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REGULAR ARTICLE

Nutrients and environment influence arbuscular mycorrhizal colonization both independently and interactively in *Schizachyrium scoparium*

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Abstract
Background and aims Arbuscular mycorrhizal fungi (AMF) are important for plant nutrient and water acquisition. Much is known about how nutrient addition and environment affect AMF, but little is known about nutrient by environment interactions. We measured AMF colonization with nutrient additions and along an environmental gradient to assess these interactions.
Methods We measured AMF colonization in roots of little bluestem (*Schizachyrium scoparium* (Michx) Nash) with nutrient addition and across an environmental gradient. We assessed how AMF colonization changed across different fertilization treatments, and used ridge regression to determine nutrient, environment, and nutrient by environment interaction variables that predicted AMF colonization.
Results The addition of nitrogen decreased AMF colonization, while mean annual temperature (MAT) and

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soil pH both positively predicted the percentage of AMF colonization in *Schizachyrium scoparium*. Additionally, we found an interaction term between MAT and phosphorus treatments that significantly affected percent AMF colonization.

Conclusions Our results show the importance of understanding environmental conditions on AMF as well as nutrient by environment interactions when assessing how AMF respond to nutrient addition. Here we present a full-factorial nutrient addition study along an environmental gradient to assess how AMF root colonization is influenced by abiotic factors in addition to nutrients.

Keywords Arbuscular mycorrhizal fungi (AMF) · *Schizachyrium scoparium* · Mycorrhizal root colonization · Ridge regression · Long-term nutrient fertilization

Abbreviations

AAI	Annual aridity index
AMF	Arbuscular mycorrhizal fungi
MAT	Mean annual temperature
N	Nitrogen
P	Phosphorus
$K_{+\mu}$	Potassium with micronutrients treatment

Introduction

Arbuscular mycorrhizal fungi (AMF) are important for nutrient acquisition (Smith and Read 2008) as well as non-nutrient related factors that increase plant fitness (Delavaux et al. 2017). The plant-mycorrhizae association is dependent on multiple factors including soil nutrient availability, climate, and environmental variables; however, root AMF colonization is generally reduced by the addition of plant-limiting nutrients (primarily nitrogen (N) and phosphorus (P)); Treseder and Allen 2002; Johnson et al. 2003; Treseder 2004; Johnson 2010; Liu et al. 2012). Nutrient loading has also been shown to destabilize mutualisms more generally (Shantz et al. 2016). However, plants, mycorrhizae, and their relationship can be constrained by other climate and environmental factors including water, temperature and soil properties. In general, levels of mycorrhizal colonization are impacted by temperature (Kim et al. 2015; Soudzilovskaia et al. 2015), pH (Postma et al. 2007), and soil nutrient stoichiometry (Soudzilovskaia et al. 2015). Percentage of root AMF colonization is known to be hump-shaped with both temperature and soil nutrient stoichiometry (Landis et al. 2004; Soudzilovskaia et al. 2015). pH also seems to affect levels of AMF colonization with maximum colonization occurring in soils with more neutral pH (Postma et al. 2007). The effect of water and water availability on AMF has been variable, depending on geographic location and plant community (Stevens and Peterson 1996; Jacobson 1997). However, AMF are generally thought to assist plants with water acquisition under conditions of low water availability (Augé 2004). Although the magnitude and direction are not always clear, environmental conditions clearly impact AMF.

It is well established that nutrients as well as environmental factors impact plants both directly and interactively across multiple aspects (e.g. growth, physiology, phenology, community structure, etc. Lambers et al. 1998). Arguably two of the most important environmental factors on aspects of plant biology and ecology are water and temperature. Plants depend on water for structural support and basic physiological functions such as photosynthesis (Lambers et al. 1998), and temperature has a direct and substantial impact on plants and their ecology. Belowground nutrients also play a substantial role in plant physiology (Lambers et al. 1998), growth, and ecology (Harpole et al. 2016) as they are the basic building blocks of plant material. The interaction of nutrients and environmental factors can also greatly impact aspects of plant physiology and growth (Porter and Lawlor 2008) as well as communities and species diversity (Zavaleta et al. 2003). For example, the addition of water reduced species diversity in an annual plant community in Michigan, but only in plots where N was also added (Goldberg and Miller 1990).

Given that environmental conditions and nutrients directly impact both plants and AMF, and that environment by nutrient interactions have been observed in plants, it seems a likely next step to explore how AMF respond to nutrient additions under different environmental conditions. Although the response of AMF colonization to nutrient fertilization has been studied heavily (Johnson 1993; Johnson et al. 2003; Treseder and Allen 2002; Treseder 2004; Johnson 2010) it is unclear how AMF respond to nutrient additions across large-scale environmental gradients. An analysis of AMF globally revealed a multivariate unimodal relationship among AMF, temperature, and nutrient stoichiometry

(Soudzilovskaia et al. 2015), which indicates that there is a plausibility that AMF will respond to nutrient addition differently across environmental gradients. This study looks at the responses of AMF to nutrient addition across an environmental gradient by assessing levels of AMF colonization in the roots of the C4 grass little bluestem (*Schizachyrium scoparium*) along a north-south gradient of annual precipitation and temperature in the United States Central Plains grassland ecosystem.

We tested plant response to nutrient addition of nitrogen (N), phosphorus (P), and potassium with micronutrients ($K_{+\mu}$) across a range of environmental factors to determine: 1) how does nutrient addition impact AMF colonization in plants given local environmental conditions, 2) which environmental variables are able to predict the abundance of AMF colonization in plants, and 3) what interactions, if any, exist among nutrients and environmental conditions in predicting AMF colonization. The aim of this study was to understand how environmental conditions impact levels of AMF colonization and if environmental conditions alter AMF response to nutrient addition. We hypothesize that addition of nutrients, especially N and P, will decrease AMF colonization, but the decrease in colonization will depend on local soil and environmental conditions. We also hypothesize that greater mean annual temperature (MAT) and annual aridity index (AAI) will be associated with increased AMF, and that the addition of nutrients will have a stronger effect in reducing AMF colonization in locations with greater water availability (i.e. lower AAI). The reason for this last hypothesis stems from the idea that increased water availability will decrease co-limitation of water and shift limitation more to nutrients such as N or P (Sadras 2004). The environmental factors that we included in this study were: MAT, AAI, soil pH, soil texture, and soil nutrient limitation measured as the change in aboveground net primary production with the addition of N, P, and $K_{+\mu}$ (Δ-ANPP N, Δ-ANPP P, Δ-ANPP $K_{+\mu}$, respectively).

