Abiotic, Biotic, and Evolutionary Control of the Distribution of C and N Isotopes in Food Webs

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abstract: Ecosystem functioning depends on nutrient cycles and their responses to abiotic and biotic determinants, with the influence of evolutionary legacies being generally overlooked in ecosystem ecology. Along a broad elevation gradient characterized by shifting climatic and grazing environments, we addressed clines of plant N and C : N content and of $\delta^{13}C$ and $\delta^{15}N$ in producers (herbs) and in primary (grasshoppers) and secondary (birds) consumers, both within and between species in phylogenetically controlled scenarios. We found parallel and significant intra- and interspecific trends of isotopic variation with elevation in the three groups. In primary producers, nutrient and isotope distributions had a detectable phylogenetic signal that constrained their variation along the environmental gradient. The influence of the environment could not be ascribed to any single factor, and both grazing and climate had an effect on leaf stoichiometry and, thus, on the resources available to consumers. Trends in consumers matched those in plants but often became nonsignificant after controlling for isotopic values of their direct resources, revealing direct bottom-up control and little phylogenetic dependence. By integrating ecosystem and mechanistic perspectives, we found that nutrient dynamics in food webs are governed at the base by the complex interaction between local determinants and evolutionary factors.

Keywords: biogeochemical fluxes, elevation gradients, grazing, nitrogen cycle, trophic relationships, evolutionary legacy.

Introduction

Almost a century of ecological and biogeochemical research has contributed to our understanding of energy and nutrient movements within the biological sphere and between the biological sphere and environmental reservoirs (Chapin et al. 2011). These studies have shown that the distribution of carbon (C) and key nutrients (such as nitrogen [N]) in the ecosystem is uneven in both space and time, following the variability of abiotic and biological factors that govern resource production and consumption (Vitousek 1982). Evolutionary history and idiosyncrasies in local communities are additional inherent sources of variability, although ecosystem studies often overlook micro- and macroevolutionary aspects or variation within species. However, historical evolutionary explanations for nutrient cycles do not appear to be incompatible with environmental determinism because the tolerance to local physical/ecological conditions is an evolved property of populations and clades (Ricklefs 2004). Importantly, clades and their intrinsic functional roles are not distributed randomly along environmental gradients (Srivastava et al. 2012). Thus, focusing on present drivers without considering evolutionary history may limit the mechanistic interpretation of ecosystem responses.

One way to disentangle biotic, abiotic, and evolutionary heterogeneities in ecosystem studies is to extend investigations spatially, across broad biotic and abiotic gradients; functionally, across trophic levels; and evolutionarily, across taxonomic levels. Macroecological studies increasingly rely on such integrative approaches to disentangle large-scale ecological patterns, such as those involving biodiversity (Flynn et al. 2011). This approach, however, may be difficult to apply to the pathways of nutrient and energy exchanges in food webs, whose multiplicity may hinder any traceable effect of contemporary and historical factors. The natural abundances of heavy C and N isotopes (^{13}C and ^{15}N) in organisms constitute a synthetic indicator of large-scale patterns of C and N cycles and stoichiometric linkages between organisms (Peterson et al. 1986; Peterson and Fry 1987). They enable the quantification of nutrient fluxes and trace environmental influence because of the isotopic fractionation in underlying chemical and physical reactions, that is, products become depleted in heavier isotopes with respect to reagents or substrates because the lighter isotope species react more rapidly and are more weakly bonded than the heavier ones (Fry 2006). The natural abundances of carbon and nitrogen isotopes are sen-

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sitive to changes and anomalies in nutrient cycles following abiotic and biotic forces. In particular, climate is the factor most frequently associated with geographic (latitudinal, elevational) variation of isotopic content in terrestrial organisms (Körner et al. 1991; Hobson 1999; Luo et al. 2009), especially with respect to water availability and temperature, which control the microbial transformation of nitrogen and the fixation of carbon dioxide by plant photosynthetic activity (Ehleringer and Dawson 1992; Lipson et al. 1999; Huber et al. 2007). Biotic components speed nutrient cycling rates and condition the stable isotope ratios of both producers (e.g., grazing, symbiotic associations) and consumers (trophic relationships; Frank et al. 2000; Post 2002). Although less frequently addressed, evolutionary legacies may condition isotope dynamics by constraining the variation of functional traits linked to their dynamics, such as body mass (Jennings et al. 2001) and nitrogen fixation (Flynn et al. 2011).

While autotrophs are highly variable in elemental stoichiometry and natural isotope composition, with both aspects permitting the tracing of environmental influence, heterotrophs exhibit a more homeostatic elemental composition, and gain and loss of nutrients have to be inferred by the abundances of their stable isotopes (Fry 2006; Elser et al. 2010). Owing to their significance in carbon and nitrogen cycling, primary producers play an important role in the isotopic fractionation of these elements in the biosphere. For instance, in plants the amount of autotrophic carbon fractionation is primarily controlled by the photosynthetic pathway (i.e., C_3 vs. C_4 plants), which governs the balance between the fixation and diffusion of carbon dioxide (Farquhar et al. 1989). In animals, the abundance of stable carbon isotopes depends on diet and metabolic rate (Focken and Becker 1998), but the stepwise enrichment is often limited, at least in terrestrial systems (Kelly et al. 2000). The amount of autotrophic nitrogen fractionation is less predictable, and plants assimilate both isotopically enriched substrates and depleted products derived from the progressive fractionation of nitrogen isotopes in soil (Högberg 1997). Moving up the food chain, however, the nitrogen isotope ratio becomes highly informative of trophic relationships among consumers since animals excrete ^{15}N-depleted urine with respect to the nitrogen assimilated in tissues and excreted in feces, and a steady ^{15}N enrichment relative to diet is observed at each step of the food chain (Cabana and Rasmussen 1996).

Mountains represent an ideal heterogeneous system to test for the influence of abiotic and biotic factors on isotope dynamics since they are characterized by sharp physical and ecological transitions over short geographic distances. Elevation gradients integrate topographic and climatic variability in a general pattern of progressive intensification of abiotic stress with elevation (Körner 2003).

Organisms exposed to these environmental gradients have had time to develop adaptations and differentiate taxonomically, presenting evidence of their evolutionary history (Laiolo et al. 2013). Mountain plant communities are exposed to sharp gradients in soil fertility, and alpine plants cope by reducing their size and enhancing mineral nutrient concentration, especially that of nitrogen (reviewed in Körner 2003). This enhanced nutrient concentration is not exhibited by all alpine species and life forms (Raich et al. 1997; Huber et al. 2007), and species composition changes significantly with elevation (Körner 2003); thus, it is unclear whether this pattern occurs community-wide. Furthermore, the effects of these changes on higher trophic levels and the role played by evolutionary constraints have yet to be tested. These questions must be answered to understand nutrient cycling in a model terrestrial ecosystem and more generally to advance the biogeographical gradient theory with integrative community-level evidence of mechanisms.

