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Growth responses, biomass partitioning, and nitrogen isotopes of prairie legumes in response to elevated temperature and varying nitrogen source in a growth chamber experiment[1]

Heather R. Whittington[2,5], Laura Deede[3], and Jennifer S. Powers[2,4]

[2]Department of Plant Biology, University of Minnesota, 1445 Gortner Avenue, Saint Paul, Minnesota 55108 USA; [3]College of Biological Sciences, University of Minnesota, Saint Paul, Minnesota 55108 USA; and [4]Department of Ecology, Evolution, and Behavior, University of Minnesota, Saint Paul, Minnesota 55108 USA

- *Premise of the Study:* Because legumes can add nitrogen (N) to ecosystems through symbiotic fixation, they play important roles in many plant communities, such as prairies and grasslands. However, very little research has examined the effect of projected climate change on legume growth and function. Our goal was to study the effects of temperature on growth, nodulation, and N chemistry of prairie legumes and determine whether these effects are mediated by source of N.
- *Methods:* We grew seedlings of *Amorpha canescens*, *Dalea purpurea*, *Lespedeza capitata*, and *Lupinus perennis* at 25/20°C (day/night) or 28/23°C with and without rhizobia and mineral N in controlled-environment growth chambers. Biomass, leaf area, nodule number and mass, and shoot N concentration and $\delta^{15}N$ values were measured after 12 wk of growth.
- *Key Results:* Both temperature and N-source affected responses in a species-specific manner. *Lespedeza* showed increased growth and higher shoot N content at 28°C. *Lupinus* showed decreases in nodulation and lower shoot N concentration at 28°C. The effect of temperature on shoot N concentration occurred only in individuals whose sole N source was N_2-fixation, but there was no effect of temperature on $\delta^{15}N$ values in these plants.
- *Conclusions:* Elevated temperature enhanced seedling growth of some species, while inhibiting nodulation in another. Temperature-induced shifts in legume composition or nitrogen dynamics may be another potential mechanism through which climate change affects unmanaged ecosystems.

Key words: $\delta^{15}N$ signatures; *Amorpha canescens*; *Dalea purpurea*; elevated temperature; legumes; *Lespedeza capitata*; *Lupinus perennis*; nitrogen source; nodulation.

Legumes (*Fabaceae*) play an important role in the nitrogen (N) cycle of terrestrial ecosystems: they are often a significant source of N (Cleveland et al., 1999) through their symbiosis with dinitrogen (N_2)–fixing rhizobia bacteria. Ongoing increases in global temperatures due to anthropogenic releases of greenhouse gases to the atmosphere will affect this role; temperature is known to affect N_2-fixation and other plant physiological processes such as photosynthesis and nutrient uptake (Bassirirad, 2000; Aranjuelo et al., 2007). The vast majority of research investigating legume responses to temperature is focused on crop or forage species (Kessler et al., 1990; Zachariassen and Power, 1991; Lilley et al., 2001). By contrast, very little research has examined the possible effects of warming on legumes native to prairies, despite the influential role of these species in these often N-limited ecosystems (Tilman, 1984; Garten et al., 2008).

Nitrogen-fixing legumes account for 1–17% of the biomass in U.S. prairies (Piper et al., 2007) and contribute about 5% of total N input into these systems (Woodmansee et al., 1981). However, prairies are generally N-limited (Craine and Jackson, 2010), and even small changes in N inputs can have a large impact over time (Seastedt et al., 1991; Kindscher and Tieszen, 1998). At a local scale, legumes influence the surrounding plants by increasing N availability, as evidenced by increased biomass and higher N concentration in nonlegume neighbors (Mulder et al., 2002; Temperton et al., 2007) and can increase soil carbon accumulation (Fornara and Tilman, 2008). Legumes are also becoming increasingly important in restoration efforts (Graham, 2005), and greater knowledge of the consequences of climate change on these species can help inform these efforts.

Temperature affects many aspects of the N_2-fixation symbiosis. Warming can inhibit nodulation (Barrios et al., 1963; Purwantari et al., 1995), slow nodule development (Piha and Munns, 1987), and reduce nodule activity (Meyer and Anderson, 1959; Hungria and Franco, 1993; Aranjuelo et al., 2007). In a recent literature synthesis, Houlton et al. (2008) found that optimal nitrogenase activity occurs in the temperature range of 20–30°C. The authors used these data to include a soil temperature component to a terrestrial biogeochemical model explaining global patterns of N_2-fixation and legume abundance and found that temperature limits N_2-fixation in northern forests (Houlton et al., 2008). The optimal temperature for plant growth and N_2-fixation is often species-specific, however, and depends on both the rhizobia and the legume species (Piha and Munns, 1987; Purwantari et al., 1995).

In unmanaged environments, legumes commonly rely on N_2-fixation only when mineral N is relatively unavailable in the

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[5]Author for correspondence (e-mail: whitt092@umn.edu)

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soil, because N_2-fixation is energetically costly from a physiological standpoint (Gutschick, 1981). Additionally, compatible strains of rhizobia may not be present in the environment, forcing legumes to rely solely on mineral N (Larson and Siemann, 1998). Because both fixation and uptake of N_2 are affected by temperature (Hatch and Macduff, 1991), we can speculate that the source of N to the plant (i.e., N_2-fixation or mineral N) may mediate the effects of elevated temperatures. For example, if N_2-fixation is more sensitive to changes in temperature than uptake of mineral N, plants that rely solely on N_2-fixation may show greater responses to temperature increases.

