of Welch's two-sided *t* test on four leaf traits on the N-fixer (*Ceanothus prostratus*) and the parasite (*Castilleja applegatei*) depending on whether they were growing near the parasite and N-fixer, respectively

Species	Parameter	Response variable	<i>t</i>	Df	<i>P</i> value
<i>Castilleja applegatei</i> (parasite)	With <i>C. prostratus</i> (N-fixer)	$\delta^{15}\text{N}$	6.590	35.657	< 0.0001
		$\delta^{13}\text{C}$	− 7.343	36.770	< 0.0001
		%N	− 6.826	21.231	< 0.0001
		C/N	9.579	36.899	< 0.0001
<i>Ceanothus prostratus</i> (N-fixer)	With <i>C. applegatei</i> (parasite)	$\delta^{15}\text{N}^{\text{a}}$	<i>Z</i> = 3.0993		0.0019
		$\delta^{13}\text{C}$	2.884	14.421	0.0117
		%N	0.537	17.363	0.5981
		C/N	−0.378	17.685	0.7099

Bold signifies significant result at $\alpha = 0.05$

^aAsymptotic Wilcoxon–Mann–Whitney test

suggest that leaf N and consequent indirect effects may be host-mediated.

A limitation of this work is that there is only one parasitic species and one N-fixing species represented. As such, any differences in leaf traits related to each of these species may be a result of other characteristics of the species rather than their parasitism and N-fixing, respectively. A second limitation is that direct host–parasite connections are not established. As mentioned previously, haustorial connections are delicate, difficult to find, and destructive to dig up. Previous work has used transfer of alkaloids to identify host connections, but this is not possible for plants without easily detected chemical markers (Adler 2003). One advantage of using stable isotopes was the possibility of detecting host–parasite connections through the distinctive signature of N-fixers; however, the results are mixed. That the parasite's increased N is derived directly from the N-fixer is potentially evidenced by the significant shift in parasites $\delta^{15}\text{N}$ values toward the $\delta^{15}\text{N}$ signature of the N-fixer. However, a shift of similar magnitude and direction is also observed in *W.*

mollis in the presence of the N-fixer, so the effect could be driven by litter deposit (i.e., the N-fixer is dropping leaves and altering the local $\delta^{15}\text{N}$ signature in the soil pool). On the other hand, it seems unlikely that the increase in leaf N in *C. applegatei* is derived entirely from leaf litter for several reasons. First, neither of the other two species, *A. tridentata* nor *W. mollis* showed the same increase in leaf N (indeed *A. tridentata* showed a decrease in leaf N). Second, while hemiparasites may develop functional roots when cultivated without a host, there is some evidence that their root systems are reduced and poorly developed when suitable hosts are available, thus the amount of mineral absorption directly from the soil is potentially small (Matthies 2017). Last, leaf N in *C. prostratus* was relatively similar to the other species present and thus unless there were large differences in resorption prior to senescence the resulting leaf litter from the N-fixer likely would not be particularly N-rich either. This third point calls into question why an N-fixing host would result in higher leaf N in the parasite if the host's leaf N is not particularly high. Given that

Table 6 Results of two-way ANOVAs on four leaf traits from two non-N-fixing, non-parasitic species (*Artemisia tridentata*, *Wyethia mollis*) depending on whether they were growing near the N-fixer (*Ceanothus prostratus*) and/or the parasite (*Castilleja applegatei*)

