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Browsing impacts on the stable isotope composition of chaparral plants

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Citation: Koch, P. L., and L. R. Fox. 2017. Browsing impacts on the stable isotope composition of chaparral plants. Ecosphere 8(2):e01686. 10.1002/ecs2.1686

Abstract. We assessed the effects of herbivory on competition, water stress, and potentially biological nitrogen fixation on three species of endemic shrubs using variations in the stable isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in leaves of chaparral shrubs in a long-term field experiment. While variations in isotopic ratios of plants are often attributed to abiotic stresses, impacts of biotic interactions are rarely evaluated. Our site was a low-nutrient, chaparral community on the central California coast. In this system, deer browsing on *Ceanothus rigidus*, which have symbiotic N-fixing bacteria (*Frankia*), was intense and suppressed growth, while the two non-fixing shrubs (*Arctostaphylos pumila* and *Ericameria ericoides*) were not browsed heavily. For *Ceanothus*, excluding deer increased both plant size and the $\delta^{15}N$ value to ~0‰; $\delta^{13}C$ values also increased as the plants increased in mass. In *Arctostaphylos* and *Ericameria*, stable isotope values did not change, while plant sizes remained the same or even declined when deer were excluded. We interpret the change in *Ceanothus* $\delta^{15}N$ values as due to increased N fixation after evaluating possible alternative explanations. The increase in *Ceanothus* $\delta^{13}C$ values may be due to increased water stress with substantial shrub growth. More broadly, herbivore suppression of N fixation may impact ecosystem processes such as productivity and N cycling, as well as an ecosystem's ability to respond to increased CO_2.

Key words: biological nitrogen fixation; *Ceanothus;* herbivory; maritime chaparral; *Odocoileus;* stable isotopes.

Received 1 September 2016; revised 13 December 2016; accepted 15 December 2016. Corresponding Editor: Jason West.
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INTRODUCTION

Herbivory can have cascading impacts on the structure and functioning of ecological communities as well as ecosystem processes that include nutrient dynamics and biogeochemistry (Schmitz 2008). Herbivores affect plants directly by reducing biomass, carbon fixation, and survival, or by increasing fertilization through liquid and solid waste. They also have indirect impacts through interactions with other species that include plants, symbiotic microbes, and other herbivores, and through changes they create in habitat structure and soil nutrient dynamics (Schmitz 2008, Tao and Hunter 2011, Strickland et al. 2013). For example, the symbiosis between nitrogen (N)-fixing bacteria and plants may be responsive to herbivory, since the dynamics of this mutualism are modulated by plant allocation of compounds to support the symbiotic bacteria (Denison 2000, Bronstein 2001, Ness et al. 2009), which might be reduced if plants are stressed (Thomas and Berry 1989).

Herbivory may indirectly modify ecosystem-wide N-fixation by reducing abundance, sizes, and growth rates of N-fixing plants, or by changing local microclimates (e.g., opening the canopy increases light and temperature), all of which may affect the competitiveness of N-fixing plants (Johnson et al. 1987, Ritchie and Tilman 1995,

Knops et al. 2000, Bishop 2002, Walker et al. 2003). Furthermore, changes in the abundance of N-fixing plants may ultimately alter the N content of soils, although outcomes can be species-specific. For example, excluding deer from an oak savanna increased the abundance of only one of three N-fixing legumes, *Lathyrus venosus*, enhancing its productivity and increasing [N] in surrounding soil (Ritchie and Tilman 1995, Knops et al. 2000). Such studies demonstrate that herbivory affects plant growth and/or abundance, but they do not show direct effects on N-fixation rates per se because biological N-fixation (BNF) rates were not measured. Impacts of biotic interactions such as herbivory on BNF rates are largely unexplored in natural terrestrial systems.

We have studied interactions among three species of endemic shrubs in a low-nutrient, chaparral community on the central California coast since 1996, and assessed how herbivores affect plant traits and interactions in a long-term experiment that compared control plots with those that prevented deer (*Odocoileus hemionus*), and in some treatments also rabbits (*Sylvilagus bachmani*), from browsing shrubs. One species, *Ceanothus rigidus*, has symbiotic bacteria (*Frankia*) that can fix atmospheric N_2, while adjacent *Arctostaphylos pumila* and *Ericameria ericoides* are non-fixers. At our site, both non-fixing species compete with *Ceanothus*, whereas *Ceanothus* sustains intense browsing by deer (Fig. 1; Deveny and Fox 2006, Pittermann et al. 2014). *Ceanothus* and *Arctostaphylos* are both considered threatened or endangered species (CNPS 2016).

In this paper, we specifically evaluate the hypotheses that browsing reduces plant growth, BNF, and general "plant health" in *Ceanothus*. We address these issues using stable isotope ratios of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$), which reflect aspects of plant health and plant responses to their environments. Stable nitrogen isotopes vary with sources of plant N (e.g., different soil pools, or the atmosphere via symbiotic fixation of N_2 or uptake of other gaseous sources through stomata), mycorrhizal symbioses, microbial cycling, and the signature of the N lost from ecosystems; at a more synthetic level, all these factors generate global patterns associated with climate and N availability (Huss-Danell 1997, Paschke 1997, Lambers et al. 2008, Craine et al. 2009, 2015). Stable carbon isotope composition indicates whole plant health, reflecting the biochemical discrimination of $^{13}CO_2$ vs. $^{12}CO_2$ during gas exchange and C fixation (Dawson et al. 2002). Factors affecting stomatal conductance or carboxylation rate (e.g., light, temperature, nutrients, water availability, relative humidity) may change plant $\delta^{13}C$ values (Farquhar et al. 1989, Dawson et al. 2002).

Materials and Methods

Study system

We worked in a protected maritime chaparral community at the Fort Ord Natural Reserve, in Marina, California. This 242-ha reserve, on a former U.S. Army base, is now part of the University of California Natural Reserve System and lies ~2 km inland from Monterey Bay at an elevation of 21–58 m. The site has a Mediterranean climate, with late autumn and winter rains and dry (though cool and foggy) summers. The soils are aeolian dunes, 10^4–10^5 years old (Dupré 1990), composed mainly of quartz sands with low levels of most nutrients (e.g., at 0–10 cm depth, total N = 0.084%; P = 21.8 ppm; organic matter = 1.9%; Fox et al. 1998). The fire history of the reserve is not known, although analyses of sporadic aerial photographs since 1941 suggest that the study site has not burned in >80 years (L. Fox, *unpublished data*).