Direct quantification of whole-plant N_2-fixation rates in field studies is currently not possible; thus, many studies use the natural-abundance isotopic method to estimate nitrogen fixation (Boddey et al., 2000; Garten et al., 2008). This method takes advantage of the difference between the $\delta^{15}N$ of atmospheric N_2 (defined as 0‰) compared with that of mineral nitrogen available to the legume from the soil (which is typically enriched in the heavy stable isotope of N) (Shearer and Kohl, 1991). In these calculations of the percentage of N derived from fixation ($\%N_{dfa}$), it is necessary to account for isotope fractionation that occurs during the fixation process and transfer of nitrogen throughout the plant. This *B* value is obtained by growing the legume of interest without mineral N, such that N_2-fixation is the sole N source. *B* values of shoot tissue range from –3.61‰ to +1.9‰ and depend on the legume species and rhizobial strain (Boddey et al., 2000; Unkovich et al., 2008). It is possible that this discrimination may be altered by temperature, but to our knowledge no studies have reported on this possibility. Understanding whether *B* values are altered by growing temperatures is essential for application of the natural-abundance method to predict how N_2-fixation may respond to ongoing climate change.

In addition to potential effects on N nutrition, temperature can also affect growth, physiology, and morphology of legumes, with feedbacks for plants, rhizobia, and ecosystem N cycling. Temperature-induced changes in photosynthesis and respiration may affect plant growth and productivity, and will also affect N_2-fixation rates because both processes determine the amount of carbon that is available for fixation. In turn, temperature-induced changes in N_2-fixation can affect carbon gain processes and growth by determining N levels. At the ecosystem scale, any changes to legume productivity and fixation rates will alter the total amount of N that is fixed and, thus, the amount of N that is added to the community.

In the present study, we used a growth chamber experiment to examine the effect of warming on prairie legume growth and function. Four legume species, *Amorpha canascens*, *Dalea purpurea*, *Lespedeza capitata*, and *Lupinus perennis* were selected because they are common in North American prairies and also are present in a manipulative field experiment to quantify the responses of prairie plants to increased temperatures (H. R. Whittington, unpublished data). These species were grown at two temperatures, with or without rhizobia and with or without mineral N. Using highly controlled conditions in a growth chamber allowed us to isolate the effects of temperature and N source, without confounding effects of soil moisture or other factors that may vary in field experiments. Our specific goals were to determine (1) whether seedling growth, morphology, and N chemistry depended on species, temperature, and N source; and (2) whether nodulation and isotopic discrimination factors were affected by species and temperature. We expected the plants that relied solely on N_2-fixation to be the most sensitive to temperature differences, because N_2-fixation would be affected more than uptake of mineral N. We expected the plants that relied solely on N_2-fixation to display slower growth than individuals given mineral N, because N_2-fixation is more costly than uptake.

MATERIALS AND METHODS

***Species*—**The focal study species were *Amorpha canescens* Pursh, *Dalea purpurea* Vent., *Lespedeza capitata* Michx., and *Lupinus perennis* L. (hereafter referred to by genus). All four of these perennial species are native and common to the grasslands of central North America; they differ in phenology and other traits. *Lupinus* is active in the spring and early summer and flowers in late spring. *Lespedeza* flowers in late summer. *Amorpha* and *Dalea* flower in early to middle summer. *Lespedeza* forms nodules with determinate growth and translocates fixed nitrogen as ureides. *Amorpha* and *Dalea* form indeterminate nodules that maintain a meristem and transport nitrogen as amides. *Lupinus* forms unique indeterminate nodules and also transports nitrogen as amides (Sprent, 2001).

***Experimental setup*—**Plants were grown in stacked 350-cm^3 Magenta vessels (PhytoTechnology Laboratories, Lenexa, Kansas, USA) that allowed us to manipulate nutrient solution and rhizobial inoculation. The bottom vessel held nutrient solution, the middle vessel was filled with silica sand, and the top vessel acted as a lid (Fig. 1). A cotton wick between the bottom and middle vessels transferred nutrient solution from the bottom unit to plant roots, giving unlimited access to water and nutrients. The assembled Magenta vessels were autoclaved with nutrient solutions before planting. The bottom vessels containing nutrient solutions were clear, and we cannot rule out the possibility that photosynthetic organisms were growing there. However, we saw no indications over the course of the experiment that this was occurring.

Seeds from Prairie Moon Nursery (Winona, Minnesota, USA) were surface-sterilized with either bleach (*Amorpha* and *Dalea*) or sulfuric acid (*Lespedeza* and *Lupinus*). The sulfuric acid also acted as a scarifying agent. Three seeds of a species were planted into the sterile sand of each growing unit and were thinned to one plant per vessel 3 wk after germination.

***Treatments*—**Plants were assigned to six treatments that consisted of factorial combinations of growing temperatures (25/20°C or 28/23°C [day/night]) and three N-source treatments. The daytime low temperature represents the average growing-season temperature in southern Minnesota (Cedar Creek LTER data; see http://cedarcreek.umn.edu/research/weather/). The high-temperature regime represents a 3°C increase, which is within the predicted range of temperature increases by 2100 (IPCC, 2007) and is similar to the warming treatment in a field experiment at Cedar Creek (East Bethel, Minnesota, USA) that contains these legume species (H. R. Whittington, unpublished data). The three N-source treatments were presence of rhizobia without mineral N, presence of rhizobia with mineral N, and absence of rhizobia with mineral N. The combination of no rhizobia and no mineral N was not included in the experimental design because plants would not be expected to survive. We note that these three N-source treatments encompass the full range of possibilities that may occur in the field (i.e., compatible rhizobia strains may be present or absent, and mineral N may be sparingly available or present in nonlimiting amounts). We contend that the "true situation" in the field is likely to lie between these extremes; thus, our results constrain the possible responses of the species that we studied to variation in N and/or rhizobia. Each treatment was replicated 10 times.

