American Journal of Botany
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RESEARCH ARTICL

INVITED SPECIAL ARTICLE

For the Special Issue: Plant–Environment Interactions: Integrating Across Levels and Scales

Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis

Rebecca T. Batstone[1,2,5], Madeline A. E. Peters[1], Anna K. Simonsen[3], John R. Stinchcombe[1,4], and Megan E. Frederickson[1]

Manuscript received 17 May 2019; revision accepted 4 November 2019.

[1] Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, M5S 3B2, Canada

[2] Carl Woese Institute for Genomic Biology, University of Illinois at Champaign-Urbana, Urbana IL 61801 USA

[3] Research School of Biology, Australian National University, Canberra, ACT, 2601, Australia

[4] Koffler Scientific Reserve, University of Toronto, King, ON, L7B 1K5, Canada

[5] Author for correspondence (e-mail: rtbats98@illinois.edu)

Citation: Batstone, R. T., M. A. E. Peters, A. K. Simonsen, J. R. Stinchcombe, and M. E. Frederickson. 2020. Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis. *American Journal of Botany* 107(2): 195–208.

doi:10.1002/ajb2.1432

PREMISE: The ecological outcomes of mutualism are well known to shift across abiotic or biotic environments, but few studies have addressed how different environments impact evolutionary responses, including the intensity of selection on and the expression of genetic variance in key mutualism-related traits.

METHODS: We planted 30 maternal lines of the legume *Medicago lupulina* in four field common gardens and compared our measures of selection on and genetic variance in nodulation, a key trait reflecting legume investment in the symbiosis, with those from a previous greenhouse experiment using the same 30 *M. lupulina* lines.

RESULTS: We found that both the mean and genetic variance for nodulation were much greater in the greenhouse than in the field and that the form of selection on nodulation significantly differed across environments. We also found significant genotype-by-environment (G × E) effects for fitness-related traits that were generated by differences in the rank order of plant lines among environments.

CONCLUSIONS: Overall, our results suggest that the expression of genotypic variation and selection on nodulation differ across environments. In the field, significant rank-order changes for plant fitness potentially help maintain genetic variation in natural populations, even in the face of directional or stabilizing selection.

KEY WORDS common garden; *Ensifer* spp.; field experiment; genotype-by-environment interactions; *Medicago lupulina*; quantitative genetics; selection-by-environment interactions; symbiosis.

Mutualisms are often described as context dependent and vulnerable to changing environmental conditions (Bronstein, 1994; Kiers et al., 2010; Chamberlain et al., 2014; Hoeksema and Bruna, 2015; but see Frederickson, 2017). The costs and benefits of mutualism often depend on the environment where the interaction is studied (Hoeksema, 2010; Simonsen and Stinchcombe, 2014a), and there is growing evidence that global environmental change is altering the net outcome of mutualism to interacting partners (e.g., Shantz et al., 2016). The environment not only affects the net benefit to each individual, but has the potential to drastically alter the magnitude, direction, or type of selection acting on traits, as well as the expression of genetic variation in those traits (Wood and Brodie, 2016; Hayward et al., 2018; Lau and terHorst, 2019). For example, under more favorable conditions, the strength of selection acting on a trait often decreases (e.g., Garant et al., 2007), while the expression of genetic variance tends to increase (Charmantier and Garant, 2005). Such selection-by-environment (i.e., S × E) and genotype-by-environment (i.e., G × E) interactions shape the evolutionary trajectories of populations, constraining or facilitating adaptive evolution (Lau et al., 2012; Des Marais et al., 2013; Wood and Brodie, 2015, 2016; Hayward et al., 2018). While S × E and G × E interactions have been documented for traits underlying plant–pollinator mutualisms

(e.g., Anderson et al., 2012), less is known about whether these interactions are present in traits underlying plant–microbe symbioses (but see Wagner et al., 2014). Here, we assessed G × E and S × E interactions for a key trait underlying the legume–rhizobium symbiosis, nodulation, across one greenhouse and four field environments using 30 maternal lines of the symbiotic legume *Medicago lupulina*.