Arctostaphylos pumila Nutt. (Sandmat Manzanita) is the dominant shrub, while *C. rigidus* (Nutt.) Hoover (syn. *C. cuneatus rigidus*) (Monterey Ceanothus) and *E. ericoides* (Less.) Jepson (California Goldenbush) are distributed patchily among the manzanita. Black-tailed deer, *O. hemionus columbianus* (Rafinesque), are the major browsers. Woodrats (*Neotoma fuscipes* (Baird)) clip branches of woody shrubs, especially *Arctostaphylos*, near patches of oak where they nest, and brush rabbits (*S. bachmani* (Waterhouse)) browse herbaceous plants and low shrubs throughout. Unidentified insect stem borers attacked some, primarily unbrowsed, *Ceanothus* for several years during the study. Apart from woodrat damage, which was very patchy in time and space, there was little evidence of herbivory on *Arctostaphylos* and *Ericameria.*

In *Ceanothus*, atmospheric N_2 is fixed by actinorhizal bacteria (*Frankia*) that induce symbiotic nodules on roots. Recent molecular—and



Fig. 1. *Ceanothus rigidus* at Fort Ord Natural Reserve showing the topiaried form of a heavily browsed plant (A) and close-up of the web of branches (B). (C) shows a *Ceanothus* inside a deer exclosure one year after the experiment began; by this time, $\delta^{15}N$ were ~0‰. After several years, *Ceanothus* were larger and had several dominant branch systems (D), such as in (E).

particularly genomic—studies show at least 19 "genospecies" (variously strains, groups, or clusters) that may be related to host plants or free-living life styles (Normand and Fernandez 2009, Gtari et al. 2013). *Frankia* living in host plants with narrow geographical ranges, including *Ceanothus,* have small genomes and low genetic diversity (Gtari et al. 2013), with possible local differentiation across host species (Oakley et al. 2004). Nodulation varies with local conditions, for example, soil moisture or nutrient balance (including [N] and [P], and plant age and health [Walls and Zamora 2000, Tobita et al. 2013]); but overall, both abiotic and biotic factors that affect plant photosynthesis also affect the *Frankia*/plant symbiosis (Huss-Danell 1997). In general, actinorhizal plants get more of their N from fixation than legumes based on the ^{15}N natural abundance method (Andrews et al. 2011); 36–69% of plant N came from N-fixation in *C. cuneatus*, which is very closely related and until recently conspecific with *C. rigidus,* in the California Sierras (Shearer and Kohl 1986), but field data are very limited.

Field experiment and laboratory methods

Design.—The experiment began in August 1996 in two areas of the reserve (~1 km apart), with three treatments arranged in a randomized block

design, and replicated four times in each area (24 plots in total). Treatments were control plots with natural levels of browsing by deer and rabbits (+D+R); plots that excluded deer but allowed rabbits to enter (−D+R); and plots that excluded both deer and rabbits (−D−R). Each plot was 9 m^2 and initially contained *Ceanothus, Arctostaphylos*, *Ericameria*, open space, and occasionally other shrubs. Deer were excluded by 2 m high cages, using 20 cm × 10 cm mesh. Rabbits were excluded by 1.5-cm mesh on the lower 1 m of half of the deer exclosures. Treatment × area effects were not statistically significant.

Many control *Ceanothus* suffered considerable dieback and some died completely, probably as a consequence of intense deer browsing over a long period of time (Coale et al. 2011). We replaced them as needed by sampling similar but healthy plants as close to the original control plot as possible. One caged *Ceanothus* died suddenly, due to rabbit girdling on the main stem, just prior to the 1999 leaf collections.

Leaf processing and analyses.—We took initial leaf samples in autumn 1996, two months after the cages were complete, and then sampled each species every autumn through 1999 and again in 2003. We clipped relatively recent branches (5–10, depending on plant size) on all parts of each plant, and removed obviously senescent leaves and all branches in the laboratory. We sampled the largest *Ceanothus* and *Ericameria* individuals in each plot and the largest *Arctostaphylos* patch, since individual plants are difficult to identify because of their multi-stemmed and amorphous form. We only analyzed *Ericameria* samples from 1996 and 2003.

For each species, all leaves from each plot were combined for analyses. We air-dried all leaf samples in the laboratory, ground them coarsely in a Wiley Mill, and further ground the samples to a fine powder with either a mortar and pestle or a cryogenic impact grinder (Spex Freezer Mill). Samples were sealed in Sn capsules, and then isotopic values and elemental concentrations were measured using a PDZ Europa Scientific 20/20 mass spectrometer coupled with an ANCA-SL C and N analyzer in the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. Leaf weight percent (wt %) N was measured at the same time. Nitrogen isotope composition is expressed as $\delta^{15}N$ value, the deviation of the sample isotopic ratio from that of a standard (atmospheric N_2) $\delta^{15}N = (({}^{15}N/{}^{14}N_{sample} \div {}^{15}N/{}^{14}N_{standard}) - 1) \times 1000$, where units are parts per thousand, or per mil (‰). Carbon isotope composition (expressed as $\delta^{13}C$ value) is calculated similarly relative to the Vienna-PDB standard. Standard deviations for repeated measurements ($n = 35$) on an internal standard (oak leaf) were 0.2‰ and 0.1‰ for $\delta^{15}N$ and $\delta^{13}C$ values, respectively, and 0.01% for wt % N. The average absolute values for differences between replicate analyses of *Ceanothus* samples ($n = 17$) were 0.2‰ for both $\delta^{15}N$ and $\delta^{13}C$ values and 0.04% for wt % N; average differences for *Arctostaphylos* replicates ($n = 14$) were 0.2‰ and 0.1‰ for $\delta^{15}N$ and $\delta^{13}C$ values, respectively, and 0.01% for wt % N. All concentration and isotopic data are presented in Data S1.

Nitrogen isotope values and N-fixation.—Plants can obtain N from the soil, either directly as NH_4^+, NO_3^-, or organic N, or indirectly via mycorrhizal fungi. Some plants obtain N from the atmosphere by symbiotic bacteria that fix N_2 (Vitousek et al. 2002, Persson et al. 2003, Lambers et al. 2008), as well as in nitrogen dioxide gas through stomata (Vallano and Sparks 2008). The $\delta^{15}N$ values of soil N sources (and the plants that grow on them) vary with soil type, climate, rooting depth, and other factors (Amundson et al. 2003). Reallocation of N among different tissues may also affect $\delta^{15}N$ values, but this process has been most fully examined in deciduous plants (Kolb and Evans 2002, Evans 2007); all the plants in our study are evergreen.