Plants that did not receive supplemental N received the following N-free nutrient solution: 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 μM NaFeEDTA, 1 mM K_2SO_4, 100 μM KH_2PO_4, 0.01 μM, $(NH_4)_6Mo_7O_{24}$, 0.16 μm $ZnSO_4$, 0.04 μM $CuSO_4$, 2μM H_3BO_3, and 0.4 μM $MnSO_4$ (modified from Franco and Munns 1982). Seedlings in the mineral N treatments received the above nutrient solution that was supplemented with NH_4NO_3 to a concentration of 100 mg N/L. The quantity of N available in the nutrient solution was sufficient to provide N in excess of plant demand over the course of the experiment (i.e., we did not expect N-limitation of plant growth in the treatments that received mineral N over the duration of the experiment, and is within the range of N added in similar studies of legumes (Legros and Smith, 1994; Plies-Balzer et al., 1995).

Five days after planting, seedlings in the rhizobia treatments were inoculated with 1 or 2 strains of compatible rhizobia. Species-specific rhizobia strains were obtained from the Rhizobia Research Laboratory at the University of Minnesota.

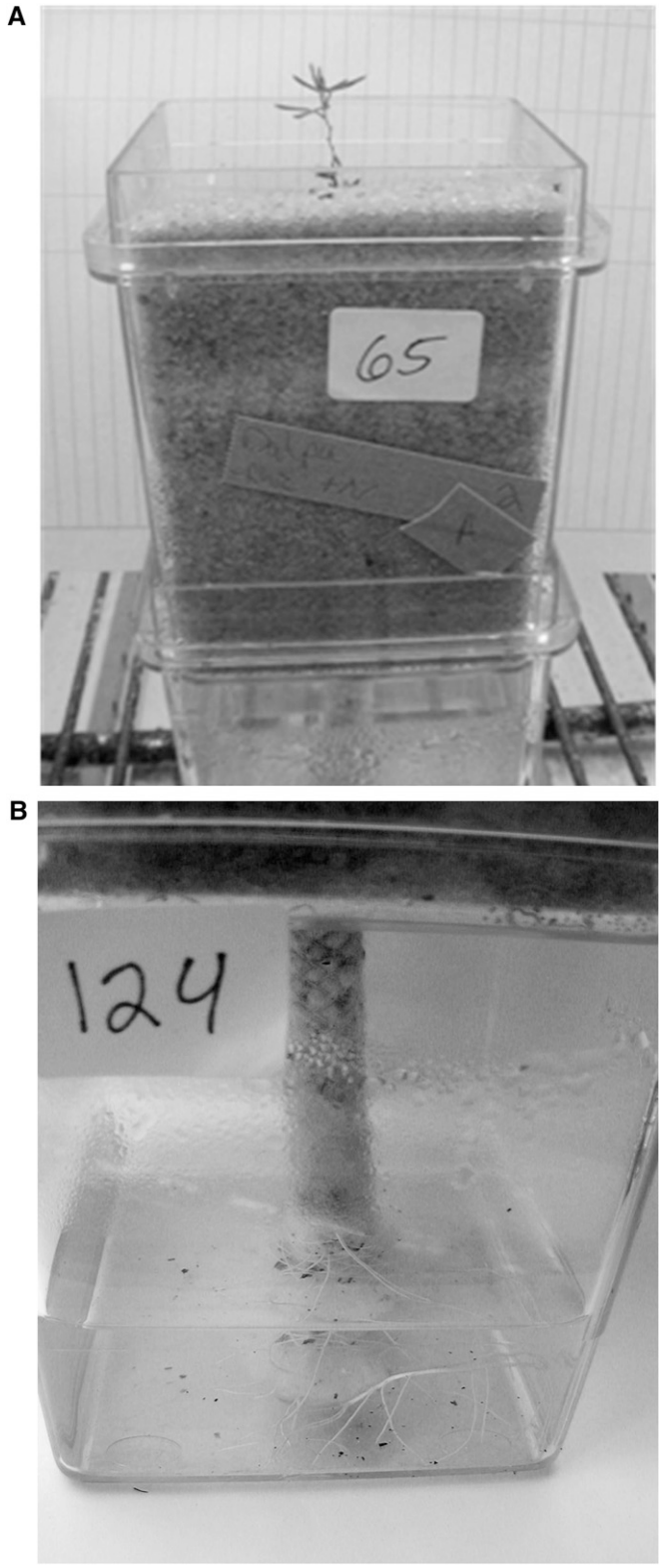

Fig. 1. The assembled Magenta units used to grow the seedlings. (A) A seedling growing in sand. (B) The cotton wick that transferred nutrient solution from the bottom vessel to plant roots.

Strains were originally isolated from Minnesota soils and were chosen for their known ability to nodulate the focal species (P. Graham, personal communication). *Amorpha* was inoculated with a strain of *Mesorhizobium amorphae*, and *Dalea* was inoculated with two *Rhizobium* spp. strains that are genetically similar to *R. etli* and *R. leguminosarum* (Tlusty et al., 2005). The identity of the strains used to inoculate *Lespedeza* and *Lupinus* is unknown. Strains were subcultured on yeast extract mannital agar (BYMA) plates for 1 wk, then transferred to BYMA broth culture. Broth cultures were shaken for 5 d and then centrifuged. The resulting pellets were resuspended in dilution fluid to an approximate concentration of 5.5×10^5 cells mL^{-1}. One milliliter of inoculum was pipetted on the sand surface of the vessel containing the corresponding species to give a rhizobia concentration of ~10^3 cells per gram of soil.