Medicago lupulina and most other legumes engage rhizobia in a nutritional symbiosis in which they exchange plant-fixed carbon for rhizobium-fixed nitrogen. Rhizobia reside in nodules that form on a legume's root system, and the number of nodules on a root is both easy to count and directly correlated with rhizobium fitness (Heath and Tiffin, 2009; Heath et al., 2010), making it a suitable trait for quantifying genetic variation and selection. Both nodule formation and symbiotic nitrogen fixation are costly to the legume (Layzell et al., 1981); thus, nodule number not only represents rhizobium fitness, but also the level of legume investment in the symbiosis. Changes in abiotic nitrogen can affect the evolution of this mutualism (West et al., 2002; Heath et al., 2010; Akçay and Simms, 2011; Weese et al., 2015; Keller and Lau, 2018) because acquiring N abiotically is cheaper for the legume, leading to a reduction in investment into the symbiosis (Glyan'ko et al., 2009; Reid et al., 2011). Furthermore, rhizobia can vary considerably in their quality as mutualists by fixing different amounts of nitrogen or demanding different carbohydrate rewards from their hosts (Parker, 1995; Burdon et al., 1999; Thrall et al., 2000; Sachs et al., 2010; Heath et al., in press in this special issue), meaning that the rhizobia community present in an environment may affect investment in and selection on nodulation. Variation in other environmental conditions, such as herbivory or mycorrhizal associations (Simonsen and Stinchcombe, 2014a; Ossler et al., 2015), can also be important for shaping selection on key mutualism-related traits.

Environmental conditions that modulate the availability of resources important to the legume–rhizobium symbiosis, such as nitrogen and light, could affect not only the outcome of the interaction, but also trait expression even within a genotype, as well as the direction, form, or magnitude of selection acting on key mutualism-related traits. At low light, nodulation and N fixation become more costly to legumes because carbon supply is limited (Lau et al., 2012), and the expression of genetic variance in nodulation is greatly reduced (Heath et al., in press in this issue).

Legumes also exhibit high phenotypic plasticity in response to nitrogen availability, which can fluctuate considerably over short temporal or spatial scales. For example, legumes typically respond to an increase in soil nitrate by downregulating nodulation (Streeter, 1988; Glyan'ko et al., 2009) as a means to optimize investment in N acquisition (Caetano-Anollés and Gresshoff, 1991; Ferguson et al., 2010; Reid et al., 2011). This plasticity is not equal across legumes, however; Heath et al. (2010) found that a legume's nitrate response depended on the combination of interacting legume and rhizobium genotypes, in addition to the level of nitrate in the soil (i.e., G × G × E for nodulation). Many legumes also have ways to deal with the patchy distribution of N in the soil environment, modulating root growth and nodule formation at very fine spatial scales, even within the root system of an individual plant (Zhang et al., in press). In addition to influencing trait expression, the environment can have profound effects on selection. Studies often find strong positive selection for nodulation under N-limiting conditions (Simonsen and Stinchcombe, 2014b; Ossler et al., 2015; Batstone et al., 2017), whereas high N availability causes legumes to become less dependent on their rhizobial partners, potentially resulting in an adaptive decrease in nodulation (Sachs et al., 2018) and rhizobial quality (Weese et al., 2015; Klinger et al., 2016).

Controlled greenhouse experiments are powerful for understanding how changing environmental conditions affect both the expression of and selection on mutualism-related traits. Many of these experiments have focused on traits that allow legumes to preferentially associate with or reward higher-quality rhizobia, often termed partner choice or host sanctions, respectively (Bull and Rice, 1991; Frederickson, 2013). These traits are thought to be important for preventing rhizobia from "cheating" and using legumes as a carbon source without fixing nitrogen (Kiers et al., 2003, 2006).

In one such greenhouse experiment, Simonsen and Stinchcombe (2014b) found significant standing genetic variation for partner choice, or the proportion of nodules formed with an ineffective N-fixing rhizobium, and that plant lines exhibiting stronger partner choice were selectively favored.

In contrast, under field conditions when many rhizobia strains and species are likely present, rhizobia may be patchily distributed or locally scarce (Simonsen et al., 2017), and variation in quality may be more evenly distributed. A trait such as partner choice could therefore have contrasting effects under conditions when few or many partners are available (Batstone et al., 2018).