While the natural abundance of N isotopes in plants has been used to assess biological N-fixation, this and all other methods for assessing BNF in the field have shortcomings (Högberg 1997, Dawson et al. 2002). In theory, the $\delta^{15}N$ values of plants that obtain all their N from N-fixing symbionts should be close to that of atmospheric N_2 (~ 0‰), but actual values for such plants range from −2 to +2‰, depending on bacterial strain and other factors (Högberg 1997). For potential N-fixers, leaf $\delta^{15}N$ values close to that of the atmosphere may indicate that they depend on N-fixation (Högberg 1997). In contrast, if potential N-fixers and adjacent non-fixers have $\delta^{15}N$ values that are similar and significantly different than 0‰, then both groups may obtain their N from the soil pool. In

chaparral and other ecosystems with recurrent fires, $\delta^{15}N$ values of non-fixing plants and soils are often negative and may vary with time since the last fire (Herman and Rundel 1989).

We used the natural abundance of N isotopes to assess BNF because it does not destroy roots or bacterial nodules and integrates N-fixation over time, in contrast to older methods such as acetylene reduction (Dawson et al. 2002, Andrews et al. 2011). Estimating BNF by dilution with a ^{15}N-enriched tracer (e.g., Busse 2000) would have permanently altered the exclosures, reducing their utility for other experiments, so we did not use this approach. One way to control for variations that complicate interpretation of natural abundance $\delta^{15}N$ values is to compare values from potentially N-fixing plants with those of adjacent non-fixers. We used both *Arctostaphylos* and *Ericameria* shrubs in the experimental plots as non-fixing comparisons to *Ceanothus*.

Carbon isotope values, carboxylation rate, and water-use efficiency.—The $\delta^{13}C$ values of C3 plants like *Ceanothus*, *Arctostaphylos*, and *Ericameria* vary in response to any factor that affects either stomatal conductance or the rate of carboxylation by the enzyme RuBisCo (Farquhar et al. 1989). Higher (i.e., less negative) $\delta^{13}C$ values, which indicate less net discrimination against ^{13}C during photosynthesis, are associated with decreased stomatal conductance (due to water or osmotic stress) and/or increased rates of carboxylation (due to higher light or heat, or high nutrient conditions) (Tieszen 1991, Dawson et al. 2002).

Arctostaphylos–Ceanothus distances.—If N from decomposition of N-fixing *Ceanothus* was used by non-fixers, we would expect the effect to diminish with distance from the nearest *Ceanothus* plant. To assess this, in 2007 we measured leaf $\delta^{15}N$ values of 20 haphazardly selected *Arctostaphylos* (in both areas of FONR used in our study) that ranged from 0 to 14 m from any *Ceanothus*. We processed the leaves in the same way as in the main experiment, and the data are presented in Data S2.

Plant size and biomass.—We estimated live cover area from annual measurements of shrub length, width, and proportion of the surface area in leaf. We estimated biomass for all experimental plants at the start of the experiment by comparing plant size with non-experimental plants that we cut down for wood and leaf biomass; our estimates of biomass were strongly correlated with actual biomass ($r > 0.90$ for all three species) in test samples. We were able to use the regressions from these comparisons for control *Ceanothus*, as well as all *Arctostaphylos* and *Ericameria*, throughout the experiment, because these plants did not change growth form. For *Ceanothus* that were protected from deer browsing, however, plant growth changed from a compact hemi-ellipsoid to a more open plant with dominant branch systems. To estimate 2003 biomass for these *Ceanothus*, we cut one branch from selected plants in 2007 and then calculated wood and leaf biomass of the entire plant based on a count of the number of branches on each plant and on plant cover areas described above. For all biomass estimates, we dried wood and leaf samples at 40°C for at least two days.

Soil nitrogen and carbon.—To assess whether observed shifts in plant $\delta^{15}N$ values might be explained by changes in rooting depth after browsing was removed, we measured the concentration and isotopic composition of soil N and C in two locations, one 2 m outside an exclosure and one inside a −D−R exclosure. We collected samples with a hand auger in spring 2010. As coring in the sandy soil was challenging, we combined materials from 25 to 30 m depth intervals into single samples for isotopic and concentration analysis, collecting samples to a maximum depth of ~ 1.3 m.

Soil samples were air-dried in the laboratory, then coarsely sieved (1.7-mm mesh) to remove large plant and root fragments. We measured soil wt % N and % C on this bulk fraction. Samples were ground to a fine powder with an agate mortar and pestle, then sealed in Sn boats for analysis. Because bulk samples were so sandy, they typically had N concentrations that were too low for reliable $\delta^{15}N$ analysis. As a consequence, we sieved samples to isolate a fine-grained fraction (which passed through 150-μm mesh) that is more organic-rich that we used for $\delta^{15}N$ analysis. For several samples, we compared material isolated at the different size fractions to those for bulk $\delta^{15}N$ and $\delta^{13}C$ values. We detected no conspicuous changes for either isotope system across the size fractions, indicating that we were analyzing roughly the same material as particle size decreased, just with less dilution by sand (Data S3). Concentration and stable isotope