Growing conditions—Plants were grown in controlled-environment chambers (Conviron, Winnipeg, Canada) under a 12-h photoperiod at two different day/night temperature regimes: 25/20°C (low) or 28/23°C (high). Two chambers were used for each temperature regime to ensure that temperature differences were not simply due to chamber effects. Light levels ranged from 210 to 250 μmoles m^{-2} s^{-1} within each chamber and were similar among chambers. Plants were randomly moved within their chamber on a weekly basis.

Measurements—After 12 wk of growth, plants were harvested and separated into roots, stems, leaves, and nodules. Leaves were placed in wet paper towel and scanned within 12 h to calculate leaf area using ImageJ software (Abramoff et al., 2004). Nodules were counted and weighed immediately after harvest. The mass per nodule of each individual was calculated by dividing the total fresh nodule weight by the total number of nodules. All tissue was then dried at 65°C for >48 h and weighed. The average specific leaf area (SLA in units of $cm^2 \cdot g^{-1}$) of each individual was calculated by dividing the total leaf area by the total dry weight of leaves.

^{15}N analysis—To calculate the isotopic discrimination due to fixation (*B* value) and shoot nitrogen concentrations (shoot [N]), all of the aboveground plant tissue (stems and leaves) was ground in a ball-mill grinder, and a subset was sent to the Stable Isotope Laboratory at UC-DAVIS for ^{15}N analysis. The *B* value is defined as the δ^{15}N value of individuals whose sole source of N was N_2-fixation (i.e., individuals that did not receive added mineral N). Shoot tissue of plants in the mineral-N-only treatment was also analyzed for ^{15}N. Last, we analyzed the ^{15}N composition of NH_4NO_3 to help us interpret the isotopic signatures of plants grown with both mineral N and rhizobia. We had originally intended to use the ^{15}N data to estimate the percentage of N derived from fixation ($\%N_{dfa}$) for plants in the treatments receiving both mineral N and *rhizobia*, but we were unable to do so because of poor nodulation. However, determining whether the *B* values of plants that rely on fixation as their sole N source differ among species and between temperature regimes is also an important goal of the present study.

Statistical analysis—Differences in the response variables of biomass, leaf area, leaf number, SLA, shoot [N], nodule number, and nodule weight among the treatments of species, temperature regime, and N-source were determined with analyses of variance (ANOVAs) using JMP 9 software (SAS Institute, Cary, North Carolina, USA). The experiment was analyzed as a split-plot design, with temperature as the whole-plot factor and species and N-source as the split-plot factors. Species, temperature, and N-source were included as fixed effects in the models, and chamber and its interaction with species and N-source were included as random effects to account for the split-plot design. When an effect was significant, post hoc Tukey HSD tests were used to determine significant differences between individual treatment levels. All biomass, leaf area, leaf number, SLA, and shoot [N] variables were transformed with the natural logarithm to improve normality and decrease nonconstant variance. Nodule number and weight were square-root transformed for the same purpose. Histograms of the data indicated that transformations sufficiently improved normality.

RESULTS

Overall, though species differences accounted for the majority of variation in morphological variables, both temperature and N-source also affected many of these traits in a species-specific manner (Table 1). N-source was the largest source of variation in shoot [N] and nodule variables, but these responses were also affected by temperature in some species. An interaction between temperature and N-source was detected

only in leaf chemistry and nodule variables, but not in growth or morphology.

Growth and morphology—Both temperature and N-source affected total, aboveground, and belowground biomass in a species-specific manner (Fig. 2). Temperature significantly affected the biomass of *Lespedeza* only. In this species, aboveground biomass was 109% larger at 28°C than at 25°C. Root biomass was not affected by temperature in any species. *Amorpha* plants grown without mineral N (i.e., that relied solely on N_2-fixation) had 65% less total and root biomass than plants that received mineral N. Shoot biomass was 40–70% lower in *Amorpha* and *Lupinus* individuals that relied solely on N_2-fixation than in those that received mineral N.

Lespedeza plants growing at the high-temperature regime had twice the total leaf area and twice as many leaves as those at the lower temperature. *Dalea* individuals in the warmer treatment also exhibited 28% more leaves at 28°C than at 25°C. *Amorpha* individuals that relied solely on N_2-fixation exhibited 40% fewer leaves and 53% less leaf area than individuals given mineral N. Specific leaf area (SLA) was the only morphological variable that was unaffected by temperature and/or N-source (Table 1).

Nitrogen concentrations—Shoot N concentration was 45–57% lower in plants that relied only on N_2-fixation than in those given mineral N for all species but *Dalea* (Table 2). Temperature affected shoot [N] in *Lupinus* individuals that relied solely on N_2-fixation, with 51% less shoot [N] at high temperature. There was a trend for 34% and 17% lower shoot [N] at 25°C than at 28°C for *Lespedeza* and *Amorpha* individuals that relied solely on N_2-fixation, respectively, but this difference was not statistically significant.

For all species but *Dalea*, the smaller shoot biomass and lower shoot [N] displayed by individuals that relied solely on N_2-fixation compared with those given mineral N led to a 72–81% lower total N content in shoots (data not shown). For *Lespedeza*, total shoot N content was 49% lower at 25°C than at 28°C, with a trend for the largest difference occurring in individuals that relied solely on N_2-fixation. There was a trend among *Amorpha* and *Lupinus* individuals that relied solely on N_2-fixation to display 50% higher or 54% lower total shoot N content values, respectively, at high than at low temperature, but this difference was not statistically significant.