We tested the above research questions by assessing the percentage of root AMF colonization in *Schizachyrium scoparium*. This is a common and widely used method for assessing AMF colonization in plants (Treseder 2013), but has not been without its criticisms (Jansa et al. 2016). Most of these criticisms have been related to differences in AMF colonization among plant species and morphological differences among AMF taxa (Koch et al. 2017) as well as staining and microscopy techniques (Vierheilig et al. 2005). All roots used in this study were collected, stained, and viewed by PNF thereby eliminating any inter-observer bias. Roots were also only collected from *Schizhacyrium scoparium* plants eliminating any plant interspecific bias in AMF colonization. Percent length root colonization in this study was therefore only subject to interspecific differences in AMF. However, percent root colonized has been shown to be proportional to total biomass of AMF within taxonomical groups (Hart and Reader 2002).

Materials and methods

Field sites

We collected root samples of *S. scoparium* at five replicated nutrient addition sites located throughout the eastern portion of the Great Plains ecoregion in the central United States representing a gradient of environmental conditions (Fig. 1, Tables 1, 2, and 3). Specifically, sites were located at: Cedar Creek LTER, Minnesota; Barta Brothers Ranch, Nebraska; Chichauqua Bottoms Greenbelt County Conservation Area, Iowa; Konza Prairie LTER, Kansas; and the Grassland Soil and Water Research Laboratory in Temple, Texas; hereafter CC, Barta, CBGB, Konza, and Temple, respectively. For further information on sites visit: http://www.nutnet.umn.edu/field_sites. Sampling sites were all a part of the Nutrient Network, which is a global research cooperative that investigates top-down and bottom-up controls on grassland plant communities by employing nutrient and herbivory treatments to grassland sites around the world (Borer et al. 2014). We chose sites based on the presence of *S. scoparium* as well as location within the tallgrass prairie ecosystem. We chose this species because of its documented association with AMF (Anderson et al. 1984) and its abundance and broad distribution across the tallgrass prairie region (Glenn and Collins 1993), which makes findings widely applicable as well as allows us to test questions about nutrient addition across a wide geographical and environmental range. In order to determine abundance of AMF colonization we collected root samples from plots located at the sites described above. Soil type information was obtained from the USDA Web Soil Survey (Soil Survey Staff 2012) and can be found in Table 2. Soil type information was obtained from the USDA Web Soil Survey (Soil Survey Staff 2012) (Table 2).

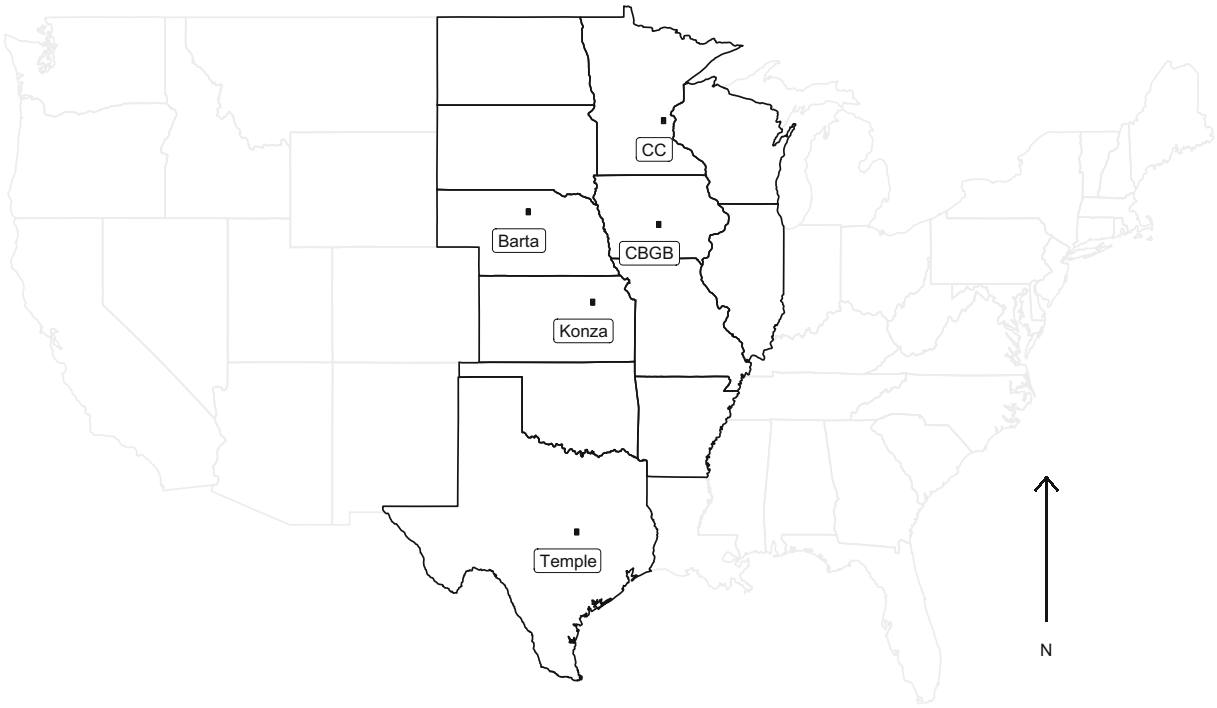

Fig. 1 Map of the United States showing study sites. Sites were selected along a north-south transect in order to sample from a wide enivronmental gradient

Sites were fertilized in a full factorial design with N, P, and $K_{+\mu}$ (the $K_{+\mu}$ being a treatment of potassium with micronutrients). All nutrients were added as pellet fertilizers and at a rate of 10 g m^{-2} $year^{-1}$ by elemental mass. N was added as time release urea (which is a polymer-coated fertilizer that releases N slowly across a growing season), P as triple super phosphate ($Ca(H_2PO_4)_2$) and $K_{+\mu}$ as a combination of potash (KCl) a macro/micronutrient blend (e.g. K, Ca, Mg, S, B, Cu, Fe, Mn, Mo, Zn) added in the first year. For more detailed information on the experimental nutrient addition protocol visit: http://www.nutnet.umn.edu/nutrients. Note while

Table 1 Location, site information, and soil and environmental data for each site