In this study, we analyze the spatial variation in the natural abundance of carbon and nitrogen isotopes in grassland plants (legumes, grasses, and sedges), primary consumers (grasshoppers), and secondary consumers (birds). We aim to identify the determinants of stable isotope distribution along a 1,500-m elevation gradient characterized by shifting topoclimatic and grazing conditions (i.e., the abiotic and biotic factors that primarily determine soil nutrient availability in grasslands; Frank and Groffman 1998) within a food web that includes producers with diverse modalities of nitrogen uptake or assimilation and consumers with diverging thermal physiology (ecto- and endotherm). First, we examine broad-scale elevation clines in stable isotope ratios for the three trophic levels, focusing on variation within species and among species in phylogenetically controlled scenarios. Then, we address the potential ecological processes underlying elevation clines by analyzing the link with C and N plant stoichiometry and its deviation along grazing and climate gradients. This permits us to (1) outline the impact of abiotic and biotic forces on plant nutrient pools and, hence, nutrient sources for higher trophic levels; (2) assess whether patterns generated at different evolutionary timescales (intra- and interspecific) bear similarities with respect to their environmental determinants; and (3) test the hypothesis that groups at the base of the trophic chain display greater evolutionary dependence on their isotopic ratios than their consumers. The isotopic composition of producers is in fact expected to be strongly dependent on species physiology (influenced by phylogenetic origin), abiotic control, and the impact of consumers (Nadelhoffer et al. 1996; Frank et al. 2000), while that of consumers should largely match or magnify changes in their resources (the stoi-

chiometric paradigm "You are what you eat plus a few per mil"; DeNiro and Epstein 1976; Post 2002).

Material and Methods

Study Area and Community

We conducted this study in mountain grasslands at 870–2,450 m asl in five massifs of the Cantabrian Mountains, northern Spain (fig. A1; figs. A1, A2 are available online). Owing to historical forest clearings, the treeline in this mountain chain is currently as low as 1,600 m, and pseudoalpine grasslands and shrublands cover almost 80% of the landscape in the montane belt (García et al. 2005). The climate ranges from alpine in the highlands, through humid Atlantic on the northern slopes, to oro-Mediterranean in the south. Study grasslands mainly grow on calcareous substrata with interspersed dolomite outcrops and sparse gorse (*Genista*, *Ulex* spp.) and heath (*Erica*, *Calluna* spp.). They are often grazed by free-ranging domestic livestock from spring to autumn and by wild ungulates when not covered by snow (mainly *Rupicapra pyrenaica* and in one massif by the introduced *Dama dama*).

We focused on plant species that dominate the local grassland community; one of their consumers, acridid grasshoppers; and passerine birds that are insectivorous during their breeding (table A1; tables A1–A12 are available online). The study plants include legumes, sedges, and grasses, all of which are C_3 plants. These are consumed by grasshoppers, although in different proportions depending on the species (Kaufmann 1965; Isern-Vallverdú et al. 1995; Unsicker et al. 2008). Grasshoppers are the most abundant arthropods in mountain grasslands and form part of the insectivorous diet of many alpine birds in the summer (Brodmann et al. 1997; Laiolo and Rolando 1999).

Sampling was performed at 29 sites across a gradient in mean annual temperature of 6°C and a gradient in accumulated precipitation of 600 mm. Plants were collected at 22 of these sites (elevation range: 900–2,450 m asl), grasshoppers at 23 (900–2,385), and birds at 12 (870–2,200; table A2). At plant sampling sites, the annual accumulated precipitation does not vary (linear regression: $t_{21} = 0.45$, $P = .65$), but the annual average temperature ($t_{21} = 21.9$, $P < .001$) and the occurrence of domestic livestock grazing (generalized linear model with a binomial distribution: $z_{21} = 2.15$, $P = .03$) steeply decline with elevation.

Field Sampling

Leaves were collected from individual plants of the dominant species (i.e., those covering >50% of area) or set of species (i.e., several plants covering >20% of area) in areas of ~1 ha that were sampled individually in 2011 or 2012 (May–November). At each sampling site, we collected one leaf per individual tussock from 7–9 randomly located tussocks per species. Leaves were collected in grazed and ungrazed plots, the former corresponding to grasslands regularly grazed by domestic livestock during three to five summer months (plus wild ungulates), the latter to grassland plots protected year-round by enclosures or only occasionally grazed by wild ungulates in low numbers. Only leaves from fully sunlit plants with no sign of wilting were collected, stored in labeled plastic bags, and refrigerated upon return to the laboratory. Adult grasshoppers were collected by hand or by means of sweep nets according to their abundances in areas of ~1 ha per site during August and November 2011–2013 (in keeping with Laiolo et al. 2013). Individuals were collected alive and stored in 50-mL centrifuge tubes and frozen upon return to the laboratory. Hind femurs were separated for stable isotope analysis (in keeping with Fry et al. 1978). Open habitat passerines were captured and ringed by means of mist nets and clap nets positioned at 12 ringing stations (each 16–40 ha) from May to July 2009–2011. Blood samples (30–50 μL) were collected from the jugular vein with an insulin syringe, diluted in 1,000 μL of 98% ethanol in a screwcap microfuge tube, and then transferred and stored in the laboratory at room temperature. The sex of adult birds of monomorphic species was determined using the molecular methods described in Griffiths et al. (1998).

In principle, the stable isotope approach can be applied to study local nutrient fluxes when organisms acquire their stable isotope values in situ. In the case of grasshoppers, we made the assumption that their tissues tracked natural variations in stable isotopes within capture sites because even macropterous species disperse only over very short distances (Laiolo et al. 2013). In the case of birds, we focused on their whole blood, which integrates the isotopic composition of resources consumed over short time periods—thus, likely near the time they were caught (see Hobson and Bairlein 2003). Moreover, birds were sampled during breeding, when they limit movements to the territory or nest area (Heer 1996; Laiolo and Rolando 2001). As evidence, almost all adult females caught showed active brood patches, and the males of many species were lured by playbacks of homospecific songs; therefore, they exhibited typical breeding and territorial behaviors. Under these premises, we are confident that consumers' tissues also mirrored local nutritional and isotopic conditions.

Overall, we obtained data on 324 individuals of 15 plant species, 554 individuals of 13 grasshopper species, and 647 individuals of 15 bird species, totaling 1,525 individuals of 43 species (table A1).