$\delta^{15}N$ values—The NH_4NO_3 used in the nutrient solution had a $\delta^{15}N$ value of 1.65‰. Surprisingly, individuals that relied solely on the nutrient solution for an N source displayed negative $\delta^{15}N$ values, whereas the $\delta^{15}N$ values of individuals given both rhizobia and mineral N were more similar to that of the nutrient solution (Table 3), although our ability to detect treatment effects and interactions may be limited by sample size.

Species and N-source caused significant differences in shoot $\delta^{15}N$ values, whereas temperature did not. *Lespedeza* displayed significantly lower $\delta^{15}N$ values than the other species overall. *Amorpha* individuals grown with both rhizobia and mineral N had significantly higher $\delta^{15}N$ values than those that relied solely on N_2-fixation or on uptake of mineral N. *Dalea* individuals given both sources of N had significantly higher $\delta^{15}N$ values than those that relied solely on N_2-fixation.

Nodules—Only three individuals in the mineral-N-only treatment developed nodules, indicating minimal contamination. Only 12.5% of plants with both rhizobia and mineral N had nodules, whereas 92.5% of individuals with rhizobia but no mineral N possessed nodules.

TABLE 1. *F*-ratios and degrees of freedom (df) from analyses of variance on variables measured on legume seedlings grown at two temperatures (Temp) and varying sources of nitrogen (N).

	Source of variation							
Variable	Species	Temp	N-source	Species × Temp	Species × N-source	Temp × N-source	Species × Temp × N-source	Denominator df [a]
Height at harvest	31.80 ***	11.89 $	17.82 ***	4.95 **	1.44	1.34	0.67	23, 2
Total biomass	35.88 ***	1.14	20.85 ***	3.49 *	5.06 **	0.30	1.04	21, 2
Shoot biomass	100.27 ***	16.80 $	40.13 ***	4.79 *	5.21 **	0.35	0.67	21, 2
Root biomass	6.35 **	0.03	6.06 **	3.48 *	4.62 ***	0.04	1.43	21, 2
Total leaf biomass	87.74 ***	11.45 $	36.16 ***	4.66 *	5.36 **	0.41	0.80	22, 2
Stem biomass	153.52 ***	52.45 *	62.99***	5.22 **	3.95 **	0.77	0.33	21, 2
Total leaf area	62.05 ***	22.75 *	25.58 ***	3.93 *	3.29 *	0.15	0.43	23, 2
SLA	30.15 ***	0.40	2.26	1.32	0.95	0.13	0.08	23, 2
Leaf number	471.40 ***	25.70 *	35.63 ***	9.31***	3.51 *	2.62	1.14	23, 2
Shoot [N]	14.79 ***	0.03	104.89 ***	1.14	4.90**	0.39	2.96 *	22, 26, 2
Shoot N content	57.51 ***	34.18 ***	99.40 ***	5.95 **	5.21 **	0.50	1.77	27, 33, 12
$\delta^{15}N$	9.63 ***	2.60	17.41 ***	1.34	3.35 *	0.28	0.53	22, 27, 3
Nodule number	7.28 **	0.42	265.49 ***	5.36 **	8.00 ***	0.27	4.32**	22, 2
Total nodule fresh mass	6.45 **	2.58	252.95 ***	10.82 ***	3.49 *	3.61 *	8.50 ***	22, 2
Mass per nodule	6.65 **	9.39 $	221.53 ***	5.79 **	2.31 $	4.03 *	5.05 **	22, 2
Numerator df	3	1	2	3	6	2	6	

Notes: The significant species × temperature interaction for total and root biomass arose because species differed at one temperature but not the other. Within a species, there was no effect of temperature on total or root biomass. $, *, **, *** indicate significance at the $P < 0.10$, 0.05, 0.01, and 0.001 levels, respectively.

[a] For all variables except shoot [N], shoot N content, and $\delta^{15}N$, the first value denotes df for all effects except "Temp," and the second value is the df for the "Temp" main effect. For nitrogen variables, the first value indicates df for all effects containing "N-source," the second value denotes df for remaining effects that contain "Species," and the third value indicates the df for the "Temp" main effect.