Species	Response variable	Parameter	Df	F	P value
<i>Artemisia tridentata</i>	$\delta^{15}\text{N}$	W/ <i>C. prostratus</i>	1	1.957	0.181
		W/ <i>C. applegatei</i>	1	0.042	0.840
		Interaction	1	0.931	0.349
		Residuals	16		
	$\delta^{13}\text{C}$	W/ <i>C. prostratus</i>	1	0.046	0.833
		W/ <i>C. applegatei</i>	1	1.097	0.310
		Interaction	1	0.002	0.966
		Residuals	16		
	%N	W/ <i>C. prostratus</i>	1	6.116	0.025
		W/ <i>C. applegatei</i>	1	1.727	0.207
		Interaction	1	0.112	0.743
		Residuals	16		
	C/N	W/ <i>C. prostratus</i>	1	7.226	0.016
		W/ <i>C. applegatei</i>	1	1.478	0.242
		Interaction	1	0.036	0.852
		Residuals	16		
<i>Wyethia mollis</i>	$\delta^{15}\text{N}$	W/ <i>C. prostratus</i>	1	6.638	0.020
		W/ <i>C. applegatei</i>	1	0.004	0.953
		Interaction	1	0.842	0.372
		Residuals	16		
	$\delta^{13}\text{C}$	W/ <i>C. prostratus</i>	1	0.857	0.368
		W/ <i>C. applegatei</i>	1	0.882	0.362
		Interaction	1	1.029	0.326
		Residuals	16		
	%N	W/ <i>C. prostratus</i>	1	0.000	0.984
		W/ <i>C. applegatei</i>	1	1.331	0.266
		Interaction	1	0.907	0.355
		Residuals	16		
	C/N	W/ <i>C. prostratus</i>	1	0.029	0.867
		W/ <i>C. applegatei</i>	1	1.165	0.296
		Interaction	1	0.574	0.460
		Residuals	16		

Bold signifies significant result at $\alpha = 0.05$

N-fixation rates are at least partially controlled by the plant's N demand, one possibility is that N loss to the parasite triggers higher N-fixation rates in compensation (Hartwig 1998). The amount and composition of N in xylem sap are variable, and thus it may also be that the N concentration in xylem sap relative to leaf N may be higher in *C. prostratus* (Bollard 1960).

The significant increase in the parasites' $\delta^{13}\text{C}$ values when growing with N-fixers supports the N-parasitism hypothesis (Fig. 1) that parasites with more access to N (indicated here by the availability of N-fixers and the associated increase in leaf N) have a

higher WUE. This is further supported by the positive correlation between leaf N and $\delta^{13}\text{C}$ among the parasites (Fig. 3). Although this does not resolve whether the increase in the parasite's WUE is due to a decrease in transpirational water loss because N needs are met or increased photosynthetic rates brought on by increased N, it adds support to the former explanation. It is also possible that the shift in $\delta^{13}\text{C}$ reflects the distinct isotopic signature of source C (i.e., the N-fixer), rather a physiological shift in the parasite (Richter et al. 1995).