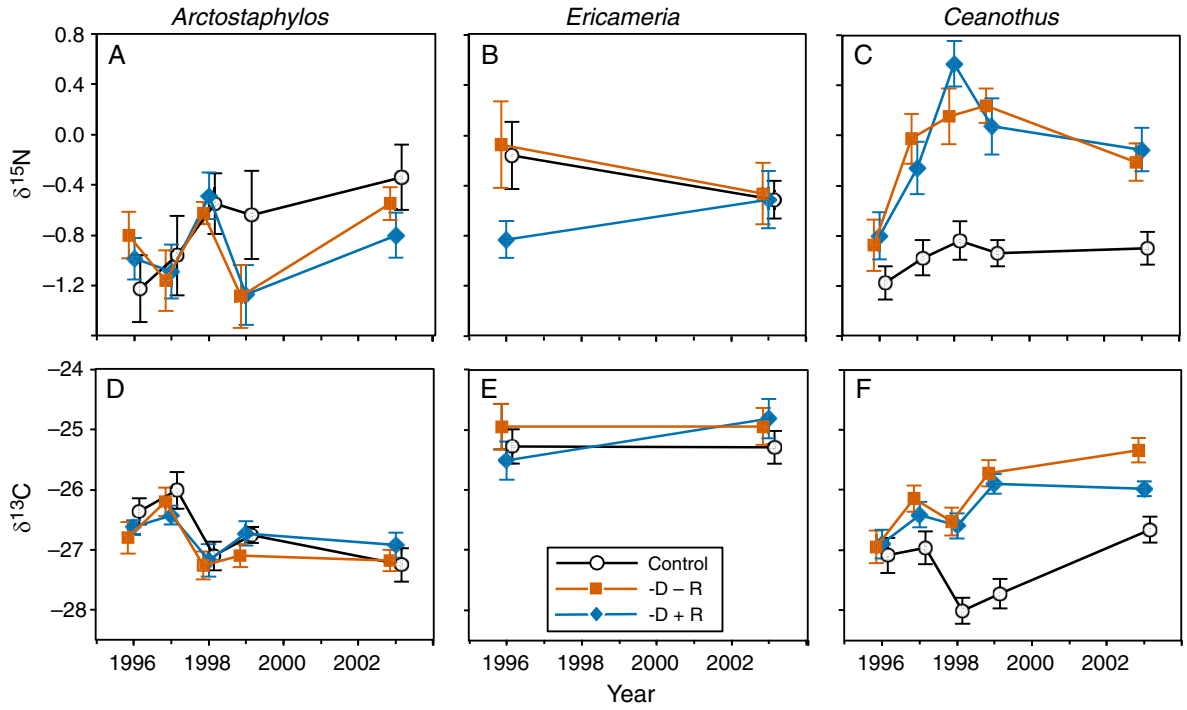


Fig. 2. Changes in foliar δ^{15}N (A–C) and δ^{13}C (D–F) values for N-fixing shrubs, *Ceanothus rigidus*, and adjacent non-fixing shrubs, *Ericameria ericoides* and *Arctostaphylos pumila*, for seven years of a field experiment that excluded only deer (−D+R, blue diamonds) or both deer and rabbits (−D−R, orange squares) from some plots. Controls are +D+R (black open circles). Data are $\bar{x} \pm$ SE.

values on soil samples were measured at the Stable Isotope Laboratory at University of California, Santa Cruz, on a Carlo Erba 1108 elemental analyzer coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer.

Data analyses.—The data for changes in leaf properties over time were analyzed with SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA). For δ^{15}N and δ^{13}C values throughout the experiment, we used ANOVAs (Proc Glimmix), with area as a random variable and plot as the unit of replication, and evaluated specific contrasts (Proc PLM) with the Holm-Sidak adjustment for multiple comparisons (Appendix S1). Because of the curvilinear responses, we included time as both linear and quadratic functions in these analyses. We used Proc Reg for regression, and Proc Means to compare plants with vs. without herbivory other than deer. We tested for temporal autocorrelation of δ^{13}C values and relative plant cover with Moran's I in R (v3.2.2, using the package "APE"; Paradis et al. 2004).

Results

Leaf nitrogen

Ceanothus had the highest leaf wt % N of the three species although leaf N levels in *Ceanothus* (1.49%) were not significantly higher than in *Ericameria* (1.3%; $P = 0.13$). Overall, wt % N did not change with time or treatment in either species. *Arctostaphylos* had the lowest wt % N of the three species ($P < 0.03$); leaf N was 0.84% in 1996 and declined slightly to 0.76% over time ($P = 0.04$) in the −D−R treatments (Data S1).

Leaf δ^{15}N values and browsing

In autumn 1996, two months after the start of the exclusion experiment, δ^{15}N values for all three species were close to −1.0‰ (Fig. 2A–C). There were no initial differences among treatments within species ($P > 0.11$ for all except in *Ericameria* control leaves, which had slightly higher δ^{15}N values than −D+R leaves [$P \sim 0.06$]). One year later (1997), values for unbrowsed

Ceanothus had increased and were significantly higher than for control plants ($P < 0.0001$ for both −D+R and −D−R); they remained close to 0‰ through the 2003 leaf collections. Since $\delta^{15}N$ values for unbrowsed *Ceanothus* in −D+R and −D−R treatments differed from browsed controls, but not from each other ($P = 0.98$), the increased $\delta^{15}N$ values of protected plants are due entirely to eliminating deer.

The $\delta^{15}N$ values for both browsed and unbrowsed *Ceanothus* were high in 1998, a very wet El Niño year (Fig. 2C). Despite this slight increase, the $\delta^{15}N$ value of control *Ceanothus* remained at ~ −0.8‰ throughout the experiment ($P > 0.15$).

Arctostaphylos $\delta^{15}N$ values increased over time ($P < 0.045$), with the largest change for control plants (Fig. 2A), but with no significant differences among treatments ($P = 0.45$). *Ericameria* $\delta^{15}N$ values did not vary with time or treatment (both $P > 0.45$; Fig. 2B).

Finally, $\delta^{15}N$ values for *Arctostaphylos* did not change with distance (0–14 m) from the nearest *Ceanothus* (adjusted $r^2 = 0.11$, $P = 0.085$; Data S2). This suggests that *Ceanothus* did not affect the $\delta^{15}N$ value of the soil N pool used by *Arctostaphylos*, even when these shrubs were in very close proximity.

Leaf $\delta^{15}N$ values and other herbivores

Excluding deer affected interactions between *Ceanothus* and other herbivores. Between 1999 and 2003, woodrats clipped branches of some caged *Ceanothus* (particularly in −D+R plots, which may have been easier to access), while some plants were attacked by stem-boring insects. Despite these sometimes intense biotic interactions, $\delta^{15}N$ values of damaged vs. healthy caged plants did not change ($P = 0.47$; Data S1). Woodrats occasionally clipped *Arctostaphylos* branches in both control and caged plots, but because of its sprawling growth form, we could not associate individual plants with browsed sections to test for isotopic effects.

Plant $\delta^{13}C$ values

The $\delta^{13}C$ values were lower in *Ceanothus* and *Arctostaphylos* leaves (−26.8‰ and −26.5‰, respectively) than in *Ericameria* (−25.3‰) at the start of the experiment ($P = 0.0001$; Fig. 2D–F). Over time, the mean $\delta^{13}C$ value of unbrowsed *Ceanothus* rose to −25.5‰ ($P < 0.0001$); for browsed plants, there was an initial decline to −27.1‰ (e.g., by 1998 $P < 0.05$) and then an increase to previous values by 2003.

The $\delta^{13}C$ values of *Arctostaphylos* varied over time, but declined to a stable value from 1998 to 2003 without treatment effects ($P > 0.90$). The $\delta^{13}C$ values for *Ericameria* in 2003 were the same as at the start of the experiment ($P = 0.93$). The lower leaf $\delta^{13}C$ values for *Arctostaphylos* and *Ceanothus* in 1998 suggest reduced stomatal resistance in wetter conditions.