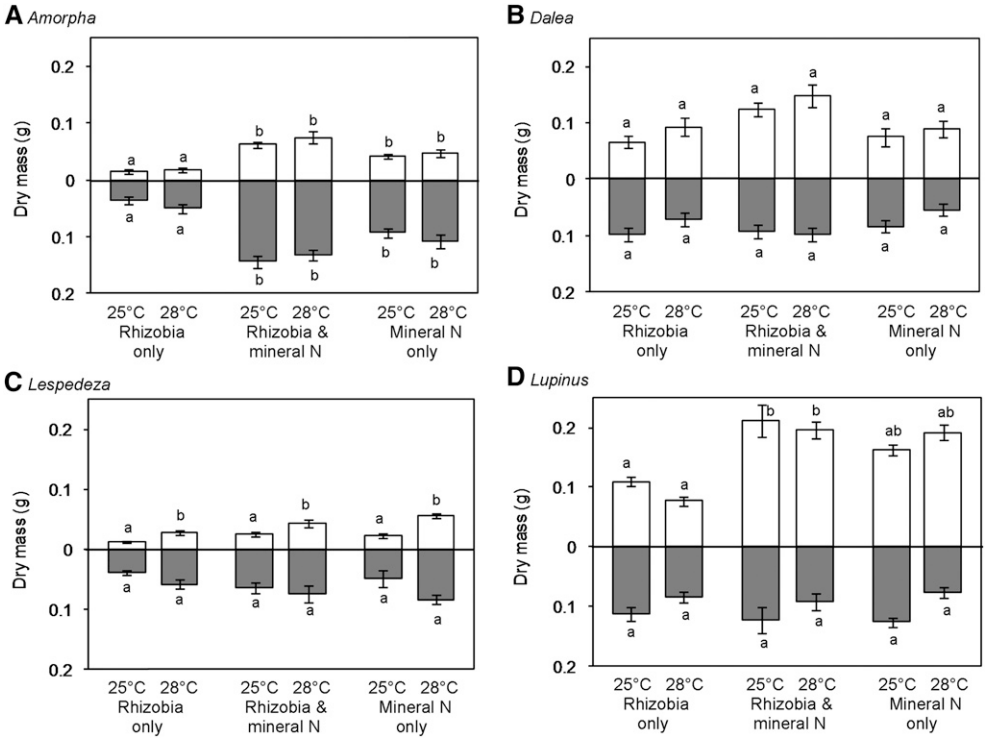

Fig. 2. The effect of temperature and nitrogen source on shoot and root dry mass of four legume species (A) *Amorpha canescens*, (B) *Dalea purpurea,* (C) *Lespedeza capitata*, and (D) *Lupinus perennis*. White bars above zero display shoot biomass, and gray bars below zero show belowground biomass. Error bars indicate ±1 SE. Within each species and variable, letters indicate significant differences among treatments at $P < 0.05$. Significant differences were determined from post hoc Tukey HSD tests after analyses of variance (see Table 1 for ANOVA results).

Lupinus individuals that relied only on N_2-fixation possessed 86% fewer nodules at 28°C than at 25°C, and nodules were 63% smaller at the high temperature. These two trends led to 87% less total nodule fresh weight in the warmer treatment of *Lupinus* (Fig. 3). One reason for this trend was the strong effect of temperature on the proportion of plants containing nodules in *Lupinus* individuals that relied solely on N_2-fixation. Nodulation decreased from 100% of plants containing nodules at 25°C to 40% at 28°C. Other species did not show a significant effect of temperature on nodule number or mass. Differences in nodule number and fresh weight between species were apparent only in individuals that relied solely on N_2-fixation.

TABLE 2. Shoot [N] (mg/g) of seedlings grown at two temperatures (Temp) with varying sources of N. Values are means ± SE; n = 2–10 individuals per treatment combination.

		N-source		
Species	Temp	Rhizobia only	Rhizobia & mineral N	Mineral N only
Amorpha	25°C	17.04 ± 1.05 ^{a}	35.22 ± 2.31 ^{c}	35.49 ± 2.20 ^{c}
	28°C	20.55 ± 1.56 ^{ab}	33.87 ± 3.38 ^{bc}	38.19 ± 2.90 ^{c}
Dalea	25°C	33.74 ± 1.28 ^{ab}	33.86 ± 3.56 ^{ab}	47.96 ± 4.39 ^{a}
	28°C	31.26 ± 0.89 ^{b}	38.94 ± 7.15 ^{ab}	43.77 ± 3.93 ^{ab}
Lespedeza	25°C	17.73 ± 0.80 ^{a}	48.90 ± 8.73 ^{b}	56.93 ± 4.60 ^{b}
	28°C	26.76 ± 1.15 ^{ab}	44.37 ± 7.74 ^{b}	47.61 ± 2.53 ^{b}
Lupinus	25°C	21.45 ± 1.84 ^{b}	32.52 ± 1.72 ^{a}	35.56 ± 1.30 ^{a}
	28°C	14.11 ± 1.19 ^{c}	32.84 ± 2.92 ^{ab}	36.30 ± 1.98 ^{a}

Notes: Letters indicate significant differences of the means at $P < 0.05$ within each species that were determined from a post hoc Tukey's HSD test after analysis of variance (see Table 1 for ANOVA results).

DISCUSSION

Prairie legumes often play an influential role in the dynamics of their surroundings by adding nitrogen (N), enriching soil organic matter, and stimulating N mineralization (Fargione et al., 2007; Fornara and Tilman, 2008; Fornara et al., 2009). Despite this importance, very little is known about the effects of elevated temperature on prairie legumes. Knowledge of how climate change will affect these species is important for predicting prairie functioning in the future and will help inform preservation and restoration practices. We grew four prairie legume species at two temperatures that mimicked climate change (25/20°C and 28/23°C) and with or without rhizobia and mineral N to determine whether these factors affected (1) seedling growth, morphology, and N chemistry, and (2) nodulation and nitrogen isotope discrimination. We found that temperature and/or N-source affected many of these traits in a species-specific manner. *Lespedeza* showed enhanced growth under the high temperature, whereas *Lupinus* displayed detrimental effects of warming on nodulation.

***Temperature effects*—**Previous studies that examined legume growth and temperature have shown that the temperature response can depend on species (Zachariassen and Power, 1991; Purwantari et al., 1995). It should be noted, however, that most studies compare species across relatively large temperature gradients (i.e., of 20°C; Purwantari et al., 1995). By contrast, we examined a smaller temperature range (3°C difference) that is similar to forecasted temperature increases and still found species-specific responses. Warming affected the growth and morphology

TABLE 3. Shoot $\delta^{15}N$ values (‰) of seedlings grown at two temperatures (Temp) with varying sources of N. Seed $\delta^{15}N$ values are also shown. Values are means ± SE; n = 2–10 individuals per treatment combination. The mineral N solution was 1.65 ± 0.08‰.