Site	Acronym	Lat	Long.	First Yr. Trt.	# of Blocks	Site PI
Barta Brothers Ranch, NE	Barta	42.24	−99.65	2007	3	David Wedin
Chichauqua Bottoms Greenbelt, IA	CBGB	41.79	−93.39	2009	6	Lori Biederman
“	“	“	“	“	–	Paul Frater
“	“	“	“	“	–	W. Stanley Harpole
“	“	“	“	“	–	Brent Mortenson
“	“	“	“	“	–	Lauren Sullivan
Cedar Creek LTER, MN	CC	45.43	−93.21	2007	5	Elizabeth Borer
“	“	“	“	“	-	W. Stanley Harpole
“	“	“	“	“	–	Adam Kay
“	“	“	“	“	–	Eric Seabloom
Konza Prairie LTER, KS	Konza	39.07	−96.58	“	3	Melinda Smith
“	“	“	“	“	–	Kimberly J. LaPierre
Temple, TX	Temple	31.04	−97.35	“	3	Philip Fay

Table 2 Site information on soil type and principal investigator for each nutrient network site

Site	Soil Type	Soil Series	Soil Description
Cedar Creek LTER, MN	Sandy Outwash Plain	Zimmerman loam fine sand	Mixed, frigid Lamellic Udipsamment
Barta Brothers Ranch, NE	Sandhills	Valentine fine sand Farrar fine sandy loam	Mixed, mesic Typic Ustipsamment
Chichauqua Bottoms Greenbelt, IA	Sandy loam	Ankeny fine sandy loam	Mixed, superactive mesic Typic Hapludolls
Konza Prairie LTER, KS	Silty clay loam	Florence silt loam	Mixed superactive mesic Typic Hapludoll
Temple, TX	Black clay soil	Austin clayloam	Carbonatic thermic Udorthentic Haplusoll
		Houston clay loam	Smectitic, thermic Oxyaquic Hapludert

that N, P, and K were added to plots every year, the macro/micronutrient blend was only added in the first year of the study. However, these K plus micronutrient plots will still be referred to as $K_{+\mu}$. All sites began fertilization in 2007 except CBGB which received its first treatment in 2009. The temporal difference in initial fertilization treatment could add a confounding factor to this study; however, CBGB was the only site to have a within-site significant AMF by nutrient effect. Therefore, we deemed this not to be an issue. Control and treatment plots were set up as 5 × 5 m plots in a randomized block design (Borer et al. 2014). All sites had at least 3 replicated blocks, with Cedar Creek LTER having 5 blocks and the CBGB site having 6 blocks.

Field and lab methods

We collected root samples from *S. scoparium* plants in August 2010. Samples were collected from every treatment plot (Control, N, P, $K_{+\mu}$, NP, $NK_{+\mu}$, $PK_{+\mu}$, $NPK_{+\mu}$) by taking a root core using a 1.75 cm diameter soil push-probe to a depth of 30 cm directly adjacent to the center of the plant to minimize damage to meristem tissue. We sealed root and soil samples in plastic bags, transported in a cooler with ice, and stored in a freezer at −20 °C until processing. We retrieved roots by sieving soil samples through a 2 mm soil sieve. To detect AMF presence, we stained a subsample of fine roots from each sample using a trypan blue staining procedure modified from Robertson et al. (1999). Roots were cleared in a 2.5 to 5% KOH solution at 90 °C for 3 to 4 h, soaked in this solution at room temperature for 6 to 8 h, treated in alkaline H_2O_2 for 1 h, then acidified in 1% HCl for 1 h. We soaked roots in a trypan blue solution made of 1:1:1 (*v:v:v*) glycerol, lactic acid, water and 1% (v) of 0.2 μm filtered trypan blue solution for 1 h on a hot plate set to 90 °C. Modifications deviating from Robertson et al. (1999) consisted of tailoring clearing and staining times to suit the roots of our study specimen as well as using filtered trypan blue solution instead of trypan blue powder.

We then assessed the percentage of root sample colonized by AMF using the grid line intercept technique (Giovannetti and Mosse 1980). This technique consists of placing the roots on a petri dish marked with vertical and horizontal lines spaced every 13 mm. We viewed roots under a dissecting microscope set to 40× magnification

Table 3 Soil, climatic, and nutrient limitation data for each site

site	% sand	% silt	% clay	pH	AAI	MAT (°C)	MAP (mm)	PET (mm)	Δ-ANPP N	Δ-ANPP P	Δ-ANPP $K_{\mu+}$
Barta Brothers Ranch, NE	96.00	3.50	0.50	5.89 ± 0.041	0.08	9.80	593.00	45.79	68.74 ± 9.9	64.94 ± 6	4.28 ± 16
Chichauqua Bottoms Greenbelt, IA	88.00	8.00	4.00	5.84 ± 0.065	0.05	9.70	917.00	49.21	91.87 ± 6	6.33 ± 10	37.46 ± 9.5
Cedar Creek LTER, MN	90.00	7.00	3.00	5.6 ± 0.027	0.05	7.00	817.00	41.75	93.7 ± 2.6	62.84 ± 2.4	48.05 ± 3.5
Konza Prairie LTER, KS	19.00	56.00	25.00	5.98 ± 0.044	0.06	12.80	904.00	57.54	167.15 ± 4.4	88.14 ± 0.49	64.76 ± 7.7
Temple, TX	31.00	31.00	38.00	7.51 ± 0.013	0.08	18.90	916.00	76.07	−15.76 ± 6.3	18.89 ± 8.2	−1.42 ± 3.7

Means ± SE are presented. Variables with only a single number had only one observation per site

AAI is annual aridity index, *MAT* is mean annual temperature, *MAP* is mean annual preciptiation, *PET* is potential evapotranspiration, and Δ-ANPP N, Δ-ANPP P, Δ-ANPP K_{+u} is the change in above ground net primary productivity with addition of N, P, and K_{+u}, respectively

and determined presence/absence of fungal organs (i.e. hyphae/vesicles) for each instance that a root intersected with a gridline. While this technique has been criticized as biased giving observer-dependent results (McGonigle et al. 1990), this is not an issue as all samples were assessed by PNF. Percent AMF colonization was calculated by dividing the number of AMF intersections by the total number of root intersections and multiplying by 100.