Analyses of δ¹³C and δ¹⁵N in Plants, Grasshoppers, and Birds and of C and N Content in Plants

Analyses of $\delta^{13}C$ and $\delta^{15}N$ in Plants, Grasshoppers, and Birds and of C and N Content in Plants

Blood samples, hind legs, and leaves were oven-dried at 50°C for 48–72 h, and each was then finely ground. No preliminary extraction of lipids was performed since the study tissues incorporate low proportions of these compounds (Fry et al. 1978; Bearhop et al. 2002; Post et al. 2007). After grinding, samples were weighed to the nearest microgram, and 1 mg of animal and 2 mg of plant tissue powder were then separated, packed in 6 × 4-mm capsules, and dispatched to the University of California, Davis, Stable Isotope Facility for analyses. A continuous-flow isotope ratio mass spectrometer measured $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, which were reported in per mil units using the standard delta notation $\delta\text{\textperthousand} = [(R_{sample} - R_{standard})/R_{standard}] \times 1,000$, where R_{sample} is the ratio between the heavier and lighter isotope in the sample and $R_{standard}$ is the same ratio calculated in international standards of Vienna Pee Dee belemnite limestone for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$. The analytical errors (SD) based on repeated analyses of 122 individual samples were on average $\pm 0.09\text{\textperthousand}$ for $\delta^{13}C$ and $\pm 0.2\text{\textperthousand}$ for $\delta^{15}N$, and correlation coefficients between repeated measurements were $r = 0.96$ for $\delta^{13}C$ and $R = 0.97$ for $\delta^{15}N$ (all $P < .001$). From leaf samples, we also analyzed the relative abundances of carbon and nitrogen to derive a rough measure of nutrient content (%N and C : N ratio). Raw data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.g4p92 (Laiolo et al. 2014).

Molecular Procedures and Phylogenetic Relationships

To test whether closely related species exhibited similar environment-isotope relationships, interspecific analyses controlling for their phylogenetic relationships were performed. Gene trees were constructed from sequences of a region of the mitochondrial cytochrome oxidase subunit I (COI) for birds and grasshoppers and from the chloroplast DNA of the megakaryocyte-associated tyrosine kinase gene (*MatK*) for plants. Sequences were downloaded from GenBank (table A3) except for *Carex asturica*, *Carex brevicollis*, *Carex caudata*, *Carex sempervirens*, *Helictotrichon sedenense*, *Chorthippus jacobsi*, *Oedipoda fuscocinta*, *Gomphocerus sibiricus*, *Calliptamus barbarus*, and *Prunella collaris*, which were newly determined for this study (GenBank codes are provided in table A3). Details on DNA extraction methods and how phylogenetic relationships were inferred are provided in the appendix (available online).

Interspecific Variation of Isotopic Values along the Elevation Gradient

We tested whether species isotope values changed with elevation within plants, grasshoppers, and birds, analyzing the relationships between the average $\delta^{13}C$ and $\delta^{15}N$ of species against the average elevation at which they were found by means of generalized least squares (GLS) regressions and by phylogenetic GLS (PGLS) regressions controlling for the phylogeny of the group. Two alternative mathematical expressions of divergence in isotopic ratios were considered in PGLS regressions: a neutral model (Brownian motion model [BM]), in which variation between species is expected to grow proportionally to the time since they shared a common ancestor because of randomly fluctuating natural selection or genetic drift, and a deterministic model, which assumed that selection may have influenced changes toward one or more selective optima (Ornstein-Uhlenbeck model [OU]; Butler and King 2004). We first compared the fit of GLS versus PGLS regressions under BM or OU by means of log-likelihood tests; we then estimated the magnitude of the phylogenetic signal (λ) by means of maximum likelihood in PGLS regressions to account for phylogenetic independence ($\lambda = 0$), pure drift as predicted by BM ($\lambda = 1$), and deviations from both occurrences ($0 < \lambda < 1$). We assumed that phylogeny was not conditioning elevation clines when the λ value did not differ significantly from 0.

To highlight whether grasshopper and bird elevational trends tracked the isotope composition of local resources or in fact depended on dietary shifts occurring along the gradient, we ran models analogous to those detailed above but entered data for $\delta^{13}C$ and $\delta^{15}N$ corrected for fractionation rather than their raw values (Post 2002). Values of $\delta^{13}C$ and $\delta^{15}N$ of plants (producers) were used to obtain values of the C and N sources at the base of the grasshopper food web. Although plants represent the direct food of grasshoppers, the use of autotrophs as basal resources in stable isotope research has been questioned, especially in aquatic systems, where autotroph assemblages (phytoplankton) undergo marked seasonal oscillations (Post 2002). In this study, however, we collected grasshoppers and the plant tussocks that hosted them at the same time or, in a few cases, plant preceded grasshopper sampling by only a few weeks; thus, comparable fluctuations are excluded (for the use of perennial grasses as baselines, also see Peterson et al. 1986). Grasshopper $\delta^{13}C$ and $\delta^{15}N$ values were therefore corrected by subtracting the average values of plant individuals collected at each grasshopper sampling site (on the assumption that both were collected according to their natural abundances; Laiolo et al. 2013). We collected between 8 and 48 individual plant leaves per grasshopper site. To obtain

Table 1: Performances of regressions testing for interspecific elevation clines of $\delta^{13}C$ and $\delta^{15}N$ in 15 species of plants, 13 species of grasshoppers, and 15 species of birds

	AIC			
Variable	GLS regression	PGLS regression (BM)	PGLS regression (OU)	λ (*P*)
Plant $\delta^{13}C$	58.5	58.7	54.8*	.960 (.035)
Plant $\delta^{15}N$	69.7	65.6*	64.9*	.946 (.003)
Grasshopper $\delta^{13}C$	35.4*	48.7	38.4	.000 (1.000)
Grasshopper $\delta^{15}N$	41.2	40.9	42.7	.693 (.204)
Bird $\delta^{13}C$	26.4	27.6	27.1	.944 (.069)
Bird $\delta^{15}N$	60.4	62.1	62.5	.000 (1.000)

Note: Generalized least squares (GLS) regressions do not incorporate phylogeny; phylogenetic GLS (PGLS) regressions incorporate it under assumptions of either a Brownian motion model (BM) or an Ornstein-Uhlenbeck model (OU). For each test the Akaike information criterion (AIC) is given, and asterisks indicate models that better describe variation according to log-likelihood ratio tests; in the case of grasshopper $\delta^{15}N$, bird $\delta^{13}C$, and bird $\delta^{15}N$, no significant differences emerged between models. We tested for λ departure from 0; *P* values are provided in parentheses.