Plant growth

Both wood and leaf biomass and plant cover area increased in unbrowsed *Ceanothus*, but generally declined in browsed plants, during this experiment (Table 1). In 1996, Ceanothus cover area was 1.18 ± 0.3 m^2 for all plants. But the substantial reduction in leaf and wood biomass and cover area of healthy leaves (by about one-third) in control plants reflects significant leaf dieback and plant mortality (L. Fox, *unpublished data*). In contrast, unbrowsed *Ceanothus* grew steadily and substantially; by 2003, plants in −D−R and −D+R plots (combined) were ~3× larger (Table 1; $P < 0.0013$ for both) than at the start.

Table 1. Estimated dry weights (kg/plant) of live *Ceanothus* leaves and their supporting wood in 1996, and the changes in dry weight of leaves and wood by 2003.

	1996		2003	
Treatment	Leaves	Wood	Change in leaves	Change in wood
Control	1.51 ± 0.29	13.10 ± 2.53	−0.48 ± 0.37	−4.14 ± 3.21
No deer	1.52 ± 0.17	13.17 ± 1.47	0.54 ± 0.12	1.62 ± 0.34

Notes: Data are means ± SE. The −D+R and −D−R treatments are combined as no deer. Only plants surviving between 1996 and 2003 are included; many control plants died during this time. The 2003 biomass for all plants in the deer exclosures represent completely new leaf and wood production; the original portions of the plant died back completely during this time. Biomass loss of living leaves and their supporting branches of control plants represents dieback associated with continued browsing.

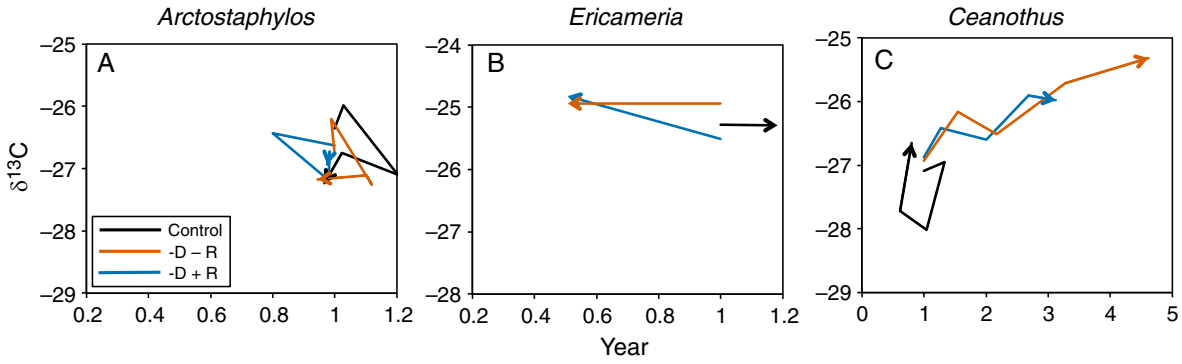


Fig. 3. Trajectories of mean $\delta^{13}C$ values for (A) *Arctostaphylos*, (B) *Ericameria*, and (C) *Ceanothus* with changes in shrub (area) cover over time. The x-axes of the graphs are drawn to different scales. To account for initial size differences, the relative size is expressed as the proportion of each plant's annual cover area relative to cover area (=1) at the start of the experiment; the trajectories are indicated by an arrow in 2003. Treatments are control (+D+R, black line), −D+R (blue line), and −D−R (orange line). The regression of $\delta^{13}C$ values on relative plant sizes for unbrowsed *Ceanothus* (−D+R and −D−R combined) is as follows: −26.8579 + 0.3038*X* (adjusted $r^2 = 0.25$, $P < 0.0001$).

The $\delta^{13}C$ values of unbrowsed *Ceanothus* increased with plant size over time (Fig. 3C; $P < 0.002$ for both), while changes (both increases and decreases) in plant size did not affect leaf $\delta^{13}C$ values of browsed plants ($P = 0.30$). *Ceanothus* in the −D+R plots grew more slowly than those in the −D−R treatments (to ~60% of the −D−R cover area by 2003); this slower growth was due to branch removal by woodrats, which was initially higher in the −D+R exclosures (Data S1).

Fluctuations in cover area and $\delta^{13}C$ values of *Arctostaphylos* and *Ericameria* were not related to treatment (Fig. 3A, B; $P > 0.20$ for all).

Soil nitrogen and carbon

Soil N and C concentrations changed with depth, but were not related to treatment (Data S3; Fig. 4). The wt % N and C in soils dropped by at least a factor of two from the upper soil layer (0–25 cm) to all deeper layers (Fig. 4A, C). Upper soil layer element concentrations and C:N ratios were slightly higher outside than inside the exclosure. The $\delta^{15}N$ values for the fine soil fraction increased with depth by 2–3‰, and even in upper soil layers, values were ^{15}N-enriched relative to plants by at least 2‰ both inside and outside of exclosures (Fig. 4B). Lower $\delta^{15}N$ values in plants relative to soil N and an increase in soil $\delta^{15}N$ values with depth are common characteristics of many systems (Evans 2007). In contrast, within the exclosure, the $\delta^{13}C$ value for bulk soil C was ~1‰ lower in the upper layer compared to other depths, whereas outside the exclosure, $\delta^{13}C$ values did not change with depth (Fig. 4D).

Isotopic mass balance models of potential changes in rooting depth

Because deep soil layers have greater $\delta^{15}N$ values than surface layers, higher $\delta^{15}N$ values in unbrowsed *Ceanothus* could be due to increased N uptake by roots at greater depths as plants increase above-ground biomass. We explore this idea using concentration-dependent isotope mass balance models (Phillips and Koch 2002) to estimate the change in N uptake by shallow (f_S) vs. deep (f_D) roots needed to explain an ~1‰ rise in unbrowsed *Ceanothus* (Appendices S2–S4). With these changes in proportional N uptake, and assuming the total amount of shallow root uptake is maintained, we estimate the increase in deep root uptake (a proxy for root volume) entailed by each model.

The models use $\delta^{15}N$ and [N] values for different soil layers, as well as $\delta^{15}N$ values for browsed and unbrowsed *Ceanothus* averaged across all years (except 1996 for unbrowsed plants). *Ceanothus* has shallow lateral roots, typically <60 cm at Fort Ord, though some roots may penetrate more deeply to access water (Kummerow et al. 1977, Miller and Ng 1977, Holl 2002, Egerton-Warburton et al. 2003, Holl and McStay 2014).