Soil analysis and plant nutrient limitation calculations

Soils that were sieved to 2 mm size for root removal were analyzed for various soil physical and chemical properties. We measured the percentage of sand and clay in sampled soils by using the standard hydrometer method for measuring particle size in soils (Robertson et al. 1999). Soil pH was measured in a slurry of 5 g of air dried soil combined with 10 ml of distilled water and then shaken for 5 min. We then measured pH of this slurry using a Fisher-Scientific Accumet Basic AB15/15+ pH reader.

To test for plant nutrient limitation, we quantified increases in live total aboveground net primary production (ANPP) of all species present at sites with the addition of a nutrient as indicative of limitation by that nutrient (Chapin III et al. 1986; Aerts and Chapin III 1999). We used ANPP data for the 2008–2016 growing seasons which was measured by clipping all live biomass from two 10 cm by 100 cm strips within plots, dried at 60 °C for 48 h and weight to the nearest 0.01 g (Nutrient Network 2017). We used treatment effect on plant ANPP measured as the difference in aboveground net primary production between fertilized plots and control plots for each added nutrient (N, P, or $K_{+\mu}$) within each block at each site which provided a metric for limitation of each nutrient (i.e. Δ-ANPP N, Δ-ANPP P, Δ-ANPP $K_{+\mu}$, respectively). Higher values of calculated differences were interpreted as a greater limitation for that particular nutrient at that site (i.e. if adding N at a site increased ANPP a substantial amount at that site, then we interpreted this as that site as having greater limitation of N). Although sites had herbivore exclusion treatments these plots were not included in the calculation of these metrics as this treatment only occurred on control and fully fertilized plots (i.e. the only exclusion treatments were control + fence and $NPK_{+\mu}$ + fence). Data on the nutrient limitation metric and other site characteristics can be found in Table 3. For further information on the calculation of nutrient limitation metrics see Supporting Documents.

Environmental variables

We used 30 year climate normals (annual averages from the past 30 years) from the National Oceanic and Atmospheric Administration National Climatic Data Center to obtain climate variables from the different sites (NOAA NCDC 2012). The climatic variables that we used were mean annual temperature (MAT, °C) and mean annual precipitation (MAP, mm). Potential evapotranspiration (PET, mm) values were obtained from the EOS-WEBSTER library of earth science data (EOS-Webster 2012). At each site, annual aridity index (AAI) was calculated as a measure of water availability for plants using the formula AAI = PET / P, where PET is potential evapotranspiration (in mm) and P is total precipitation (in mm) (Middetone and Thomas 1997). Therefore, a higher AAI is representative of a more arid site. We did not include MAP directly in statistical analyses since it introduced singularities due to its information redundancy with AAI.

Statistical analysis

We performed an analysis of covariance (ANCOVA) to test for the effects of site, nutrient additions (control, N, P, $K_{+\mu}$, along with interactions) with soil pH as a covariate on percent AMF colonization in *S. scoparium*. pH was included as a covariate as it can have a strong influence on percent AMF colonization (Abbott and Robson 1985), and this particular analysis was meant to test for the effect of nutrient addtions on percent AMF colonization. All analyses were performed using R 3.0 (R Core Development Team 2013). To obtain the most parsimonious model we used stepwise Akaike's Information Criteria with stepAIC()in R package "MASS" (Venables and Ripley 2002). To analyze environmental variables along with nutrients, we used ridge regression (RR), which fits a biased regression to account for correlations between explanatory variables. Ridge regression is similar to a multiple linear regression using ordinary least squares except that the calculation adds a shrinkage parameter, *k*, that prevents fitted coefficients from becoming too large, a sometimes unwanted result of correlated independent variables. The RR approach prevents a false inflation in coefficients that may come about with high multicollinearity among independent variables. For more details on RR, see Appendix A. The variables included in the RR were soil pH, soil % sand, soil % clay, MAT, AAI, Δ-ANPP N, Δ-ANPP P,

and Δ-ANPP $K_{+\mu}$, and N, P, or $K_{+\mu}$. MAT and AAI were treated as ordered factors in the RR to account for pseudo-replication of these variables. Multicollinearity existed among the environmental variables, so bivariate correlation coefficients were computed for each pair of explanatory variables and an assessment of multicollinearity was done by computing a condition number for the regression matrix.

We used both lm.ridge in the R package "MASS" (Venables and Ripley 2002) and linearRidge in the R package ridge (Cule 2014) in our analysis. We used lm.ridge to select an optimal *k* value from a sequence of 50,000 real numbers evenly spaced between 0 and 500. We then used linearRidge with the optimal *k* value to fit that particular linear model. The summary output from linearRidge contains RR coefficients fit to the original data and RR coefficients fit to standardized (mean zero, variance one) explanatory data. We used two packages to fit the RR models as lm.ridge in package "MASS" provided an efficient method for obtaining an optimal *k* using generalized cross-validation and linearRidge in package "ridge" provided estimates for standard errors, t-values, and *p*-values, computed using the methodology of Cule et al. (2011). We began with a RR model that included all nutrient addition (represented as dummy variables 0 or 1 for control or addition, respectively) and their interactions as well as all environmental variables and their interaction with each nutrient added. This resulted in quite a large model, so we used bootstepAIC package (Rizopoulos 2009) in R to find the most parsimoniuous model, which uses bootstraps the stepwise algorithm of the stepAIC algorithm found in package MASS. The results shown are those of a final model that only included significant coefficients from this original model. All coefficients significant in the initial model remained significant in the final model. *P*-values less than 0.05 were taken to indicate statistical significance of an explanatory variable.

Results

Percent AMF colonization changed significantly with the main effects of Site ($F_{4,179} = 10.21$, $P < 0.0001$), N ($F_{1,179} = 9.39$, $P < 0.01$), and pH ($F_{1,179} = 3.98$, $P < 0.05$; Table 4). In general, mean root colonization by AMF was greatest at southern sites and decreased with increasing latitude. Cedar Creek, the most northerly site, had the lowest mean AMF colonization, and Temple, the furthest south, had the highest mean levels of AMF. Across all sites, the addition of N decreased AMF colonization by over nine percentage points in N-fertilized treatments (30.0%) compared to control plots (39.4%, Fig. 2). The only within-site treatment that had a significant effect was the addition of N at CBGB which significantly decreased AMF colonization (Fig. 3).