* $P < .05$.

values of the C and N sources at the base of the bird food web, we adopted the common practice of using $\delta^{13}C$ and $\delta^{15}N$ values of primary consumers (here grasshoppers) to parse the potential contribution of direct food sources in each local population (Vander Zanden and Rasmussen 1999; Matthews and Mazumder 2003; Quevedo et al. 2009). The same procedure described above was followed to obtain bird corrected $\delta^{13}C$ and $\delta^{15}N$ values, with between 8 and 63 grasshoppers per bird site used as a source baseline.

Intraspecific Variation of Isotopic Values along the Elevation Gradient

We studied intraspecific isotopic shifts along elevation in 16 species that were collected in at least 20 samples across an elevation distribution of >300 m to embrace a substantial portion of the gradient (table A1). We ran generalized linear mixed models (GLMMs), where $\delta^{13}C$ and $\delta^{15}N$ values of individuals were dependent variables and elevation was entered as a continuous factor. In addition, year of sampling was entered as a continuous covariate to control for temporal variation, and age and sex were entered as fixed factors in the case of birds and grasshoppers (yearling vs. adult female vs. adult male for birds; adult female vs. adult male for grasshoppers) to control for age- or sex-dependent variation. The identity of the massif where the sample sites were located was entered as a random factor to account for data pseudoreplication and unequal sampling among massifs. Model explanatory power was determined by conditional R^2 in keeping with Nakagawa and Schielzeth (2012). In the case of grasshoppers and birds, we ran models with raw data and with corrected data for isotopic baselines.

Associations with Plant Nutrients, Climate, and Grazing

To test whether plant isotope values traced their nutritional features and whether this relationship was conditioned by their evolutionary history, we analyzed the relationships between plant $\delta^{13}C$ and $\delta^{15}N$ and their %N and C : N ratios by means of PGLS regressions, estimating λ by means of maximum likelihood to account for deviations from both phylogenetic independence and pure drift models as described above. To explore the potential for climate-driven variation, we obtained climate characteristics of plant sampling sites from the digital layers of the Climate Atlas of the Iberian Peninsula, which were built with a resolution of 200 m by modeling 15 years of meteorological data from the local stations of the Spanish National Meteorological Institute (Ninyerola 2005). At each plant sampling site, we estimated accumulated precipitations and mean monthly temperatures from May through September to capture variability in the most influential climate variables for grassland vegetation during the growing season (He et al. 2008); these variables were reduced and summarized by a principal component analysis to two uncorrelated factors, the first (PC1) increasing with temperature and the second (PC2) increasing with precipitation (table A4). To approximate the grazing pressure on the study plant species, for each individual collected we assigned the category of "grazed plot" (leaves collected in pastures grazed by domestic livestock from spring to autumn) or "ungrazed plot" (leaves collected in enclosures or on slopes only occasionally grazed by a few wild ungulates). Both climate and grazing characteristics of sampling plots were therefore associated pairwise with the foliar chemistry of plants collected therein, in a data set presenting individual $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios, the PC1 and PC2 values corresponding to the site of collection, and the presence of grazing (grazed vs. ungrazed plot).
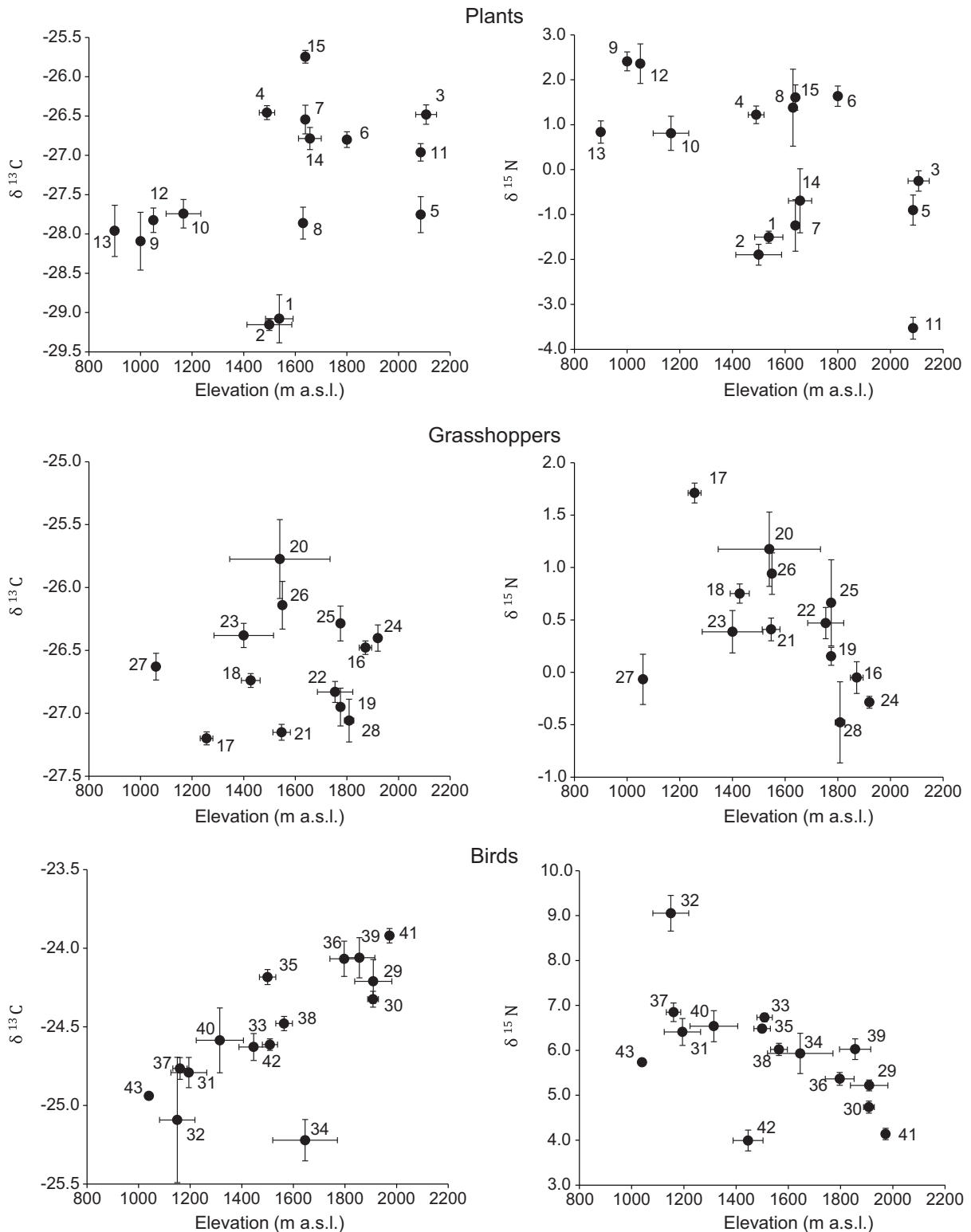
Figure 1: Elevation clines of $\delta^{13}C$ (*left*) and $\delta^{15}N$ (*right*) in 43 species from the Cantabrian Mountain grasslands. The average elevations where the species were found and their paired isotopic values are shown, together with SEs. Bird and grasshopper $\delta^{13}C$ and $\delta^{15}N$ were uncorrected for baseline values (see the appendix, available online, for corrected values). Phylogenetic effects are not controlled for in these