Burghardt et al. (2018) inoculated legumes with either a multistrain community of 101 rhizobia strains or singly inoculated legumes with each of the 101 strains and found that nodulation in the single-inoculation experiment was only weakly correlated with strain fitness in the multistrain experiment, suggesting that strain competition for host access and/or host preference for particular strains influences selection on nodulation. Whether greenhouse experiments can be used to predict how populations evolve in the field depends on the degree to which G × E interactions are predominately due to changes in rank order, whereby the most-fit genotype in the greenhouse is not the most fit in the field. Quantifying selection on and the expression of genetic variance in mutualism-related traits across multiple environments is necessary for a better understanding of how populations evolve in nature.

We planted 30 lines of the legume *Medicago lupulina* in four plots in old fields in southern Ontario. The same 30 lines were also used in a previous greenhouse experiment (Simonsen and Stinchcombe, 2014b), allowing us to test how the environment impacts the expression of genetic variance (G × E interactions) in and selection (S × E interactions) on a key mutualism-related trait, nodulation. The greenhouse environment differed from the field in several important ways: the only source of nitrogen available to plants was that fixed by rhizobia, only two strains of rhizobia were present in the soil, and the spatial and temporal heterogeneity of key resources (e.g., light, water, nutrients other than N) were minimized. Thus, we expected to find stronger selection and a reduction in the expression of genetic variance for nodulation in the greenhouse compared to the field. By comparing S × E and G × E between the greenhouse and field, as well as among different field plots, we can better understand the role that environmental variation plays in shaping evolutionary responses within the legume–rhizobium mutualism.

MATERIALS AND METHODS

Study system

Medicago lupulina is an annual legume native to eastern Europe and western Asia that was first introduced to North America in the 1700s (Turkington and Cavers, 1978). Being a weedy plant,

M. lupulina typically occurs in disturbed areas, such as along roadsides and in old fields, and reproduces predominantly by selfing. Throughout most of its range, including southern Ontario, it associates mostly with two species of rhizobia: *Ensifer meliloti* and *E. medicae* (Harrison et al., 2017a).

Experimental design

In a previous study, Simonsen and Stinchcombe (2014b) collected *M. lupulina* seeds from 11 subpopulations spanning 1.2 km^2 of the University of Toronto's Koffler Scientific Reserve (KSR, www.ksr.utoronto.ca), located in King, Ontario, Canada. Toward reducing maternal effects, seeds from each subpopulation were selfed for one generation in the greenhouse, and 10 lines from each subpopulation were randomly selected for a total of 110 lines. In the greenhouse, Simonsen and Stinchcombe (2014b) planted seeds from these lines and inoculated each plant with a 3:1 mixture of two strains of *Ensifer*, which they termed a mutualist and an exploiter. In single-strain inoculations, the mutualist promoted plant growth and tended to form pink, N-fixing nodules, while the exploiter formed mostly white, non-N-fixing nodules and decreased plant growth and survival (Simonsen and Stinchcombe, 2014b). The mutualist strain they used was originally isolated from *M. lupulina* growing at KSR, while the exploiter was isolated from *Melilotus alba* growing in a fallow field in southern Ontario where *M. lupulina* co-occurs (Bromfield et al., 2001). Simonsen and Stinchcombe (2014b) quantified partner choice in mixed inoculation by measuring the proportion of nodules on each plant that were white and non-N-fixing.

We chose 30 of these 110 *M. lupulina* lines by ranking each line based on seed mass as determined during seed-bulking in the greenhouse (range = 0.395 to 4.289 g), and selected lines that spanned the full range, resulting in 2–3 lines per subpopulation for all 11 subpopulations. Beginning in mid-May 2014, we prepared approximately 60 seeds of each line in two batches, 2 weeks apart, according to standard protocols (Barker et al., 2006; Simonsen and Stinchcombe, 2014b). Briefly, we nick-scarified seeds, placed them in 95% ethanol for 30 s, 6% v/v sodium hypochlorite for 4 min, rinsed in distilled water for 5 min, and then allowed them to imbibe in distilled water for up to 20 min. We transferred swollen seeds to water-agar plates that were covered with autoclaved filter paper and sealed. We kept seeds in the dark for 3 d at 4°C, then transferred them to room temperature for an additional day to promote radical growth. We then planted germinated seedlings individually into bleach-sterilized 4-inch Kord Lite black pots (JVK, St. Catherine's, ON, Canada) filled with autoclaved washed river sand (New Canadian Lumber and Building Supplies, Toronto, ON, Canada), keeping them in a greenhouse located at KSR. To promote growth before transplanting seedlings in the field, we gave each seedling a one-time supplement of 5 mL of 2.5 mM N fertilizer (recipe of Batstone et al., 2017), and then supplied them with distilled water at least once every 2 d.