Table 4 Results from ANCOVA of % AMF colonized by nutrients, site, and pH

Coefficient	D.f.	Sum.Sq	Mean.Sq	F	*p*-value
N	**1**	**1369.53**	**1369.53**	**9.39**	**<0.01**
P	1	194.72	194.72	1.34	0.25
$K_{+\mu}$	1	72.61	72.61	0.498	0.48
Site	**4**	**5958.00**	**1489.50**	**10.21**	**<0.0001**
pH	**1**	**580.19**	**580.19**	**3.98**	**<0.05**
N:P	1	12.81	12.81	0.09	0.77
N:$K_{+\mu}$	1	302.97	302.97	2.08	0.15
P:$K_{+\mu}$	1	396.80	396.80	2.72	0.10
P:site	4	1336.42	334.11	2.29	0.06
N:P:$K_{+\mu}$	1	338.54	338.54	2.32	0.13
Residuals	179	26,106.22	145.85		

Significant results are in bold text

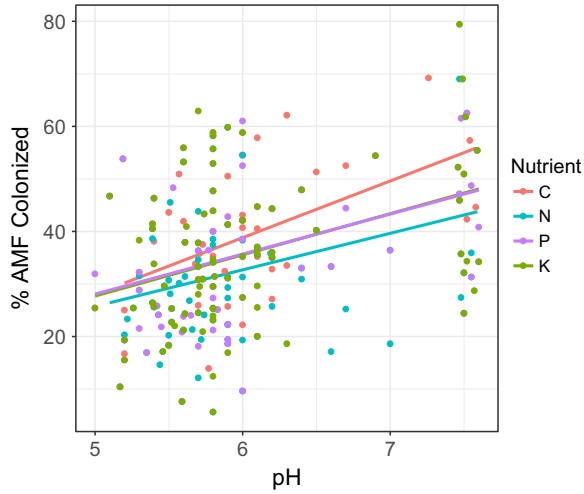

Fig. 2 Scatterplot of % AMF colonized by soil pH with added nutrients represented as different colors. % AMF colonized increased with pH under all nutrient additions ($F_{1;179} = 3.98$, $p < 0.05$), but N addition treatments still significantly lowered AMF colonization ($F_{1;179} = 9.39$, $p < 0.01$). P and K+$_\mu$ additions did not significantly alter % AMF colonization, but are included for reference

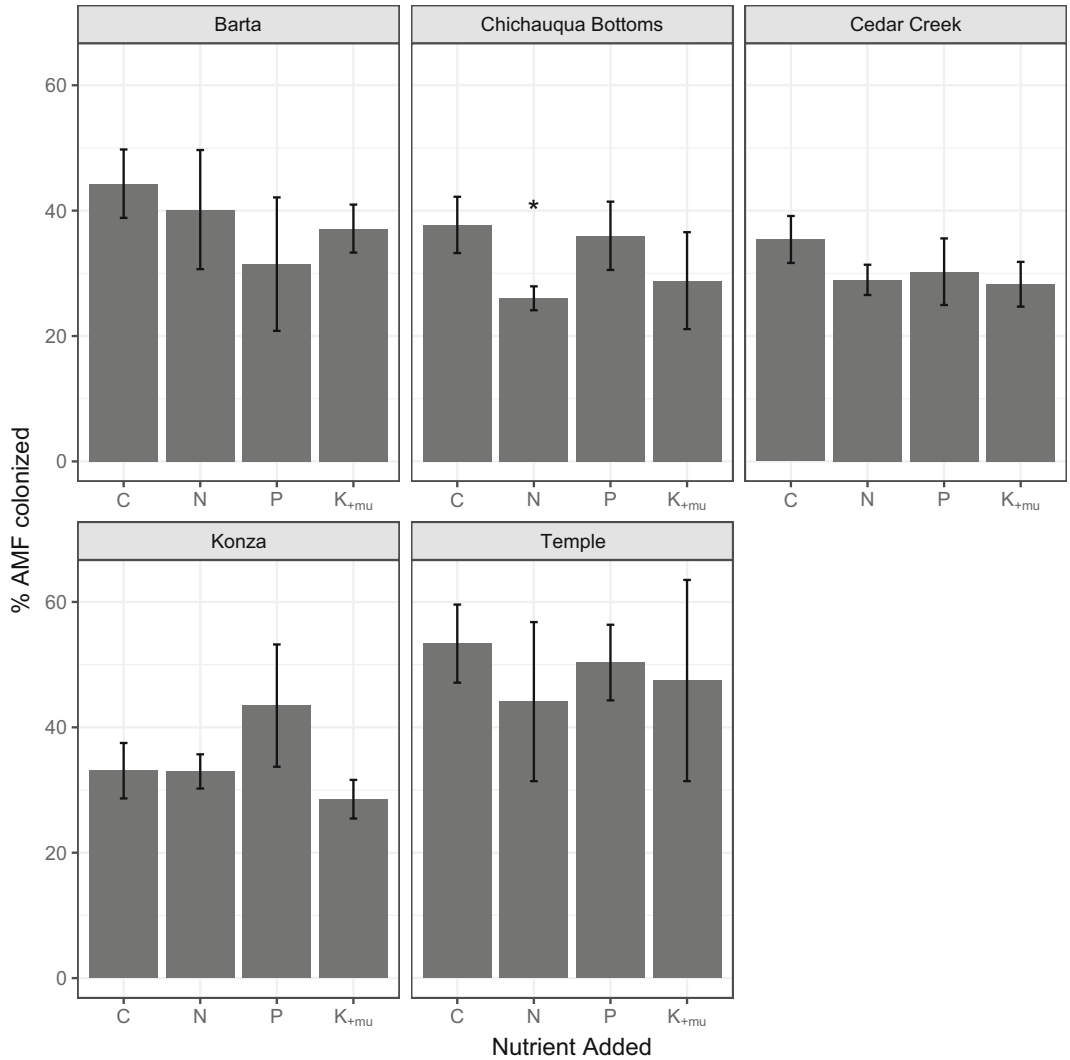

Fig. 3 Barplot showing % AMF colonized in the roots of little bluestem under control (C), nitrogen (N), phosphorus (P), and potassium with mi-cronutrient ($K+_{\mu}$) treatments. When analyzed within sites N only significantly decreased AMF at Chichauqua Bottoms

Almost all pair-wise comparisons of environmental variables were correlated (Table 5), which justified our use of ridge regression. Using ridge regression we found significant positive correlations between AMF colonization and pH ($P < 0.0001$) and MAT ($P < 0.0001$) and AMF colonization significantly decreased with addition of N ($P < 0.01$, Fig. 4, Table 6).