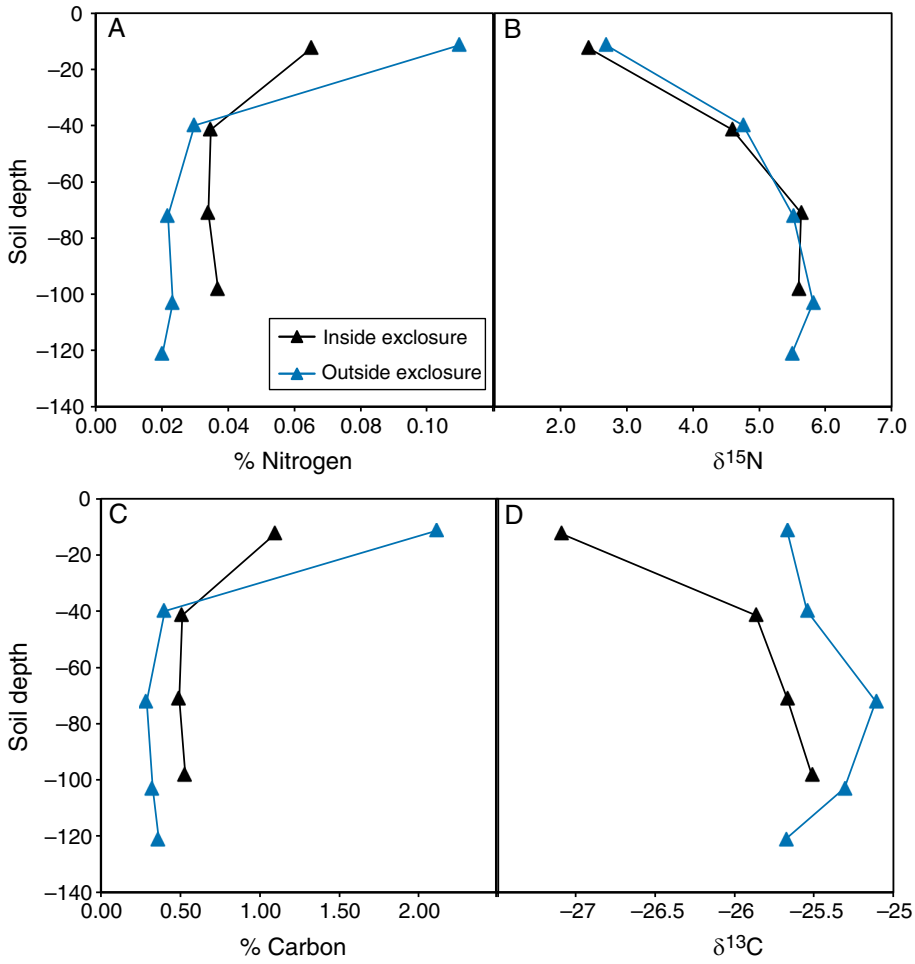


Fig. 4. Profiles of weight % N (A) and δ^{15}N values (B) of soil nitrogen, and weight % (C) and δ^{13}C values (D) of soil carbon, both outside (blue triangles) and inside (black triangles) of deer exclosures. Values are for depth ranges in Data S3 and reported for the midpoint depth of each range.

Given these observations, we consider three scenarios: Model I (Appendix S2), where unbrowsed *Ceanothus* changes its rooting depth within soils <60 cm deep; Model II (Appendix S3), where unbrowsed *Ceanothus* mines soils >60 cm deep; and Model III (Appendix S4), which is similar to Model II, but includes a small and constant proportion of fixed N in both browsed and unbrowsed plants.

We sampled these sandy soils in relatively long core segments. Model I considers the balance of soil N uptake from the upper two core segments (0–25 cm vs. 25–60 cm). For Models II and III, we treat the upper two core segments (<60 cm) as a single shallow pool and all segments >60 cm as a single deep pool. Plant available N may be more concentrated in layers closer to the surface, but a model that weights soil isotope values for [N] reduces the impact of not sampling soils at finer depth resolution.

The mass balance models require an estimate of N isotope fractionation during uptake by plants. A variety of soil N pools that are dynamic in concentration and isotopic composition can contribute to plant N (Högberg 1997, Dawson et al. 2002, Evans 2007). Yet at global and regional scales, there is a surprisingly strong relationship between the δ^{15}N values of plant and bulk soil N that results from climatic and other impacts on soil N dynamics (Amundson et al. 2003, Craine et al. 2009, 2015). The strength of these relationships suggests that bulk soil N is a reasonable

proxy for soil available N. As considered in greater detail in the appendices, for an ecosystem where the mean $\delta^{15}N$ value for non-fixing plants is ~ −0.8‰ (the average for *Arctostaphylos* and browsed *Ceanothus*), the expected difference in $\delta^{15}N$ value between plant and bulk soil N ($\Delta^{15}N$ = plant $\delta^{15}N$−soil $\delta^{15}N$) ranges from ~ −3.5‰ to −4.5‰ (Craine et al. 2009, 2015).

In Model I, with $\Delta^{15}N = -3.4‰$, browsed *Ceanothus* obtains 100% of its N from 0 to 25 cm and 0% from 25 to 60 cm. Under this model, to explain the increase in *Ceanothus* $\delta^{15}N$ values in exclosures, 70% of N in unbrowsed plants would need to come from 25 to 60 cm (Appendix S2). Assuming that the absolute amount of N from shallow roots does not change and that uptake efficiency is the same at different depths, total root volume would need to increase more than three times in unbrowsed plants to explain higher $\delta^{15}N$ values in unbrowsed *Ceanothus*, with all the new growth from 25 to 60 cm. If the $\Delta^{15}N$ value is lower (e.g., −4.1‰), the required increase in deeper roots would be even greater.

Under Model II, with $\Delta^{15}N = -4.1‰$, browsed *Ceanothus* obtains all of its N from 0 to 60 cm, whereas unbrowsed *Ceanothus* obtains 60% of its N from >60 cm (Appendix S3). With a fractionation of greater magnitude ($\Delta^{15}N = -4.5‰$), browsed *Ceanothus* would obtain 30% of its N from >60 cm, whereas 70% of unbrowsed *Ceanothus* N would come from this great depth. Again, assuming no change in shallow N uptake or uptake efficiency, total root volume would need to increase by 2–2.5 times in unbrowsed plants, with all new growth >60 cm deep.