We planted 25–30 replicates of each line into four field plots at KSR (Fig. 1A); plots 2 and 3 occurred in open, grassy fields, while plot 1 was surrounded by tall plants, including milkweed and goldenrod, and plot 4 was surrounded mostly by trees. For the first week after planting, we watered plants with rainwater, and then we left plants to grow and form nodules with the existing rhizobia in the soil for approximately 12 weeks. In September, we scored whether each plant survived to harvest as a binary variable (1 = survived, 0 = died), and then we harvested surviving plants and counted their leaves, flowers, fruits, and nodules. We also collected the aboveground (i.e., shoot) and belowground (i.e., root) biomasses and dried them at 60°C for 2 d before weighing them to the nearest 0.001 g.

Nodule occupancy

A month before harvesting the field plots, in August, we harvested a single representative of each of the 30 lines in each plot, to dissect, sterilize, and plate nodules to determine nodule occupancy. Because nodules senesce as plants begin to flower and set fruit (Simonsen and Stinchcombe, 2014a), we completed this earlier harvest to ensure viable rhizobia could be collected. We removed nodules from roots and placed them in microcentrifuge tubes filled with silica gel and cotton. Back in the lab, we imbibed nodules in distilled water overnight at 4°C, surface-sterilized them in 95% ethanol for 15 s and 6% v/v sodium hypochlorite solution for 10 s, and then rinsed them five times with distilled water. We then individually crushed nodules with a sterile cotton swab and streaked their contents onto tryptone yeast (TY) agar (Somasegaran and Hoben, 1985) before incubating plates at 30°C for 3 days. To ensure we were isolating a single rhizobium clone, we repeated the same process of haphazardly picking a colony, streaking onto a new plate, and incubating for 3 d an additional four times. In the final streaking, we used a sterile 1 µL inoculation loop to transfer a single colony into TY broth, and then placed tubes containing the inoculated medium into a shaking incubator set to 30°C and 200 rpm for an additional 3 d. Once cultures grew to an optical density of 0.1–0.2 at 600 nm (ca. 10^8 cells/mL), 2 mL of each culture was pipetted into cryotubes containing 15% v/v glycerol, and tubes were flash-frozen in liquid nitrogen before being placed into a –80°C freezer for storage. Based on whole-genome sequencing, previous work (Harrison et al., 2017b) found surprisingly little nucleotide diversity in the rhizobia associating with *M. lupulina* across a large geographic range in eastern North America. To confirm that *E. meliloti* and *E. medicae* were the main rhizobia associating with *M. lupulina* in our field plots, we haphazardly chose 60 cultures for sequencing. We extracted DNA from cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) and carried out Sanger sequencing on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using universal 16S rRNA bacterial primers (Weisburg et al., 1991) and standard protocols. Sequencing was completed at the Centre for the Analysis of Genome Evolution and Function at the University of Toronto, Ontario, Canada.

Statistical analyses

We used a generalized linear model (GLM) framework in the R environment (R Core Team, 2016) to test for G × E interactions. Using the data set of Simonsen and Stinchcombe (2014b; https://doi.org/10.5061/dryad.j2063), we estimated line means for each trait using methods similar to the ones the authors described (Simonsen and Stinchcombe, 2014b), except that we included only plants from (1) the same 30 lines used in our field experiment, and (2) their mixed inoculation treatment, in which both mutualist and exploiter strains were present. By using data from the mixed-inoculation treatment only, we were able to include a measure of partner choice (i.e., the number of exploiter-occupying nodules divided by total nodule number), allowing us to test whether partner choice as measured