		N-source			
Species	Temp	Rhizobia only (*B* values)	Rhizobia & mineral N	Mineral N only	Seed
Amorpha	25°C	–1.23 ± 0.25 [ab]	1.53 ± 1.57 [b]	–3.39 ± 0.84 [a]	–1.23 ± 0.50
	28°C	–1.07 ± 0.43 [ab]	2.49 ± 1.12 [b]	–2.49 ± 1.13 [a]	
Dalea	25°C	–2.07 ± 0.19 [a]	2.39 ± 0.38 [b]	–0.20 ± 0.92 [ab]	0.81 ± 0.51
	28°C	–1.73 ± 0.11 [a]	1.13 ± 0.78 [b]	–0.12 ± 0.56 [ab]	
Lespedeza	25°C	–3.30 ± 0.70 [a]	–1.62 ± 1.49 [a]	–5.16 ± 0.79 [a]	–0.37 ± 0.21
	28°C	–2.65 ± 0.29 [a]	–0.33 ± 1.21 [a]	–2.36 ± 0.48 [a]	
Lupinus	25°C	–1.52 ± 0.32 [a]	0.58 ± 0.63 [a]	0.15 ± 0.31 [a]	0.23 ± 0.18
	28°C	–0.95 ± 0.28 [a]	0.68 ± 0.77 [a]	–0.26 ± 0.65 [a]	

Notes: Letters indicate significant differences of the means at $P < 0.05$ within each species. Significant differences were determined with a post hoc Tukey's HSD test after analysis of variance (see Table 1 for ANOVA results).

of two of the four species, *Lespedeza* and *Dalea*. *Lespedeza* plants were 60% larger and had twice the leaf area at 28°C than at 25°C. *Dalea* displayed 28% more leaves in the high temperature, but there was no temperature effect on total leaf area, indicating that leaves produced at 28°C were smaller than those at 25°C. In contrast to our findings, *Medicago sativa* (alfalfa) displayed decreased growth at 28°C than at 25°C (Aranjuelo et al., 2007).

Numerous studies have shown that temperature affects nodule number and weight in a species-specific manner (Jones, 1921; Purwantari et al., 1995; Lira et al., 2005), and we found similar results. *Lupinus* plants that relied solely on N_2-fixation had 63% smaller and 86% fewer nodules at the high temperature. This reduction in total nodule mass most likely led to decreased rates of N_2-fixation on a whole-plant basis. Indeed, while biomass was not statistically affected by temperature in these individuals, they displayed 35% lower shoot [N] at the low temperature. *Lupinus* grows in the cooler spring months, and 28°C may be above the optimal temperature for *Lupinus* and/or the rhizobia strain used in the study. Our results suggest that if *Lupinus* does not shift its phenology in response to future climate change, it may show detrimental effects of elevated temperature in relation to nodulation, possibly leading to decreases in N_2-fixation. It should be noted, however, that we used only one strain of rhizobia to inoculate *Lupinus*. Since the strain of rhizobia can affect the temperature response, the exact response in the field may depend on the rhizobia strains present (Montanez et al., 1995).

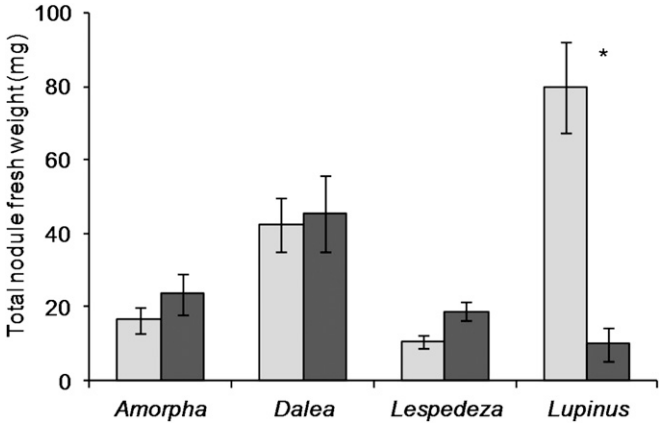

Fig. 3. The effect of temperature on the total fresh weight of nodules for individuals of four legumes species that relied solely on fixation for nitrogen. Light gray bars represent individuals grown at 25/20°C (day/night), and dark gray bars represent those grown at 28/23°C. Error bars display ±1 SE. Asterisk indicates a significant difference ($P < 0.05$) among the temperature treatments within each species. Significant differences were determined with a post hoc Tukey HSD test after analysis of variance (see Table 1 for ANOVA results).

Our expectation that plants that relied solely on N_2-fixation would display larger responses to temperature (i.e., greater warming effects on growth and morphological variables) than plants that received mineral N, because the N_2-fixation symbiosis may be more sensitive to warming than uptake of mineral N, was not seen. Previous studies in the literature that have addressed this question have yielded mixed results. In several studies, *Glycine max* (soybean) and *Phaseolus vulgaris* (common bean) that relied more on N_2-fixation than on uptake of soil N displayed larger effects of root temperature on N accumulation and biomass growth than plants that received more mineral N (Lie, 1971; Piha and Munns, 1987; Legros and Smith, 1994). However, Kessler et al. (1990) found evidence that the growth response of *Trifolium repens* (white clover) to temperature did not depend on N-source. Contrary to our expectations, we found no significant temperature by N-source interactions for the biomass or morphological variables, which suggests that source of N may not be important to the temperature response of growth for these legumes.

Only shoot [N] in *Lupinus* showed a larger temperature response in individuals that relied solely on fixation than in those given mineral N, perhaps reflecting a temperature effect on N_2-fixation, as others have shown in global literature syntheses (Houlton et al., 2008). As mentioned above, the decrease in nodulation at 28°C compared with 25°C in *Lupinus* probably led to a decrease in or lack of N_2-fixation, likely limiting the N content to that present in the seed. In *Lespedeza* individuals that relied solely on N_2-fixation, the larger total shoot N content at 28°C compared with 25°C suggests more N_2-fixation at the high temperature. Individuals grown at 28°C displayed a trend (not statistically significant) toward higher total nodule fresh mass than those grown at 25°C. Increased rates of nodule activity could have led to this difference in N content as well. However, because we did not measure N_2-fixation rates directly, we can only speculate on the causes and consequences of this pattern. We were also unable to examine the effects of temperature on the percent N derived from fixation ($\%N_{dfa}$) because of the poor nodulation of individuals given both rhizobia and mineral N. These shoot N results suggest a higher sensitivity of N_2-fixation

to temperature than uptake of mineral N for these two species, but this difference, surprisingly, did not affect the sensitivity of growth to temperature.