This data set was used to establish average values per species of $\delta^{13}C$, $\delta^{15}N$, %N, C : N ratios, PC1, PC2, and grazing intensity (percentage of samples collected in grazed plots).

We tested whether average species $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios responded to grazing intensity, PC1, and PC2 of climate variables while controlling for phylogeny with PGLS regressions. Since climate (chiefly temperature) and grazing covary along our elevation gradient, we performed two further tests to shed light on their independent contributions. First, we analyzed the response of average plant $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios to grazing in a restricted altitudinal band (1,465–1,640 m), where variation due to climate was minimal (0.9°C and 5 mm differences in growing season temperature and precipitation, respectively). The response to PC1 and PC2 of climate was instead tested along the whole elevational gradient using data from ungrazed plots only. Both analyses were performed while controlling for the effect of phylogeny in PGLS regressions, entering 7 and 13 species, respectively.

A Gaussian distribution of errors was used in all GLMMs, and type III models were run, testing each effect after controlling for the other determinants. All statistical analyses were performed using R (ver. 3.1.0), with the caper (Orme et al. 2013), APE (Paradis et al. 2004), lme4 (Bates 2013), and MuMIn (Barton 2011) packages.

Results

Elevation Clines in $\delta^{13}C$ and $\delta^{15}N$

In plants, interspecific elevation clines of foliar $\delta^{13}C$ and $\delta^{15}N$ were significantly affected by their evolutionary history ($\lambda \geq 0.95$), and models incorporating phylogeny better fit the data than did GLS regressions (table 1). In the case of animal groups, the magnitude of the phylogenetic signal did not differ significantly from 0 (and differed only marginally for bird $\delta^{13}C$; table 1), and the incorporation of phylogeny did not improve model fit.

When controlling for species phylogeny, there was a significant interspecific tendency of increasing foliar $\delta^{13}C$ and decreasing $\delta^{15}N$ with elevation in plants (fig. 1; table A5). High-elevation species were significantly enriched in ^{13}C and depleted in ^{15}N in birds, while only $\delta^{15}N$ decreased

with elevation in grasshoppers (fig. 1; table A5). However, these marked elevation trends disappeared when correcting for diet baseline values in both birds and grasshoppers (fig. A2). The incorporation of quadratic terms did not produce significant tendencies in any of the above-described relationships.

Changes in the same direction as interspecific clines were found in almost two-thirds of the 16 species in which we explored intraspecific variation, and just one species displayed opposite variation in one of the stable isotopes (tables 2, A6–A8). In the remaining species no significant tendency was found, although two showed ^{13}C enrichment or ^{15}N depletion with elevation at lower significance levels (*Trifolium pratense* and *Monticola saxatilis*; tables A6, A8), and another three showed significant and similar patterns below and above the timberline (1,600 m) for elevation as a categorical variable ($\delta^{15}N$ was higher below the timberline in *Chorthippus cazurroi* [$t = 2.40$, $P = .018$], *Omocestus kaestneri* [$t = 2.72$, $P = .008$], and *Carduelis cannabina* [$t = 2.61$, $P = .012$], and $\delta^{13}C$ was higher above the timberline in *Carduelis cannabina* [$t = 3.10$, $P = .004$]).

In grasshoppers, we found that males tended to be enriched in ^{15}N with respect to females (up to 1.65‰; table A7), and when variation in basal plant resources was accounted for, the highland populations of four species were shown to be ^{15}N enriched, in contrast to the negative trends observed with raw data (tables 2, A9). Most trends became nonsignificant when subtracting basal resource values in birds, with the exception of *Oenanthe oenanthe*, *Saxicola torquata*, and *Prunella collaris* (table A9).

Associations with Plant Nutrients, Climate, and Grazing

The abundance of stable isotopes was tied to nutrient content in plants: species with higher %N and lower C : N ratios were significantly ^{13}C depleted and ^{15}N enriched (table A10). Foliar nutrients tended to decline in species dwelling at high elevations irrespective of species evolutionary history (%N: estimate ± SE = -0.0011 ± 0.0004, $R^2 = 0.29$, $t = 2.61$, $P = .021$, $\lambda = 0.76$, $P = .29$; C : N ratio: estimate ± SE = 0.013 ± 0.004, $R^2 = 0.35$, $t = 2.92$, $P = .011$, $\lambda = 0.0$, $P = 1.0$; fig. A3). Both grazing intensity

graphs. Numbering denotes species as follows: 1 = *Trifolium repens*, 2 = *Trifolium pratense*, 3 = *Carex sempervirens*, 4 = *Carex brevicollis*, 5 = *Carex caryophyllea*, 6 = *Carex asturica*, 7 = *Carex caudata*, 8 = *Poa alpina*, 9 = *Poa pratensis*, 10 = *Festuca rubra*, 11 = *Festuca paniculata*, 12 = *Dactylis glomerata*, 13 = *Brachypodium pinnatum*, 14 = *Bromus erectus*, 15 = *Helictrotrichon sedenense*, 16 = *Chorthippus cazurroi*, 17 = *Chorthippus parallelus*, 18 = *Chorthippus yersini*, 19 = *Chorthippus jacobsi*, 20 = *Chorthippus binotatus*, 21 = *Omocestus kaestneri*, 22 = *Stenobothrus stigmaticus*, 23 = *Stenobothrus nigromaculatus*, 24 = *Gomphocerus sibiricus*, 25 = *Myrmeleotettix maculatus*, 26 = *Oedipoda fuscocinta (caerulea)*, 27 = *Calliptamus barbarus*, 28 = *Podisma carpetana*, 29 = *Alauda arvensis*, 30 = *Prunella collaris*, 31 = *Prunella modularis*, 32 = *Motacilla alba*, 33 = *Anthus spinoletta*, 34 = *Anthus trivialis*, 35 = *Oenanthe oenanthe*, 36 = *Monticola saxatilis*, 37 = *Saxicola torquata*, 38 = *Phoenicurus ochruros*, 39 = *Pyrrhocorax graculus*, 40 = *Pyrrhocorax pyrrhocorax*, 41 = *Montifringilla nivalis*, 42 = *Carduelis cannabina*, 43 = *Emberiza cia*.