We also found a positive correlation between AMF and the P x MAT interaction term ($P < 0.01$). AMF colonization still increased with MAT, but the slopes were different between the P treatment and control plots. Sites with a lower MAT experienced an increase in AMF colonization when P was added as compared to sites with high MAT, which experienced little to no increase (Fig. 5).

Discussion

Across a broad geographic gradient, we found that AMF colonization increased with MAT and pH, but decreased with the addition of N. Additionally, we discovered an interactive effect between P fertilization and MAT. We hypothesized that the addition of nutrients (especially N and P) would be associated with decreased AMF

Table 5 Pearson correlation coefficients for independent variables included in the ridge regression model

	% sand	% clay	pH	MAT	AAI	Δ-ANPP N	Δ-ANPP P	Δ-ANPP $K_{+\mu}$
% sand	1.00							
% clay	−0.93	1.00						
pH	−0.51	0.73	1.00					
MAT	−0.79	0.93	0.83	1.00				
AAI	−0.47	0.61	0.68	0.78	1.00			
Δ-ANPP N	−0.06	−0.23	−0.57	−0.39	−0.44	1.00		
Δ-ANPP P	−0.14	0.02	0.04	−0.10	0.05	0.16	1.00	
Δ-ANPP K_{+u}	−0.04	−0.06	−0.10	−0.17	−0.27	0.52	0.48	1.00

Δ-ANPP N, P, and $K_{+\mu}$ refer to the nutrient limitation variable for each of those nutrients, respectively

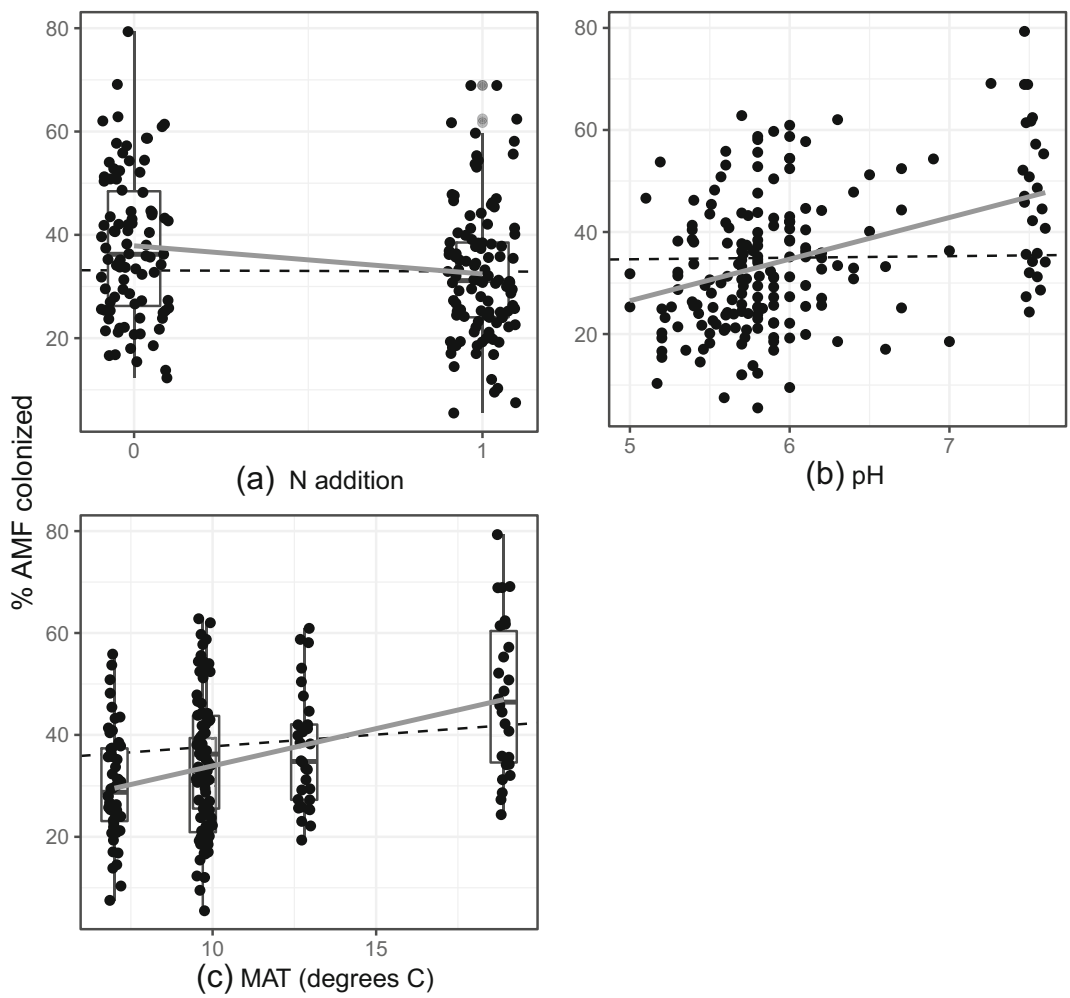

Fig. 4 Scatterplots of significant variables from our ridge regression. y-axes are % AMF colonized for all plots. x-axes are (**a**) 1 or 0 for N added (1) or not (0), (**b**) pH, and (**c**) MAT in degrees Celsius. Solid gray lines are the regression line from an ordinary least squares (OLS) regression while dashed black lines are the coefficient estimate from the ridge regression. Lines from the ridge regression appear relatively at compared to the OLS line { recall that ridge regression is a technique which shrinks coefficients in order to defend against large coefficients which might be falsely estimated due to co-linearity among variables. Hence, the RR estimate shown is biased, but provides an method for testing the desired effect given other correlated variables

Table 6 Output from most parsimonious ridge regression as determined by bootstrapped AIC