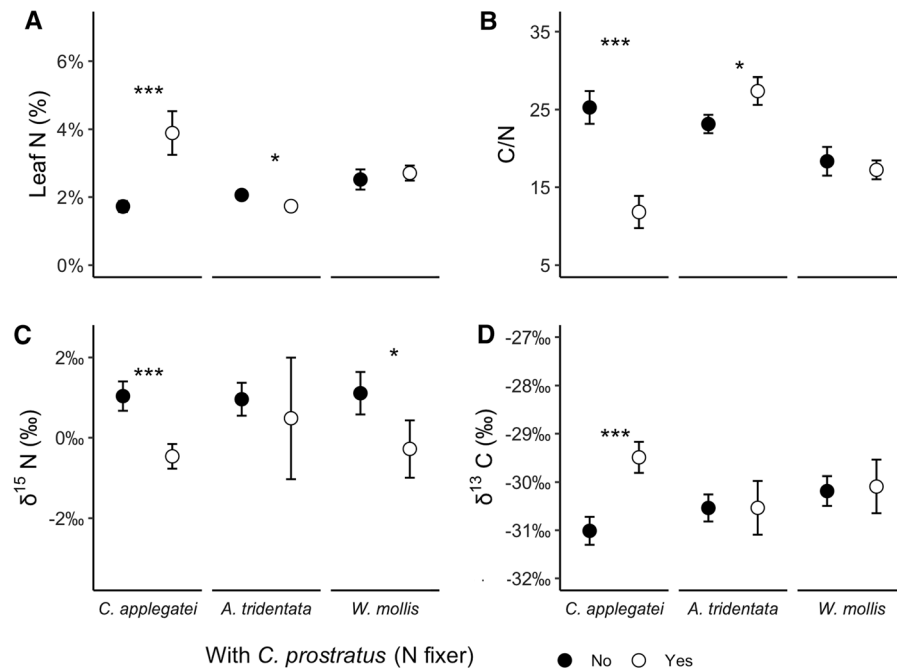


Fig. 1 Differences in four leaf traits: **A** foliar leaf N (%), **B** foliar C/N, **C** $\delta^{15}\text{N}$, and **D** $\delta^{13}\text{C}$ among two non-N-fixer plant species (*Artemisia tridentata*, *Wyethia mollis*) and a root hemiparasite (*Castilleja applegatei*) when in the presence of the N-fixer (*Ceanothus prostratus*). Leaves collected at Sagehen

Experimental Forest in 2015. Asterisks denote significant differences within a species where: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See Tables 5 and 6 for statistics. Error bars are 95% confidence intervals

Related, but not mutually exclusive, is the hypothesis that observed low WUE is a by-product of heterotrophy. The ability of parasites to obtain C from their hosts' transpiration stream biases traditional methods for estimating A/E (i.e., WUE) because A only accounts for carbon gain via photosynthesis (Marshall et al. 1994). When heterotrophic carbon gain is accounted for, estimates of WUE are more similar to host species (Marshall and Ehleringer 1990). However, estimates of the contribution of host-derived C to total C acquisition in parasites are highly variable and not well constrained. In addition, $\delta^{13}\text{C}$, used as an estimate of WUE, will reflect not only the parasite's long-term c_i/c_a but also the host's. This ultimately dilutes the signal from the parasite (Bannister and Strong 2001).

The positive correlation between leaf N and $\delta^{13}\text{C}$ is also seen in the N-fixer itself and one of the non-N-fixers, *A. tridentata* (Fig. 4). In theory, some possible drivers behind this pattern in parasitic plants also apply to non-parasites. For one, more N allows for more photosynthesis because it is a key component to RuBisCO, the main carboxylating enzyme in leaves

(Chapin et al. 1987; Evans 1989). In this case, increases in WUE would be driven by upregulation of photosynthesis rather than downregulation of transpiration. This has been observed previously in *Rhinanthus* (Těšitel et al. 2015). Alternatively, transpiration generally increases the mass flow of nutrients to plant roots (Barber 1962; McDonald et al. 2002; Matimati et al. 2014), and thus correlations between leaf N and $\delta^{13}\text{C}$ could also be driven by upregulation of transpiration when plant demand for N is higher. However, for non-parasites the potential benefit of high transpiration rates is reduced because they do not obtain N from dilute xylem streams, while the relative cost of water is higher because of investment in root architecture.

Previous work has suggested that the deposit of N-rich litter from parasites alters local plant diversity and growth (Quasted et al. 2003; Spasojevic and Suding 2011; Fisher et al. 2013). Despite evidence that the parasite has relatively high leaf N when N-fixing hosts are available, neither the presence of the parasite nor combination of the parasite and the N-fixer had an impact on plot-level diversity or cover. The presence