Table 2: Summary of results of generalized linear mixed models testing for the effect of elevation on raw $\delta^{13}C$ and $\delta^{15}N$ and on $\delta^{13}C$ and $\delta^{15}N$ corrected for the $\delta^{13}C$ and $\delta^{15}N$ of baseline resources (both grasshoppers and birds)

| | Relationships with elevation | | | | Sample size (no. individuals) |
| | Raw | | Corrected | | |
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	
Plants:					
Carex brevicollis	+	−			88
Carex sempervirens	+	NS			56
Trifolium pratense	NS	−			24
Grasshoppers:					
Chorthippus cazurroi	+	NS	−	NS	106
Chorthippus yersini	+	−	NS	+	138
Chorthippus parallelus	−	−	NS	+	134
Stenobothrus stigmaticus	+	−	NS	+	39
Omocestus kaestneri	NS	NS	NS	+	91
Birds:					
Anthus spinoletta	+	−	NS	NS	170
Oenanthe oenanthe	+	−	+	−	131
Monticola saxatilis	+	NS	NS	NS	21
Phoenicurus ochruros	+	−	NS	NS	95
Saxicola torquata	+	−	+	NS	31
Prunella collaris	+	NS	NS	+	43
Prunella modularis	NS	−	NS	NS	24
Carduelis cannabina	NS	NS	NS	NS	49

Note: Plus signs indicate significant positive trends with elevation, and minus signs indicate significant negative trends.

and climate (PC1, associated with temperature, or PC2, associated with precipitation) significantly affected average plant $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios when these variables were entered in models alone, but because of covariation between PC1 and grazing intensity their contribution was difficult to ascertain in models in which they were combined (either focusing on R^2 or the Akaike information criterion; tables A11, A12). Tests conducted in one climate band or in ungrazed plots showed that both predictors played a significant role, dismissing the idea of spurious correlations (table 3; fig. 2). In *Ceteris paribus*, grazing was associated with ^{13}C depletion, ^{15}N enrichment, an increase in %N, and a decrease in C : N ratio. At ungrazed sites, PC2 (precipitation factor) induced a similar pattern, while PC1 (temperature factor) positively influenced %N and $\delta^{15}N$. Significant phylogenetic dependence was often found for %N and $\delta^{15}N$ but not for $\delta^{13}C$ (table 3).

Discussion

By integrating ecosystem and mechanistic perspectives, we found that the nutrient and isotope dynamics in a model terrestrial ecosystem are tightly interconnected and depend on environmental as well as evolutionary influences. Significant trends of increasing heavy carbon isotope ratios and decreasing heavy nitrogen isotope ratios with elevation

were often found among populations and species, in tight association with changes in plant C and N pools. Elevation patterns in producers were reflected—and even magnified—by the isotopic ratios of the higher trophic levels, although without the strong phylogenetic dependence found in producers, and they often disappeared when controlling for isotopic baselines.

Evolutionary Control

Significant elevation clines in isotopic ratios were often found in populations within species and among species, suggesting that patterns involving individuals within species are not fully decoupled from those affecting entire phylogenetic lineages. Although population differentiation often bears less consistent messages about adaptive responses than higher-level taxa (which display larger structural and functional divergences), partial agreement of species-level and community-level responses is often found along elevation (Laiolo et al. 2013), and stable isotope ratios are not an exception (Körner 2003; Männel et al. 2007). Interspecific variation in $\delta^{13}C$ and $\delta^{15}N$ of plants across the elevation gradient was significantly constrained by phylogenetic history, and strong phylogenetic signals were often found for $\delta^{15}N$ and %N in response to climate and grazing gradients. The values of λ, often close to 1,

Table 3: Results of phylogenetic generalized least squares regressions testing for the relationship among the average values of $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios in 7 species of plants undergoing different grazing pressure but found in similar climate conditions (*top*) and for the relationship among the average values of $\delta^{13}C$, $\delta^{15}N$, %N, and C : N ratios in 13 species of plants from ungrazed slopes and the average local climate conditions in which they grew (*bottom*)

	Estimate (SE)	t	λ (P)	R^2
Effect of grazing intensity in plant species growing at middle elevations:				
$\delta^{13}C$	−3.47 (.78)	4.42**	.00 (1.00)	.76
$\delta^{15}N$	2.35 (.90)	2.62*	1.00 (.02)	.49
%N	1.84 (.66)	2.77*	1.00 (.08)	.52
C : N ratio	−31.62 (8.97)	3.52*	.57 (.58)	.66
Effect of climate variation in plant species growing in ungrazed plots:				
$\delta^{13}C$:				
PC1	...	.63	.00 (1.00)	.51
PC2	−1.10 (.29)	3.79**		
$\delta^{15}N$:				
PC1	2.74 (.79)	3.46**	.84 (.21)	.52
PC2	1.96 (.91)	2.13+		
%N:				
PC1	.62 (.31)	2.76*	1.00 (.09)	.43
PC2	.90 (.38)	3.35**		
C : N ratio:				
PC1	−5.72 (2.07)	1.99+	1.00 (.05)	.62
PC2	−8.30 (2.40)	2.40*		

Note: PC1 is positively associated with temperature and PC2 is positively associated with precipitation during growing season months; grazing intensity is expressed as the percentage of plant individuals collected in grazed plots. We tested for λ departure from 0; P values are provided in parentheses.

+ $P < .10$.

* $P < .05$.