Model III explores the interacting influences on *Ceanothus* $\delta^{15}N$ values of three variables: the proportion of fixed plant N (from 0.1 to 0.3), the $\delta^{15}N$ value of fixed N ($\delta^{15}N_F$, ranging from −2 to 2‰), and $\Delta^{15}N$ (−4.5‰, and a larger value of −5‰). Models with $\delta^{15}N_F = 0$ or 2 and $\Delta^{15}N = -4.5‰$ yield the most ecologically plausible results for browsed *Ceanothus*, with greater proportions of uptake <60 cm. Under all combinations that do not yield nonsensical results, a substantial increase in N uptake at depths >60 cm is required to explain the increase in unbrowsed *Ceanothus* $\delta^{15}N$ values, regardless of the proportion of N-fixation (Appendix S4). For example, with 20% fixation, $\delta^{15}N_F = 0‰$, and $\Delta^{15}N = -4.5‰$, browsed *Ceanothus* obtains 80% of its N from 0 to 60 cm and 0% from >60 cm, whereas unbrowsed *Ceanothus* obtains 30% of its N from 0 to 60 cm and 50% from >60 cm. If the absolute amount of shallow soil uptake is constant and total N uptake increases, then the total amount of both fixed N and deep soil N in plants must increase. In this example, total N uptake would increase by 2.7 times, with ~70% of the increase from deep soils and 30% from fixation.

Discussion

Ceanothus responded to suppression of deer browsing with (1) increased $\delta^{15}N$ values to around 0‰ that persisted for the duration of the study; (2) a very substantial increase in size (also increased reproduction and altered morphology [Deveny and Fox 2006; L. Fox, *unpublished data*]); and (3) increased $\delta^{13}C$ values highly correlated with size increase. Control *Ceanothus* showed none of these patterns. The wt % leaf N and $\delta^{15}N$ values of the non-fixing plants did not change during this study. *Arctostaphylos* sizes and $\delta^{13}C$ values were variable, but not related to treatment, whereas *Ericameria* cover area declined and $\delta^{13}C$ values increased. The cover area of both non-fixing species declined substantially in subsequent years; while neither were heavily browsed, they were out-competed by *Ceanothus* (especially *Ericameria* during the time frame considered in this paper) when deer browsing was eliminated (L. Fox, *unpublished manuscript*).

We interpret the changes in *Ceanothus* $\delta^{15}N$ and $\delta^{13}C$ values and size after the relaxation of browsing as reflecting (1) increased N-fixation and (2) increased water stress with substantial growth. Similar $\delta^{15}N$ values for *Arctostaphylos* and browsed *Ceanothus* suggest that the latter were not fixing N and were instead relying on the soil N pool. The rise in $\delta^{15}N$ value in unbrowsed *Ceanothus* to ~0‰ suggests they were fixing N_2 within only one year after browsing was removed. Such rapid response may be possible because apparently healthy *Frankia* nodules were already present on roots of heavily browsed *Ceanothus* (L. Fox, *personal observations*). Suppression of BNF with heavy browsing is consistent with physiological and biochemical observations that reduced photosynthesis, which may be caused by defoliation (among many other factors), reduces a plant's ability to maintain its

mutualism with N-fixing bacteria (Huss-Danell 1997, Vitousek et al. 2002).

Biological nitrogen fixation vs. alternative mechanisms

The hypothesis that browsing reduces BNF in *Ceanothus* explains all of our N isotope and growth data, but there are factors that complicate this interpretation. The initial *Ceanothus* (and *Arctostaphylos* and *Ericameria*) $\delta^{15}N$ values are within the range observed for N-fixers. As a consequence, the treatment effect associated with reduced browsing is small; *Ceanothus* $\delta^{15}N$ values are only $\sim -1‰$ before excluding deer, and values in unbrowsed *Ceanothus* increase to just 0‰. Nevertheless, $\delta^{15}N$ values of browsed *Ceanothus*, which are similar to the values observed in adjacent unambiguously, non-fixing plants, likely represent suppression of (or at least a substantial reduction in) N-fixation. The highly significant increase to just 0‰ (but no higher) when browsing pressure is relaxed is the expected response if BNF is occurring, with the atmosphere becoming the main source of N. Below, we evaluate several other mechanisms that may affect $\delta^{15}N$ values in plants. Several are falsified as explanations for our results, and unlike the hypothesis of reduced N-fixation under browsing, none can explain the totality of our data.

The first alternative is that if increased aboveground biomass in unbrowsed *Ceanothus* is accompanied by increased root growth, unbrowsed plants may access N from different soil depths than before, potentially altering $\delta^{15}N$ values. We cannot directly test this idea without massively disturbing *Ceanothus* shrubs, but our mass balance models suggest this hypothesis is unlikely. Whether or not *Ceanothus* fixed a small amount of N when browsed, it would need to increase the fraction of N obtained from deeper soils (>25 cm) by 40–90%, with an attendant increase in total N uptake of two to four times, mostly from deep soils. Our results assume that root uptake efficiency was the same at all depths, but given the lower [N] in deep soil layers, N uptake efficiency per unit length of root might be lower, so an even greater increase in deep root volume might be needed to supply such a large fraction of plant N. In addition, this dramatic change in root volume would need to occur in a single year, since the isotopic response to elimination of browsing occurred by 1997 (Fig. 2C). Such a rapid increase and redistribution in belowground biomass is highly unlikely, especially since above-ground plant areal cover increased more gradually in response to the cessation of browsing (Fig. 3C).

Two other alternative hypotheses relate to higher deposition of dung and urine with intense browsing, which might impact the soil N cycle and soil N loss. Dung deposition could lead to lower plant $\delta^{15}N$ values prior to deer exclusion (Frank and Evans 1997, Frank et al. 2004). This hypothesis predicts similar directional changes in $\delta^{15}N$ values for both N-fixing and non-fixing plants in exclosures, however, so it does not explain why $\delta^{15}N$ values in our exclosures change only in *Ceanothus*, not *Arctostaphylos* or *Ericameria*. Alternatively, deposition of dung and urine could have increased soil [N], which in turn suppressed BNF. However, despite intense deer browsing outside of exclosures, soil N concentrations are very low even near the surface (Fox et al. 1998) and mobile NO_3-N and NH_4-N, each typically <5 ppm in the top 5 cm, are well below concentrations that inhibit or even reduce BNF (Thomas and Berry 1989). Thus, this alternative is also highly unlikely.