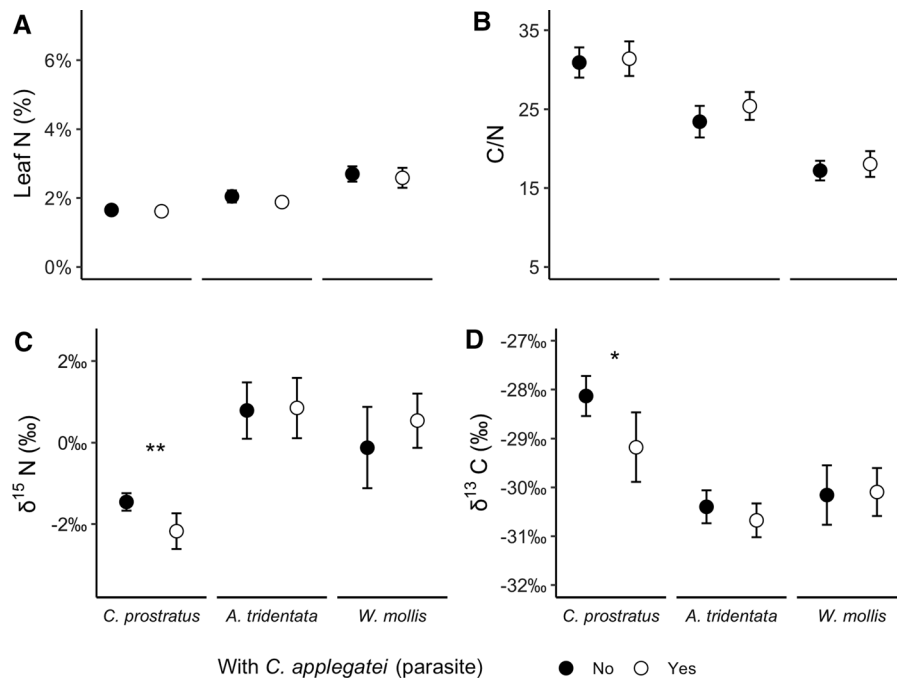


Fig. 2 Differences in four leaf traits: **A** foliar leaf N (%), **B** foliar C/N, **C** $\delta^{15}\text{N}$, and **D** $\delta^{13}\text{C}$ among two non-N-fixer plant species (*Artemisia tridentata*, *Wyethia mollis*) and the N-fixer (*Ceanothus prostratus*) when in the presence of the parasite (*Castilleja applegatei*). Leaves collected at Sagehen

Experimental Forest in 2015. Asterisks denote significant differences within a species where: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See Tables 5 and 6 for statistics. Error bars are 95% confidence intervals

of the N-fixer did have a negative impact on percent cover, likely reflecting *C. prostratus* mat-forming habit, wherein it crowds out other species. It could be that in a system such as the one observed here, the parasites do not constitute a large enough portion of aboveground biomass to have community-level effects. While I did not quantify biomass, in plots with the parasite the average percent cover of the actual parasite was 7.25%, while the average percent cover for plots with and without the parasite were 52.50% and 39.58%, respectively (including the parasite in percent cover totals). The documentation by Spasojevic and Suding (2011) of increased plant growth as a result of parasitic plant litter was in a setting where parasites made up nearly half of the biomass in plots where they were present.

Although community-level effects were largely absent, the presence of the parasite did significantly affect the N-fixer (but none of the other species present) (Fig. 2). This further suggests that the parasites were attached to the N-fixers, although it does not preclude attachments to other species. That effects

which are observed only in the N-fixer suggests either disproportionate parasitism on N-fixers or disproportionate vulnerability to parasitism in N-fixers (although the former seems more likely). The disproportionate parasitism on N-fixers could be caused by a difference in how likely the parasite is to attach to a particular host or a shift in the parasites' heterotrophy/autotrophy balance when attached to certain hosts (such as in Nge et al. 2019).

In the N-fixer, the presence of the parasite was associated with a significant decrease in $\delta^{13}\text{C}$, signifying a lower WUE (Fig. 2). Because parasitic plants access host xylem and then pull water into their own xylem with transpiration rates high enough to maintain a favorable water potential gradient, the lower WUE may be a result of the N-fixer's increased transpiration due to competition with the parasite for its own xylem stream. The N-fixer's leaf N was unchanged in the presence of the parasite, further suggesting an upregulation of transpiration rather than downregulation of photosynthesis.