** $P < .01$.

indicate that differences between species may have accumulated randomly since time to divergence, although models of neutrality do not appear to fully fit $\delta^{13}C$ elevation clines (table 1). Phylogenetic relationships capture important functional aspects of species; thus, a high phylogenetic dependence likely reveals the existence of some kind of physiological mechanism that constrains plant responses, while the absence of a signal would indicate functional convergence along the gradient or lack of differentiation. As an example, phylogeny did not affect $\delta^{13}C$ variation with climate or grazing (table 3), suggesting overall that the underlying mechanisms of variation in $\delta^{13}C$ are broadly shared by closely and distantly related plants. The sole source of carbon for green plants is air carbon dioxide, and the C_3 photosynthetic pathway is adopted by all study species—aspects that simplify fractionation pathways and determine species' convergent responses. Mixed C_3-C_4 plant assemblages typical of low latitudes in fact display abrupt divergence in this aspect (Körner et al. 1988; Luo et al. 2009). Conversely, strong phylogenetic dependence has been found for plant $\delta^{15}N$ and %N, and indeed a greater diversity of functional and physiological mechanisms govern the nitrogen compared with the carbon cycle. Plants chiefly absorb this nutrient as nitrate, am-

monium, and organic nitrogen, which have different stable isotope ratios depending on both the sources and the step of nitrogen cycle (^{15}N-enriched ammonium from nitrification, ammonia volatilization, and mineralization; ^{15}N-depleted nitrate from nitrification; ^{15}N-enriched nitrate from denitrification, etc.; Högberg 1997). The association with N_2-fixing bacteria and mycorrhizal fungi, which alter pathways of nitrogen uptake, also conditions plant $\delta^{15}N$ and N content (Craine et al. 2009). Among the study species, *Trifolium* spp. rely in variable proportions on the nitrogen provided by N_2-fixing bacteria, grasses (genera *Poa*, *Festuca*, *Brachypodium*, etc.) form mycorrhizal associations, and *Carex* spp. are often nonmycorrhizal (Michelsen et al. 1996; Welker et al. 2003), representing diverse functional strategies that constrain correlations with environmental factors. More pronounced stoichiometric clines are indeed displayed by grasses (figs. 1 and 2, species 8–15), a group known for being strongly nitrogen limited at high elevations or latitudes, where the N cycling rate is slow (Theodose and Bowman 1997). Grasses are also more palatable, at least with respect to local sedges (Busqué et al. 2010), and thus should display greater stoichiometric shifts in response to defoliation than the latter group (Obeso 1993; Mysterud et al. 2011), a difference
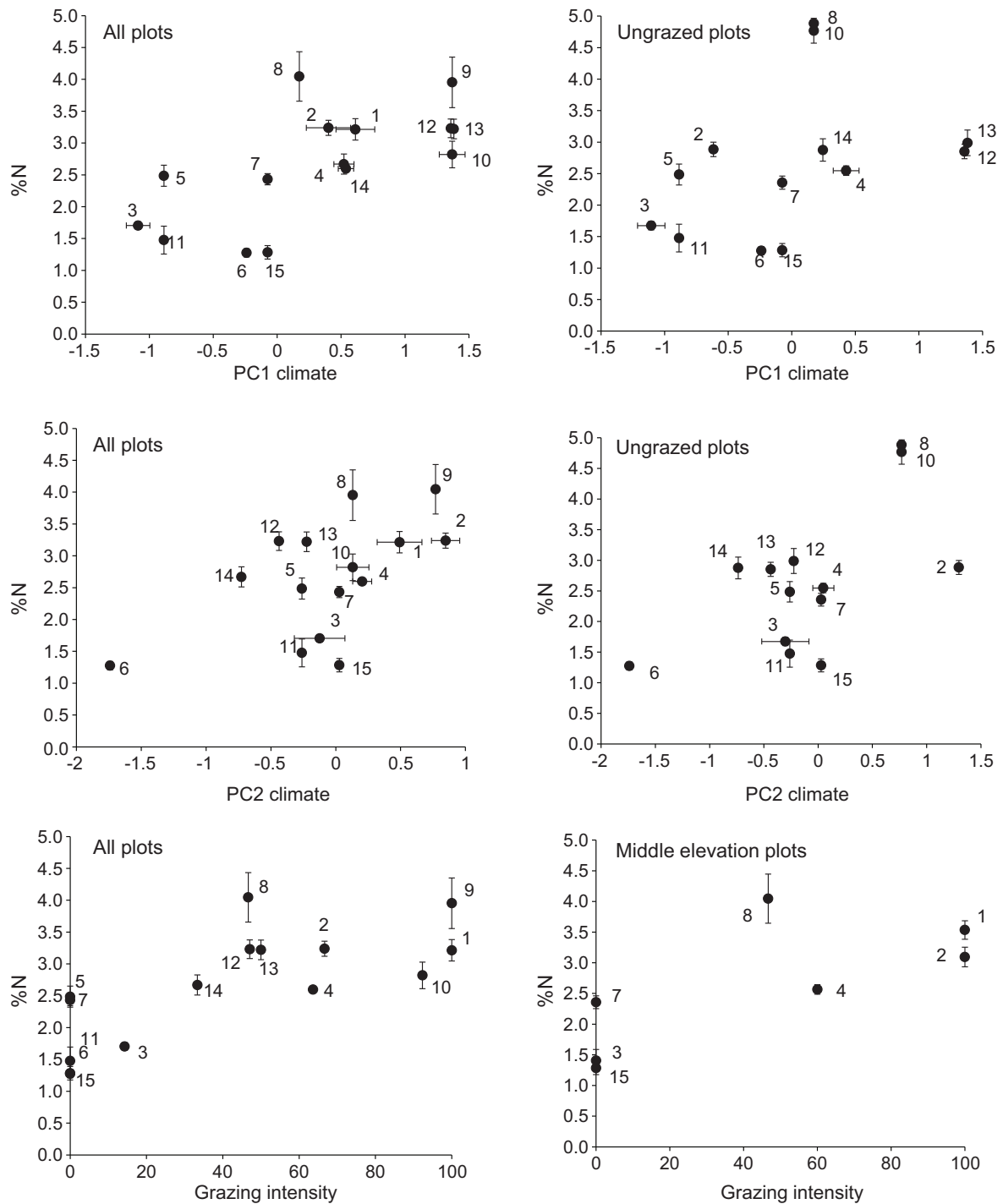
Figure 2: Relationship between average %N (± SE) and PC1 (± SE), PC2 (± SE), and grazing intensity in plant species from the Cantabrian Mountains. The average PC1 and PC2 where the species were found and the percentage of plant samples collected in grazed plots are paired to their average %N. Phylogenetic effects are not controlled for in these graphics. Left panels show relationships tested with data from all sampling sites and species. Top and middle right panels show relationships tested in ungrazed plots only. The bottom right panel shows the relationship tested at middle elevations to minimize variation in climate. PC1 is positively associated with temperature and PC2 is positively associated with precipitation during growing season months. Grazing intensity is represented by the percentage of plant individuals collected in grazed plots. Numbering is as in figure 1.

embedded in a significant phylogenetic signal in the response to grazing intensity.