The final alternatives, and the most difficult to reject, are that decreased browsing and increased production of photosynthate changed either N allocation within *Ceanothus*, the fractionation of N isotopes by *Frankia* symbionts, or the fractionation of N during uptake by *Ceanothus*, in all cases without changing the rate of N-fixation. Differences in N allocation in deciduous, non-fixing plants associated with seasonal loss of leaves do affect $\delta^{15}N$ variability (Kolb and Evans 2002), and it is possible that fractionation by microbial strains might vary with environmental conditions. The major problem with these alternative explanations is that none explain why foliar values rapidly stabilize at 0‰ and remain at that value for more than six years as plants continued to add biomass. We did not conduct alternative tests of changes in rates of N-fixation between unbrowsed and browsed *Ceanothus* because they are destructive (e.g., cutting roots to assess short-term N-fixation by acetylene reduction) or would dramatically disturb our experimental system (e.g., isotope dilution using labeled substrates, as in Busse 2000), which we have continued to use for a variety of other studies (e.g., Pittermann

et al. 2014), and because none of the alternative hypotheses adequately explain our natural abundance N isotope results.

Biological nitrogen fixation in ecological systems

Biological nitrogen fixation from *Ceanothus* spp. can be an important source of ecosystem N, with some field estimates of N-fixation rates that are similar to agricultural N inputs (e.g., >100 $kg{\cdot}ha^{-1}{\cdot}yr^{-1}$ for the *Frankia–Ceanothus velutinus* symbiosis; Hibbs and Cromach 1990, Paschke 1997). In other cases, soil N inputs from BNF are much lower (e.g., <1 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$ for *Ceanothus integerrimus*; Hibbs and Cromach 1990), while several *Ceanothus* spp. have BNF activity only in young plants or not at all (Kummerow et al. 1978, Ellis and Kummerow 1989). Nodulation may also be spatially variable within a site (Busse 2000, He et al. 2006, Busse et al. 2007, Markham 2008). It is relevant to our conclusions that despite its low rates of BNF, *C. integerrimus* is an important food of mammalian browsers in Pacific Northwest forests, where its common name is "Deerbrush" (Hibbs and Cromach 1990). Low BNF rates, including those for *C. integerrimus*, have been attributed solely to abiotic limitation (Rojas et al. 2002, Raddad et al. 2005, Khadka and Tatsumi 2006), but our results suggest that biotic effects may also be important.

Direct effects of herbivory on BNF rate in some terrestrial systems are consistent with several observations. Greenhouse defoliation of alder saplings and other N-fixing plants reduced BNF by symbiotic bacteria (Ruess et al. 2006), while field BNF rates in legume crops, measured by the invasive acetylene reduction method, declined with increased insect herbivory (Layton and Boethel 1987, Dale 1988, Hansen et al. 2002). Together with these studies, our results suggest that herbivore suppression of BNF may be very relevant to a range of terrestrial ecosystems and that BNF limitation by herbivores should be evaluated as an independent limiting factor.

Plant growth and water stress

Without deer browsing, *Ceanothus* plants grew larger and $\delta^{13}C$ values increased, despite the effects of other herbivores. Neither $\delta^{13}C$ values nor plant size increased in *Ceanothus* at natural browsing levels or in the other plant species. Higher $\delta^{13}C$ values signal increased stomatal resistance (i.e., greater water-use efficiency, WUE) and/or higher carboxylation (i.e., C fixation and growth) rates. Carbon and nitrogen isotope values are known to be correlated, possibly reflecting the impact of nutrient conditions on growth rate and water-use efficiency (Handley and Raven 1992, Heaton 1999, Dawson et al. 2002, Swap et al. 2004). Only a few studies demonstrate the effects of herbivores on WUE using $\delta^{13}C$ values (Dawson et al. 2002), where browsing reduces WUE and plants are less water stressed.

Increased water stress in unbrowsed plants was reflected in lower stomatal conductance but similar water potentials to that in browsed shrubs in spring 2011, following a year of slightly above-average rain; but after two years of drought (autumn 2013), water potentials were two to three times lower than in spring 2011, showing extreme stress (−8 to −10 MPa) in most unbrowsed *Ceanothus* and less stress (all >−7 MPa) in browsed plants (Pittermann et al. 2014). By the third year of the drought, several unbrowsed *Ceanothus*, but no browsed plants, had either lost a significant amount of leaf tissue or died (L. Fox, *personal observations*).

Implications of reduced browsing

Our study has several clear implications for plant–plant and plant–animal interactions. First, the $\delta^{15}N$ values of unbrowsed *Ceanothus* that were attacked by woodrats and stem-boring insects between 1999 and 2003 remained near 0‰, suggesting that either the damage was not severe enough to affect BNF (or potentially other physiological traits) or that without deer browsing larger plants could compensate for the loss of a (sometimes) significant proportion of their leaves and branches.

Second, the dramatic increase in the size of unbrowsed *Ceanothus* presumably led to a much greater contribution by these plants to leaf litter and soil organic matter. Yet even after a decade, this organic matter had not yet impacted the soil N pool being taken up by *Arctostaphylos* or *Ericameria*, despite the fact that all these plants are closely associated within treatment plots (i.e., the $\delta^{15}N$ values of *Arctostaphylos* and *Ericameria* did not converge on those for *Ceanothus*). However, effects of N-fixation on soils may be quite variable (Power et al. 2003, Oakley et al. 2004, Treydte et al. 2007).

Conclusion

Many abiotic explanations have been invoked to understand changes in plant $\delta^{15}N$ and $\delta^{13}C$ values and to predict ecosystem responses to climate change (Dawson et al. 2002, Vitousek et al. 2002, Hungate et al. 2004). The magnitudes of isotopic responses of *Ceanothus* to herbivory in our study are similar to those attributed to abiotic effects among and within species (Garten and Taylor 1992, Diels et al. 2001, Warren et al. 2001, Busse et al. 2007), yet such biotic factors are usually overlooked. We conclude that physiological-, community-, and ecosystem-level impacts of biotic interactions—as well as abiotic factors—need to be considered and addressed, not just to understand intrinsic ecological processes, but also to predict ecological responses to climatic and other environmental changes.

Acknowledgments

The University of California Natural Reserve System and the UC Santa Cruz Natural Reserves staff, M. Fusari and S. McStay, made the Fort Ord Natural Reserve available and provided support. The work was funded by the UC Santa Cruz Monterey Bay Regional Studies program and UC Santa Cruz COR grants to LRF and PLK. Two anonymous reviewers, Norma Fowler, Ingrid Parker, Donald Potts, and Sarah Swope reviewed and improved the manuscript; and Robert Clark, Justin Yeakel, Andrew Marcus, Brooke Crowley, Sora Kim, Katie Snell, and Patrick Wheatley provided technical assistance. Stephen Potts provided statistical support. We thank Stephania Mambelli at the Center for Stable Isotope Biogeochemistry (UC Berkeley) for overseeing analyses of plant stable isotope values.

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