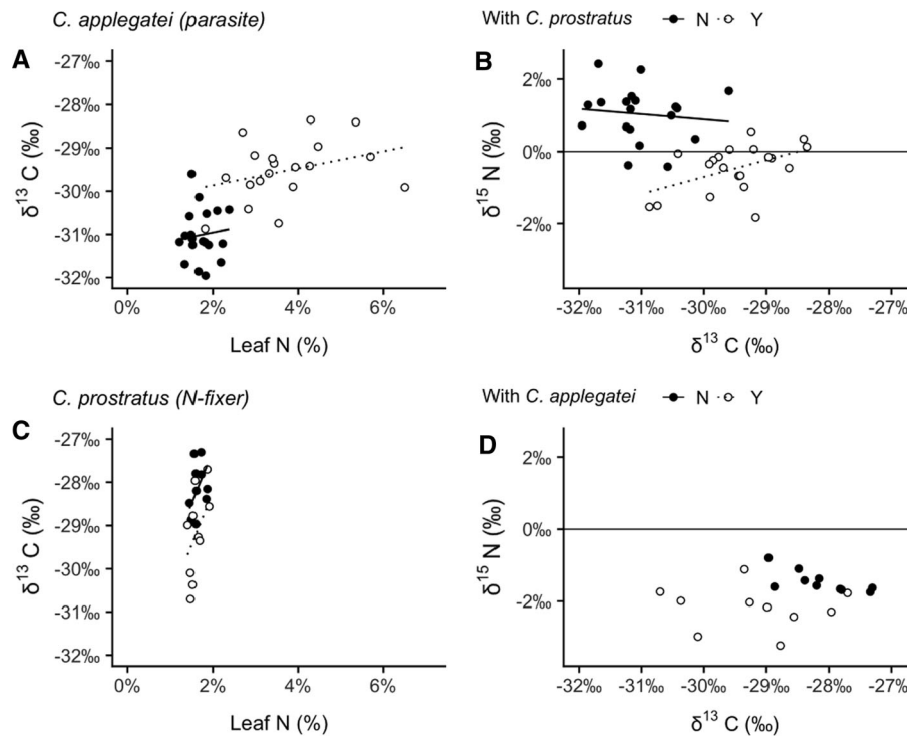


Fig. 3 Relationship between leaf traits: **A** foliar leaf N (%) vs. $\delta^{13}\text{C}$ and **B** $\delta^{13}\text{C}$ vs. $\delta^{15}\text{N}$ compared within a root hemiparasite (*Castilleja applegatei*) when in the presence of the N-fixer (*Ceanothus prostratus*) and **C** foliar leaf N (%) vs. $\delta^{13}\text{C}$ and **D** $\delta^{13}\text{C}$ vs. $\delta^{15}\text{N}$ within the N-fixer (*C. prostratus*) when in the

presence of a root hemiparasite (*C. applegatei*). Leaves collected at Sagehen Experimental Forest in 2015. Trend lines are shown where the continuous variable or interaction was at least marginally significant ($p < 0.1$) in ANCOVAs. See Table 7 for statistics

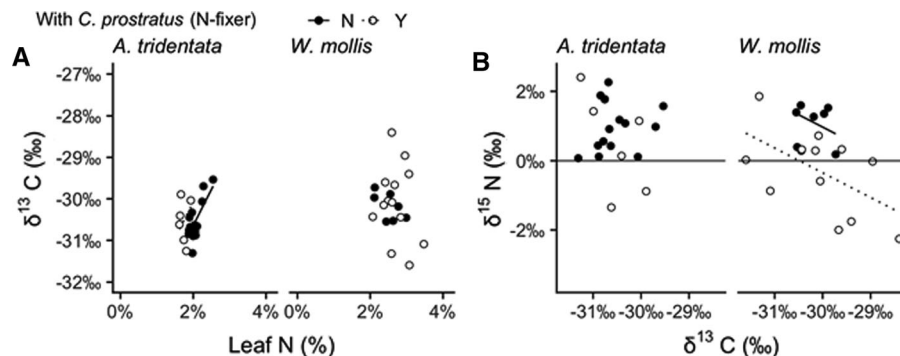


Fig. 4 Relationship between leaf traits: **A** foliar leaf N (%) vs. $\delta^{13}\text{C}$ and **B** $\delta^{13}\text{C}$ vs. $\delta^{15}\text{N}$ compared within two species, *Artemisia tridentata* and *Wyetha mollis*, when in the presence of the N-fixer (*Ceanothus prostratus*). Leaves collected at Sagehen

Experimental Forest in 2015. Trend lines are shown where the continuous variable or interaction was at least marginally significant ($p < 0.1$) in ANCOVAs. The presence of *Castilleja applegatei* was not significant. See Table 7 for statistics