Variable	β	Scaled-β	Scaled SE	Scaled t-value	p-value
Intercept	33	–	–	–	–
N	**−0.2**	**−1.4**	**0.51**	**2.8**	**<0.01**
P	0.06	0.42	0.48	0.88	0.38
K	0.048	0.33	0.5	0.67	0.5
pH	**0.3**	**2.8**	**0.5**	**5.7**	**<0.0001**
MAT L	**0.45**	**2.8**	**0.5**	**5.5**	**<0.0001**
MAT Q	0.048	0.29	0.51	0.58	0.56
MAT C	0.009	0.059	0.51	0.12	0.91
MAT4	0.15	0.9	0.51	1.8	0.078
Δ-ANPP P	0.00025	0.4	0.48	0.84	0.4
Δ-ANPP K_{+u}	0.00039	0.46	0.48	0.96	0.34
N x P	−0.072	−0.44	0.48	0.92	0.36
N x K	−0.011	−0.067	0.49	0.14	0.89
P x K	0.12	0.74	0.48	1.5	0.12
P x pH	0.017	0.72	0.48	1.5	0.14
P x MAT L	**0.39**	**1.6**	**0.5**	**3.2**	**<0.01**
P x MAT Q	0.078	0.32	0.51	0.62	0.53
P x MAT C	−0.0043	−0.019	0.51	0.037	0.97
P x MAT4	−0.11	−0.45	0.51	0.89	0.37
N x Δ-ANPP P	−0.00031	−0.38	0.49	0.77	0.44
P x Δ-ANPP P	0.00016	0.17	0.49	0.34	0.73
P x Δ-ANPP K_{+u}	0.00049	0.39	0.49	0.8	0.42
N x P x K	−0.01	−0.052	0.48	0.11	0.91

Significant variables are shown in bold. The L, Q, C and 4th power terms shown after MAT are polynomial contrasts, which R outputs when variables are treated as ordered factors. Scaled columns are those on which the ridge regression was performed to calculate the penalty term for predictor variables on the same scale

colonization and that higher MAT and AAI would lead to increased AMF.

As hypothesized the addition of N did significantly decrease percent AMF colonization among all sites. This is consistent with previous studies on AMF colonization with nutrient addition (Johnson 1993; Treseder and Allen 2002; Johnson et al. 2003; Treseder 2004; Johnson 2010; Liu et al. 2012), which tend to show that the addition of N decreases AMF colonization when N is not so low that it also limits the growth of AMF. N generally seemed to be a limiting nutrient at the sites used in this study when looking at our Δ-ANPP N metric (see Supporting Documents). The reduction of AMF colonization with N addition observed in this study is consistent with previous work on AMF colonization with N fertilization (Kim et al. 2015; Yang et al. 2016) and for nutrients and mutualisms more generally (Shantz et al. 2016). Given that grassland productivity is usually limited by N and often co-limited by a combination of N, P, and/or K (Fay et al. 2015) and the high and increasing rate of N-deposition in many grassland regions (Galloway et al. 2004) and its impact on grassland productivity (Stevens et al. 2015), our results suggest that ongoing N deposition could further reduce AMF colonization in grasslands. Another consequence of N deposition is stoichiometric induced P limitation in plants Li et al. (2016) a nutrient for which plants also depend on AMF. If increased N deposition both increases P limitation and decreases AMF then plants may have even further P limitation. Increased P limitation however will likely depend on ambient levels of P in the soil at a site. For example, the level of AMF colonization decreased at most sites with the addition of N, but did not seem to change at Konza Prairie (Fig. 3), which is known to be relatively more P-limited than other grassland sites (Johnson et al. 2010).

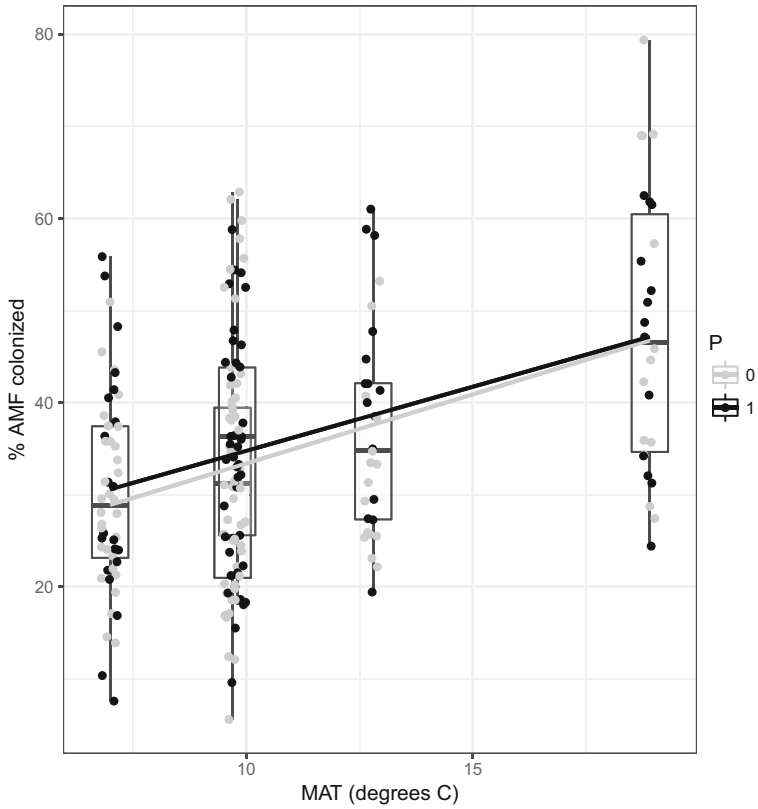

Fig. 5 Interaction plot of the significant interaction term from the ridge regression. % AMF colonized still increased with MAT, but did so at differing slopes when P was added (1). y-axis is the % of roots colonized by AMF and the x-axis is MAT in degrees Celsius

This result is in line with the trade-balance model of mycorrhizal symbiosis, which states that an N-induced P limitation should enhance the plant-AMF mutualism (Johnson 2010) as fungi are generally more limited by N than by P (Johnson et al. 2015). In other words, adding N to an already P-limited system should result in a greater stoichiometric imbalance between N and P and increase relative P-limitation, which would enhance plant-AMF mutualisms there even more.

Our findings of increased levels of AMF with MAT and pH are also consistent with previous work on environmental factors controlling colonization of AMF. Prior studies have shown that AMF are highest in soil pH close to neutral (Postma et al. 2007). Percent AMF colonization in our study only increased with pH; however, the maximum soil pH for our study sites was near neutral. Therefore, it makes sense that we did not see a unimodal relationship between AMF colonization and pH. We also observed a positive correlation between MAT and AMF similar to correlations observed in a global assessment of climatic predictors of AMF colonization (Soudzilovskaia et al. 2015). AMF only increased with MAT in our study, while Soudzilovskaia et al. (2015) found a unimodal relationship between mean temperature of the warmest month at a site and percent AMF colonization with an optimum occurring at a mean monthly temperature of 19.5 °C. We analyzed our data using MAT; however, the mean monthly temperature of the warmest month at our sites ranged between about 24 and 28 °C. This is actually counter to what was found in Soudzilovskaia et al. (2015); however, their results were also correlated with soil C:N where AMF colonization increased with decreasing soil C:N. It is possible that our gradient also happened to fall along a gradient of soil C:N ratio. We did not measure these soil characteristics; however, a gradient of nutrient stoichiometry is known to exist with latitude which is also correlated with MAT (Reich and Oleksyn 2004) and it is possible that our sites fell along the multivariate unimodal space found in Soudzilovskaia et al. (2015) in such a way that AMF increased with temperature and soil C:N. While we used changes in plant ANPP with nutrient addition as a proxy for soil nutrient limitation we did not directly test the levels of soil nutrients and cannot strictly rule out the correlation between AMF, MAT, and changes in latitudinal nutrient stoichiometry.