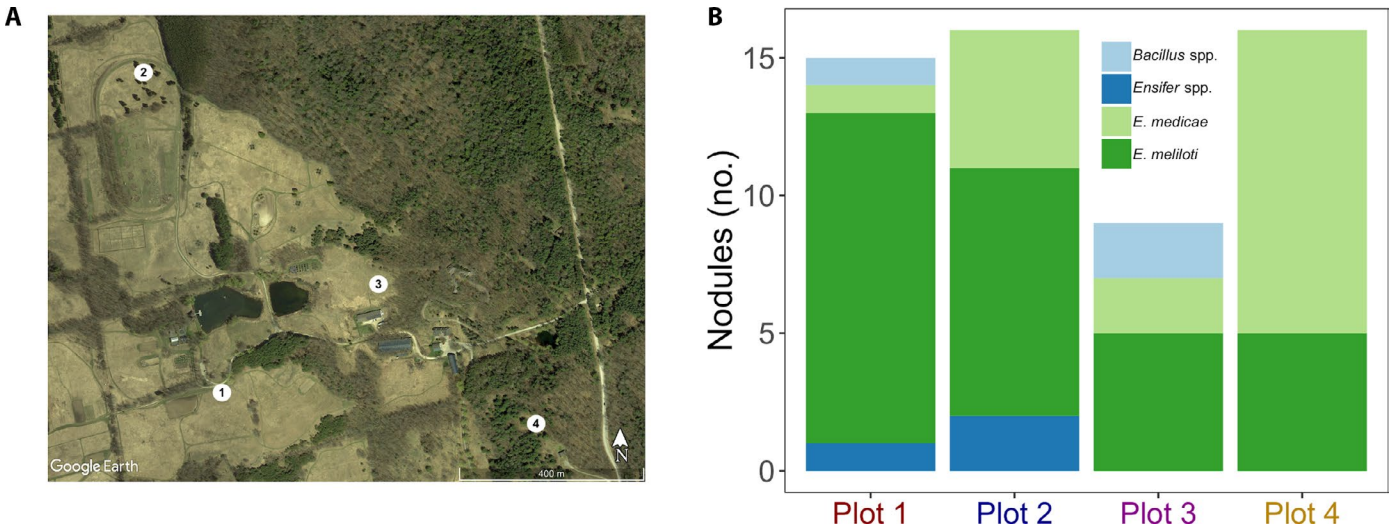

FIGURE 1. Plot locations at the Koffler Scientific Reserve (KSR) and corresponding nodule occupancy results. (A) Field plot locations (1–4) at KSR located in King, ON, Canada. (B) Proportion of nodules in each plot occupied by different rhizobia species identified through Sanger sequencing of bacterial 16S rRNA genes.

in the greenhouse correlates with performance or fitness in the field. We combined this subsetted data set (N = 282 plants) with our field-collected data (N = 1043 plants), and conducted GLMs including the main fixed effects of plant line (30 levels), environment (5 levels: greenhouse and four field plots) and their interaction (i.e., G × E). GLMs were conducted on nine traits, four measured in both experiments (shoot biomass, survival, nodule number, and leaf number), three measured in the field experiment only (flower and fruit success, fruit number), and two measured in the greenhouse only (partner choice, and N-fixing [red] nodule number). Partner choice was calculated by dividing the number of effective N-fixing nodules by total nodule number (i.e., the complement of "ineffective/total" in Simonsen and Stinchcombe, 2014b).

We determined the best-fit probability distribution for each response variable using the fitdist package in R (Delignette-Muller and Dutang, 2015), selecting the fit that gave the lowest Akaike information criterion (AIC) value (Appendix S1). We set contrasts in the R environment to "effects" before running models. For non-binary trait data (i.e., shoot biomass, count data), we used Wald χ^2 tests (car package in R; Fox and Weisberg, 2011) with type II sum of squares to assess the significance of the main effects, and used the joint tests function of the emmeans package (formerly lsmeans, v.2.25; Lenth et al., 2015) with type III sum of squares to assess the significance of the interaction terms. For binary trait data (i.e., survival, flower and fruit success), we used a penalized likelihood method from the logistf package in R (v.1.23) that implements Firth's bias-reduced logistic regression (Firth, 1993). We checked model fit using the DHARMa package in R (v 0.1.6: Hartig, 2018) to assess scaled residuals for GLMs. We were unable to calculate four of the 150 line × environment combinations due to either high mortality or insufficient data ($N \leq 2$), including DU17 in plot 3, and KA24, WR34, and WT39 in plot 4, and thus, these lines were excluded from further analysis. We did not analyze flower and fruit data from field plot 4 because legumes formed no or very few flowers and fruits in this plot.