Unlike producers, consumers showed weak phylogenetic conservationism in their isotopic ratio trends. This may be due to the lower evolutionary distances among animal taxa, which translates into smaller community niche space and more similar functions compared with plants (Srivastava et al. 2012), or to the overwhelming effect of external factors (food) over internal ones (physiology, metabolism, etc.) in determining the isotopic characteristics of animal tissues (see below). In addition, it is often through behavior that animals interact with other organisms and the environment, and behavioral traits are poorly conserved from a phylogenetic point of view (Blomberg et al. 2003). Finally, it could be argued that sampling biases induced the observed variability in phylogenetic signals among assemblages. However, mountain communities are not randomly assembled; they are strongly shaped by environmental filters and uncover phylogenetic patterns that are distinct from random subsets of regional pools, facts that may limit biases in estimating phylogenetic dependence (Cisneros et al. 2014).

Abiotic and Biotic Control

The isotopic fractionation of the carbon in leaves is tightly associated with CO_2 diffusion inward and outward and thus with stomatal activity, which in turn is controlled by short-term changes in climate (stomata close to reduce water loss in dry and warm conditions) and also depend on physiological and morphological adaptations to high-altitude climate (increased leaf thickness, stomata density, etc.; Vitousek et al. 1990). These long- and short-term strategies indirectly determine $\delta^{13}C$ variation along elevation (Luo et al. 2009) and also explain its responsiveness to changes in growing season precipitation (table 3). The value of $\delta^{13}C$ was also influenced by plant nitrogen content, possibly because of the photosynthetic requirement for this element. An increase in the intrinsic photosynthetic capacity of leaves in fact reduces the ratio of intercellular to atmospheric partial pressure of CO_2; thus, the discrimination against ^{13}C (Morecroft and Woodward 1996; Araus et al. 1997). This mechanism may help to explain the reduction in $\delta^{13}C$ values where N pools are enhanced by grazing and mild temperatures (see below) and points to the importance of soil chemistry, together with the direct influence of climate, in determining variation in this isotope.

Unlike $\delta^{13}C$, plant nitrogen content and $\delta^{15}N$ showed negative elevation clines. Although the observed decline in plant nutrients with elevation was in some aspects opposite to that described in vegetation from other mountain ridges (Körner 2003; but see Huber et al. 2007), it may still be explained by variation in abiotic and biotic conditions that affect soil microbial activity, nitrogen mineralization, and turnover along the elevation gradient. At low elevations, grazing and mild thermal conditions accelerate the decomposition rate and net nitrogen mineralization, processes that enhance soil nutrient availability for plants with direct consequences in their isotopic ratios (Mariotti et al. 1980). Conversely, in the highlands low temperatures and reduced N inputs from herbivores hamper the microbial activities underlying these processes (Mariotti et al. 1980; Schinner 1982; Frank and Groffman 1998). Changes in the plant nutrient pools along elevation were accompanied by variation in the same direction of $\delta^{15}N$ values; thus, both $\delta^{13}C$ and $\delta^{15}N$ may serve to infer patterns of N cycling across environmental gradients. This coupling has been described in ecosystems in which climate chiefly controls plant nutrient uptake and storage (Dawson et al. 2002; Craine et al. 2009) but not in systems where grazing is the main environmental perturbation. Nitrogen input from herbivore waste contains large amounts of ^{15}N-enriched ammonium and depleted nitrate (Frank et al. 2000); thus, plant communities tend to homogenize with respect to nitrogen content but split with respect to $\delta^{15}N$ values (Högberg 1997; Dawson et al. 2002). The above-described results suggest that soil chemistry is often the prime target of biotic and abiotic forces, thus representing a key component of nutrient cycles that should be addressed to fully comprehend ecosystem processes.

Isotope Clines in Consumers

The distribution of C and N isotopes in animals appears to be the direct result of the effect of elevation on vegetation, a finding that reveals the strong, passive, bottom-up control of raw isotopic variation in consumers. Although this was expected given that consumer isotopic values reflect their diet and thus the environment in which primary producers grow (Fry et al. 1978; Bump et al. 2007), few studies along terrestrial spatial gradients have demonstrated the transfer of environmental information across trophic levels characterized by broad differences in metabolism (homeotherms vs. ectotherms) and stable isotope physiology (Fry 2006). These multitrophic pathways have, however, been well scrutinized in aquatic systems (Cabana and Rasmussen 1996; Vander Zander and Rasmussen 1999; Vander Zander et al. 1999; Rasmussen 2010).

Variation of carbon isotope ratios with elevation in birds was similar to that described in *Dendroica* warblers in the Appalachian Mountains (Graves and Romanek 2009), hummingbirds in the Ecuadorean Andes (Hobson et al. 2003), and passerines in Taiwan (Chang et al. 2011), thus pointing to a general pattern in mountain birds similar to that observed in plants. In the study grasshoppers, $\delta^{13}C$ also increased with elevation but not at the interspecific

level, possibly because of the bias introduced by a few species (*Calliptamus barbarus*, *Chorthippus binotatus*) that occasionally feed on tree or shrub matter, which greatly differs from herbs in composition (Picaud et al. 2002; Benzara et al. 2003).

The $\delta^{15}N$ values of grasshoppers covaried with elevation similarly to plant values at both the intra- and the interspecific level, although many negative intraspecific trends with elevation changed their sign after the $\delta^{15}N$ of baseline resources were controlled for. This indicates that high-elevation grasshoppers showed a greater isotopic fractionation, increasing their nitrogen isotope ratio with respect to baseline more than lowland populations. Highland grasshoppers may possess some buffering behavior in the face of declining food quality with elevation, such as feeding on greater quantities of nutrient-poor plants or feeding on the most nutrient-rich plant species within the community, all adjustments that have been described in mountain grasshoppers (Berner et al. 2005; Behmer and Joern 2008). Significant elevation clines after correction for baseline have also been found in three bird species (table 2), pointing overall to the need to test whether the widespread reduction in plant quality is accompanied by substantial consumer trophic shifts along the elevation gradient.

Conclusions

Both historical and ecological/physical aspects of the local environment concur in shaping nutrient and isotopic distribution in mountain food webs and environmental gradients. Nutrient and isotope clines have a measurable phylogenetic signal detectable at the scale of the plant community, likely because phylogenetic relationships capture important functional aspects controlling plant uptake, assimilation, and turnover of nitrogen. The influence of the environment cannot be ascribed to any single factor; both biotic forces (such as grazing) and abiotic climate factors interact in shaping the C and N cycles, leaf chemistry, and resources available to consumers. Although we outlined a number of mechanisms by which the above-described factors can affect nutrient cycles, there are still few studies uncovering the phylogenetic dependence of ecological patterns in natural communities, and even fewer concerned with ecosystem processes. This study shows that synthetic approaches may provide important insights into understanding nutrient cycling and the functional role played by different groups in ecological networks.

Acknowledgments

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