Interestingly, the presence of the parasite was also associated with a significant shift in the $\delta^{15}\text{N}$ signature of the N-fixer (Fig. 2). Biologically fixed N is typically very close to atmospheric N_2 , which is by definition 0‰, whereas soil N typically has a $\delta^{15}\text{N}$

signature distinct from the atmosphere (Dawson et al. 2002). As mentioned above, it is possible that N loss to the parasite leads to increased N-fixation by the N-fixer, keeping the overall leaf N unchanged. However, the $\delta^{15}\text{N}$ values do not support that conclusion, as

Table 7 Results of ANCOVAs on the relationship between leaf traits within four species, including the N-fixer (*Ceanothus prostratus*), the parasite (*Castilleja applegatei*), and two other non-parasites (*Artemisia tridentata*, *Wyethia mollis*)

Species	Response variable	Parameter	Df	F	P value	Adjusted R ²
<i>Castilleja applegatei</i> (parasite)	$\delta^{13}\text{C}$	%N	1	3.765	<i>0.0602</i>	0.610
		W/ <i>C. prostratus</i>	1	13.538	0.0008	
		Residuals	36			
	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	1	1.474	0.2329	0.560
		W/ <i>C. prostratus</i>	1	28.140	< 0.0001	
$\delta^{13}\text{C} \times \text{w/ } C. \text{ prostratus}$		1	3.068	<i>0.089</i>		
<i>Ceanothus prostrates</i> (N-fixer)	$\delta^{13}\text{C}$	%N	1	4.472	0.044	0.402
		W/ <i>C. applegatei</i>	1	8.238	0.011	
		Residuals	17			
	$\delta^{15}\text{N}^{\text{a}}$	$\delta^{13}\text{C}$	1	0.443	0.515	–
		W/ <i>C. applegatei</i>	1	16.304	0.009	
<i>Artemisia tridentata</i>	$\delta^{13}\text{C}$	%N	1	9.753	0.007	0.309
		W/ <i>C. prostratus</i>	1	4.505	0.050	
		%N \times w/ <i>C. prostratus</i>	1	1.750	0.204	
		Residuals	17			
	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	1	0.353	0.562	0.147
W/ <i>C. prostratus</i>		1	1.200	0.292		
W/ <i>C. applegatei</i>		1	0.054	0.820		
$\delta^{13}\text{C} \times \text{w/ } C. \text{ prostratus}$		1	1.518	0.238		
$\delta^{13}\text{C} \times \text{w/ } C. \text{ applegatei}$		1	2.194	0.161		
<i>Wyethia mollis</i>	$\delta^{13}\text{C}^{\text{b}}$	%N	1	0.932	0.347	-0.004
		Residuals	18			
	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	1	8.103	0.012	0.504
		W/ <i>C. prostratus</i>	1	9.887	0.006	
		W/ <i>C. applegatei</i>	1	2.324	0.147	
Residuals	17					

Bold signifies significant result at $\alpha = 0.05$; italics signify marginally significant result at $\alpha = 0.1$

^aGeneralized least squares (gls) fit by restricted maximum likelihood with a variance structure that allows for different standard deviations in each stratum. R² not calculated for gls fit

^bLinear regression rather than an ANCOVA because of elimination of non-significant parameters

the N-fixer’s $\delta^{15}\text{N}$ signature shifts further from 0‰, which would signify a decreased reliance on N-fixation. However, a wide range of factors, beyond N-fixation rates and different source pools, could cause the shift in $\delta^{15}\text{N}$ signature within the N-fixer and any conclusions here are premature. For example, it is not currently known whether nutrient transfer through haustoria results in N isotope fractionation. The observed shift could also be a result of discrimination

as N leaves the individual plant toward the parasite, rather than simply reflecting the N-fixer’s source N.

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Declarations

Conflict of interest The authors declare they have no conflict of interest.

Availability of data and material Data are available in the online repository figshare. <https://doi.org/10.6084/m9.figshare.11965557>.

Code availability Not applicable.

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