To quantify genetic variation (Vg) and heritability (H^2 = Vg/Vp) for non-binary traits in both the greenhouse and field, we used a linear mixed model (LMM) framework, treating the main effect of line as a random effect and additionally included the random effects of tray (greenhouse data set only) to account for the spatial arrangement of plants in the greenhouse, and a researcher effect (field data set only) to account for differences among researchers in phenotypic count data. All models also included the fixed effect of block to account for spatial or temporal effects present in the greenhouse or field, respectively. We log-transformed shoot biomass and square root-transformed all count variables to improve normality and analyzed each trait within each environment separately. Heritability in this case is considered "broad sense" (Lynch and Walsh, 1998) because *M. lupulina* is a highly selfing species (Yan et al., 2009); thus, total Vg is a more relevant measure of heritability than additive genetic variance (Roughgarden, 1979). Further, the crosses required to measure additive genetic variation would not reflect natural populations due to unusually high heterozygosities. We calculated heritability by taking the variance for the random effect of line and dividing by total variance (Vp = sum of variance for line, tray, researcher, block and residual). To test whether Vg was significant, we conducted log-likelihood ratio tests comparing LMMs with and without the line term, and present the χ^2 and p-values (after dividing by two for a one-tailed test) for each trait in each environment.

To test for genetic correlations (r_g) among traits across environments, we used the raw line means across environments to calculate a pairwise correlation matrix using the cor function (stats package; R Core Team, 2016), and a corresponding p-value matrix using the corr.test function (psych package v. 1.8.4; Revelle, 2018), correcting p-values for multiple tests. To visualize the correlations, we used the corrplot package in R (v. 0.84; Taiyun and Viliam, 2017). Significant correlations from this analysis indicate that traits are genetically correlated across environments. We tested for additional correlations using the line means calculated within each environment, to test for all pairwise trait comparisons both within and across environments.

We decomposed significant G × E interactions for fitness-related traits (shoot biomass, leaf number, fruit number) into changes in scale versus changes in rank. With a change in scale, genotypic values change in magnitude across environments, but the rank order among genotypes remains constant. Conversely, a change in rank results when the order of genotypic values varies across environments. When the

genotypic value of interest is a proxy for fitness and changes in rank are largely driving G × E, this suggests that the relative fitness of the different genotypes is dependent on the environment. We quantified the relative importance of change in scale versus rank by considering their respective contributions to the G × E interaction component of variance. Specifically, we adopted the method laid out by Cockerham (1963); see Muir et al. (1992) for additional explanation.

We calculated the variance V_{IC} associated with imperfect correlation, or change in rank, for a given genotype (g) in environments 1 and 2 as $[2\times\sqrt{V_{g1}}\times\sqrt{V_{g2}}\times(1-r_{12})]$, where r_{12} is the genetic correlation of expression of genotype g in the two environments. Across e environments, this expression is generalized as

$$V_{IC}=\sum_{i<j}^{e}[2\times\sqrt{V_{gi}}\times\sqrt{V_{gj}}\times(1-r_{ij})]/e(e-1), \quad (1)$$

where, V_{gi} and V_{gj} are variances among genotypes in the i^{th} and j^{th} environments respectively. For each environment, we extracted the variance for the line term (i.e., V_g) from the LMM's used to determine heritability and additionally extracted the correlation coefficients (r) for each trait between environments as determined using the cor function described above (e.g., line means for trait 1 in environment X correlated with line means for trait 1 in environment Y). We calculated the variance associated with heterogeneous variance (V_{HV}), or change in scale, for a given genotype in environments 1 and 2 as $(\sqrt{V_{g1}}-\sqrt{V_{g2}})^2$. Across e environments, this is generalized as

$$V_{HV}=\sum_{i<j}^{e}[(\sqrt{V_{gi}}-\sqrt{V_{gj}})^2]/e(e-1). \quad (2)$$

Again, V_{gi} and V_{gj} are variances among genotypes in the i^{th} and j^{th} environments. The G × E interaction component of variance that is driven by change in rank is thus simply $\frac{V_{IC}}{V_{IC}+V_{HV}}$.