The relationship to of AMF to MAT could be driven either by increased temperatures themselves or the correlated increased growing lengths at sites with higher MAT allowing plants to acquire carbon throughout a greater portion of the year. Theoretically, plants would have more carbon for the trade-balance model with AMF and could support higher levels of AMF (Johnson 2010). A similar argument could also be made with increased metabolic reactions at higher MAT in plants (Brown et al. 2004). Alternatively, from the fungal perspective, AMF grown at higher MAT could experience less freezing, which has been shown to decrease levels of AMF (Klironomos et al. 2001), compared to AMF grown at more northerly climates. Our results also revealed a significant nutrient by environment interaction in the P x MAT interaction term.. In our study AMF increased with MAT; however, the slopes differed between sites with P added and those without. This could be interpreted in one of two ways: either a) the addition of P increases AMF colonization at sites with lower MAT or b) MAT has less of an effect on the plant-AMF relationship when P is present and abundant. To our knowledge, no interaction between temperature and nutrient addition has been observed in rates of AMF colonization, although temperature and nutrient stoichiometry (C:N) have been shown to collectively influence AMF on a global scale (Soudzilovskaia et al. 2015) so it is plausible that the relationship for MAT and P exists as well. With the scale of our study it is possible that this relationship is driven by the data from the Konza site and the substantial (though not significant) increase in AMF colonization with P addition at that site, which is known to be relatively more P limited (Johnson et al. 2010)., However, the Temple site experienced very little decrease in AMF with the addition of P (Fig. 3). Therefore, the relationship between AMF and MAT is likely not just a correlation between MAT and soil P, but rather likely a complex relationship between both soil, nutrients, and climate.

It is probably worth mentioning that there was a distinct and noticeable difference in root morphologies ranging along the climatic gradient used in our study. More northerly sites generally had very long and fine roots while those of more southerly sites tended to have thicker shorter roots. Overall root length and percent AMF colonized tended to be negatively correlated with each other (*pers. obs.*). It seemed that there was an investment tradeoff between fine roots and root hairs versus outsourcing nutrient acquisition to AMF, which is also likely a complex interaction among nutrients, soil, and climate. In addition to the criticisms associated with using percent root length colonized that were noted in the introduction section of this manuscript readers should also bear in mind the exact definition of the metric, which is a ratio of AMF colonized root segments over all root segments. Therefore, higher levels of percent root length colonized could be achieved via both parties of the symbiosis (i.e. AMF or plants) by either increased AMF growth or reduced plant root growth. This is an important morphological aspect to view these results by, especially as plant root morphology seemed to differ across sites in this study, and should not be taken lightly.

These findings demonstrate that a suite of environmental factors determining plant nutrient and water relations as well as soil factors are both strong predictors of AMF colonization rates in a dominant tallgrass prairie species spanning a large biogeographic region. Our findings are consistent with prior research, but also reveal the importance of environmental predictors in determining AMF colonization and AMF response to nutrients as well as the potential for differential responses of AMF colonization between environmental variables and nutrient addition. Our results show that, although nutrients can and do have important effects on AMF colonization in plants, it is also important to take into account environmental variables when assessing AMF colonization as well as the interplay between the two. Nutrient-related factors (in terms of nutrient addition) were statistically significant in explaining levels of AMF colonization in our study; however, our results suggest that temperature and pH may also play an important role in determining levels of AMF colonization along with N addition. Additionally, we found a significant nutrient by environmental interaction (P x MAT) that revealed a difference in how AMF responds to this nutrient along a gradient of temperature. Various limitations likely exist with mycorrhizae depending on local conditions just as they do with plants, and our results show that it is important to keep these environmental conditions in mind when assessing mycorrhizae and the plant-mycorrhizae relationship.

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Appendix - Description of Ridge Regression

We used ridge regression as opposed to a traditional multiple linear regression as a high degree of multicollinearity existed among our continous explanatory variables. Ridge Regression (RR) guards against fitting a model with extremely large fitted coefficients, which may occur inadvertently when using OLS in the presence of multicollinearity. While ordinary least-squares (OLS) regression fits a model $Y = X\beta + \epsilon$ by selecting β so as to minimize $\|Y - X\beta\|^2$, RR minimizes $\|Y - X\beta\|^2 + \|k\beta\|^2$, where k is the shrinkage parameter. The shrinkage parameter imposes a penalty on regression coefficients that are large. When k is zero RR is the same as OLS. As k increases the penalty for large coefficients increases.

The RR estimate can be obtained with the formula $(\mathbf{X}^T\mathbf{X} + k\mathbf{I})^{-1}\mathbf{X}^T\mathbf{Y}$, and it has an interpretation as a Bayes estimate (Seber & Lee 2012, p. 321). The RR estimate is biased, but there exists k a such that the mean square error of the RR estimate is less than the mean square error of the OLS estimate (Hoerl & Kennard 1970). We set the shrinkage parameter as the minimizer of prediction error with leave-one out cross validation (Golub et al. 1979).

To find the optimal shrinkage parameter we performed our initial RR using lm.ridge() in the R package MASS with k as a vector of 50,000 integers spaced evenly from 0 to 500. Package MASS provides a function select () that chooses the optimal k based on leave-one-out cross validation.

Using leave-one-out cross validation we obtained an optimal shrinkage parameter (k) of 310.5. We then used this k to fit a linear model using linearRidge() in R package ridge since the summary from this output contains t-statistics and p-values determined by the methods in Cule et al. (2011). We performed another RR using the most parsimonious model determined by boot.stepAIC() in package bootStepAIC (Rizopoulos 2009). The optimal k from this secondary RR was 25. The results from this output can be seen in Table 6.

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