The effect of temperature on N_2-fixation is often included in conceptual models of N_2-fixation regulation (Hartwig, 1998; Vitousek et al., 2002; Reed et al., 2011) but is not always explicitly included in simulation models used to examine how ecological controls on N_2-fixation and legumes lead to the observed patterns of global N_2-fixation rates (Vitousek and Field, 1999; Menge et al., 2009). Houlton et al. (2008) included temperature constraints on nitrogenase activity in a terrestrial biogeochemical model. Using data from the literature, they developed an empirical relationship between N_2-fixation rates and soil temperature. Including this relationship in the model helped to explain the observable patterns of low N_2-fixation rates in high-latitude forests. They used a single relationship, but it is known that legume and rhizobial species can vary in their response to temperature (Piha and Munns, 1987; Purwantari et al., 1995). Although our growth chamber study is on a much smaller scale than most continental modeling studies, it does provide further evidence that temperature can affect N_2-fixation differently for co-occurring legume species. Incorporating this variation may improve the representation of N_2-fixation in ecosystem models.

In general, temperature did not affect $\delta^{15}N$ values, including the *B* value of any species (i.e., the $\delta^{15}N$ value of individuals that relied solely on N_2-fixation). The *B* value adjusts for the isotopic fractionation that occurs during the fixation process and transfer of fixed N throughout the plant and is important because it serves as an end member in mixed model equations to calculate the percent N derived from fixation in field-grown plants (Unkovich et al., 2008). Our mean *B* values, –2.97‰ to –1.16‰, fall within the range of commonly reported mean shoot *B* values of –3.61‰ to 1.9‰ (Boddey et al., 2000; Unkovich et al., 2008). Isotopic fractionation can be affected by environmental factors such as nutrient availability and soil moisture, although the effects are often small (Ledgard, 1989). To our knowledge, this was the first study to examine the effect of temperature on *B* values. Our findings are encouraging for field studies of N_2-fixation that employ the natural abundance technique, because it suggests that *B* values obtained at one growth temperature can be applied to plants grown at different temperatures.

N-source effects and inferences from $\delta^{15}N$**—**In most species, individuals that relied solely on N_2-fixation were 36% smaller than those given mineral N, as we expected. Both Legros and Smith (1994) and Purwantari et al. (1995) found similar results with *Sesbania sesban* and *Glycine max*, respectively. This reduction in biomass may be due to the high carbon costs of N_2-fixation (Ryle et al., 1979; Gutschick, 1981). These plants could also have been limited by nitrogen, as suggested by the 40% lower shoot [N] values in plants that relied solely on N_2-fixation than those given mineral N, because N_2-fixation was limited. Thus, if elevated temperature affects the availability of mineral N in the soil and/or the activity of N_2-fixation, productivity of legumes is likely to be affected.

The effects of N-source on $\delta^{15}N$ values were not as we expected. Surprisingly, $\delta^{15}N$ values of those given both rhizobia and mineral N did not always display intermediate $\delta^{15}N$ values between those that relied solely on N_2-fixation (–2.97‰ to –1.16‰) and those given only mineral N (–3.60‰ to –0.07‰). Additionally, individuals that received mineral N did not display $\delta^{15}N$ values more similar to the bulk fertilizer than those that received rhizobia. The reason for these results is not clear, although we can suggest several hypotheses to explain these patterns: (1) discrimination against the heavier isotope occurring during both N_2-fixation and uptake of mineral N (Kohl and Shearer, 1980; Yoneyama et al., 2001); (2) discrimination due to translocation of N from roots to shoots being similar regardless of source; (3) varying preferences for NH_4^+ or NO_3^- among species or treatments (Clarkson et al., 1986; Macduff and Jackson, 1991); and (4) larger plants displaying higher $\delta^{15}N$ values more similar to the bulk fertilizer because of more complete use of the nitrogen pool.

We caution that our ability to extrapolate our growth chamber results to the field is limited. Our results pertain to legume seedlings, and the responses of mature plants with large root systems and growing in competition are likely to differ. Our seedlings did not experience water limitation, but seedlings in the field may be more affected by warming-induced decreases in soil moisture than by direct effects of temperature increases. Additionally, the response is likely to be mediated by the available rhizobia strains. We used one or two strains, but these species are capable of nodulating with many strains (Tlusty et al., 2004). On the other hand, the seedling stage of many plants' life cycles is often vulnerable to environmental factors and places a strong filter on the composition of the future plant community. Our study suggests that certain legume species may be favored by climate change, whereas other species may not, and this may have implications not only for the diversity of prairie communities in a warmer world, but also for N cycling.

Conclusions**—**Overall, temperature and N-source influenced growth, nodulation, and N content in a species-dependent manner. *Lespedeza* exhibited enhanced seedling growth under warming while *Lupinus* displayed decreased nodulation under warming. In response to warming as a consequence of climate change, plants can shift their range, shift their phenology, acclimate, and/or adapt (Walther, 2003). Because legumes are often dependent on specific rhizobia strains, their response may be limited by the response of rhizobia. Although, we can only extrapolate the results of a growth chamber experiment to field situations with caution, our study indicates that the seedlings of certain legume species may display beneficial effects of elevated temperature whereas other species may not.

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