Finally, we used the line means in quadratic models that test for linear (i.e., directional) and nonlinear (i.e., stabilizing, disruptive) selection on nodulation, which represents legume investment in the symbiosis. We fit ANCOVAs that included the main and interactive effects of environment, allowing us to test whether selection on nodulation differed among environments (i.e., S × E), and then conducted linear models testing for selection on nodulation within each environment separately. Specifically, we calculated the total directional selection differential (S) for nodules, which also includes indirect selection on any correlated traits (Lande and Arnold, 1983; Rausher, 1992). We used relativized survival, shoot biomass, leaf number, and fruit number as proxies for fitness (i.e., line mean divided by global mean) and standardized nodule number (i.e., (line mean – global mean)/global SD). Here, global means and SDs are calculated across (rather than within) environments, because we purposely chose environments that likely differed in mean fitness and trait values and wanted to preserve those differences (De Lisle and Svensson, 2017) and to ensure that both the mean and SD are not confounded with potential differences in selection (terHorst et al., 2017). To confirm that our results were not dependent on the method used to relativize fitness (De Lisle and Svensson, 2017), we additionally examined S × E interactions using (1) absolute fitness and (2) locally (within-environment) relativized and standardized traits.

RESULTS

Medicago lupulina associates with both *Ensifer meliloti* and *E. medicae* in the field

We harvested 1043 plants from the four field common gardens, with an average of 8–9 replicates per maternal line per plot (range: 3–25). We obtained sequence data for rhizobia from 56 nodules from 39 plants, including 23 of the 30 maternal lines. Just over 55% of nodules were occupied by *Ensifer meliloti*, 35% were occupied by *E. medicae*, and the remaining 10% by unidentified species of *Ensifer* or free-living soil microbes (*Paenibacillus* and *Bacillus* spp.) (Fig. 1B). Both *E. meliloti* and *E. medicae* were present in all four field plots, but *E. meliloti* was more abundant in plot 1 (12/15 nodules), while *E. medicae* was more abundant in plot 4 (11/16 nodules) (Fig. 1B).

Significant G × E was evident for measures of performance and fitness

Greenhouse plants made more nodules but fewer leaves and less shoot biomass than field plants, and for most variables, there was also substantial variation among field plots (Fig. 2). The GLMs revealed a significant main effect of environment for every trait measured in multiple environments (Appendix S1). There were also significant plant line × environment (G × E) interaction terms for all non-binary traits (Fig. 2, Appendix S1). Based on Cockerham's method, the percentage contribution of changes in rank order versus scale in generating G × E interactions depended on the trait examined, ranging from 95% due to rank for shoot biomass, 54% for leaf number, and 30% for fruit number. In the GLMs, the main effect of plant line was also significant for all traits. However, broad-sense heritabilities (H_2) for traits calculated within each environment were quite low (range = 0–0.380), and many were not significantly different from zero (Appendix S2).

Significant S × E evident for two fitness proxies

Based on globally relativized and standardized trait values, we found significant S × E interactions for nodulation when shoot biomass and leaf number were used as fitness proxies, but not for survival or fruit number (Fig. 3, Table 1). The magnitude of selection differed among environments (Fig. 3), with linear selection differentials ranging from 0.088 in the greenhouse to 3.778 in field plot 2 for shoot biomass (Table 2). We also detected significant nonlinear selection at field plot 2 for shoot biomass only (Fig. 3, Table 2). Additionally, S × E interactions were found when we used absolute, rather than relativized, fitness measures, indicating that interactions were not due to the scaling method employed (Appendices S3, S4). However, S × E interactions were not found when we relativized and standardized traits within each environment; we found significant positive directional selection on nodulation regardless of the environment (Appendices S3, S4).

Genetic correlations (r_g) were stronger within versus between environments

Regardless of environment, measures of plant size and fitness were always significantly positively correlated (Fig. 4); not surprisingly, lines that made more leaves also had greater shoot biomass, and similarly, lines that successfully produced flowers also produced fruits. We also found a significant positive genetic correlation

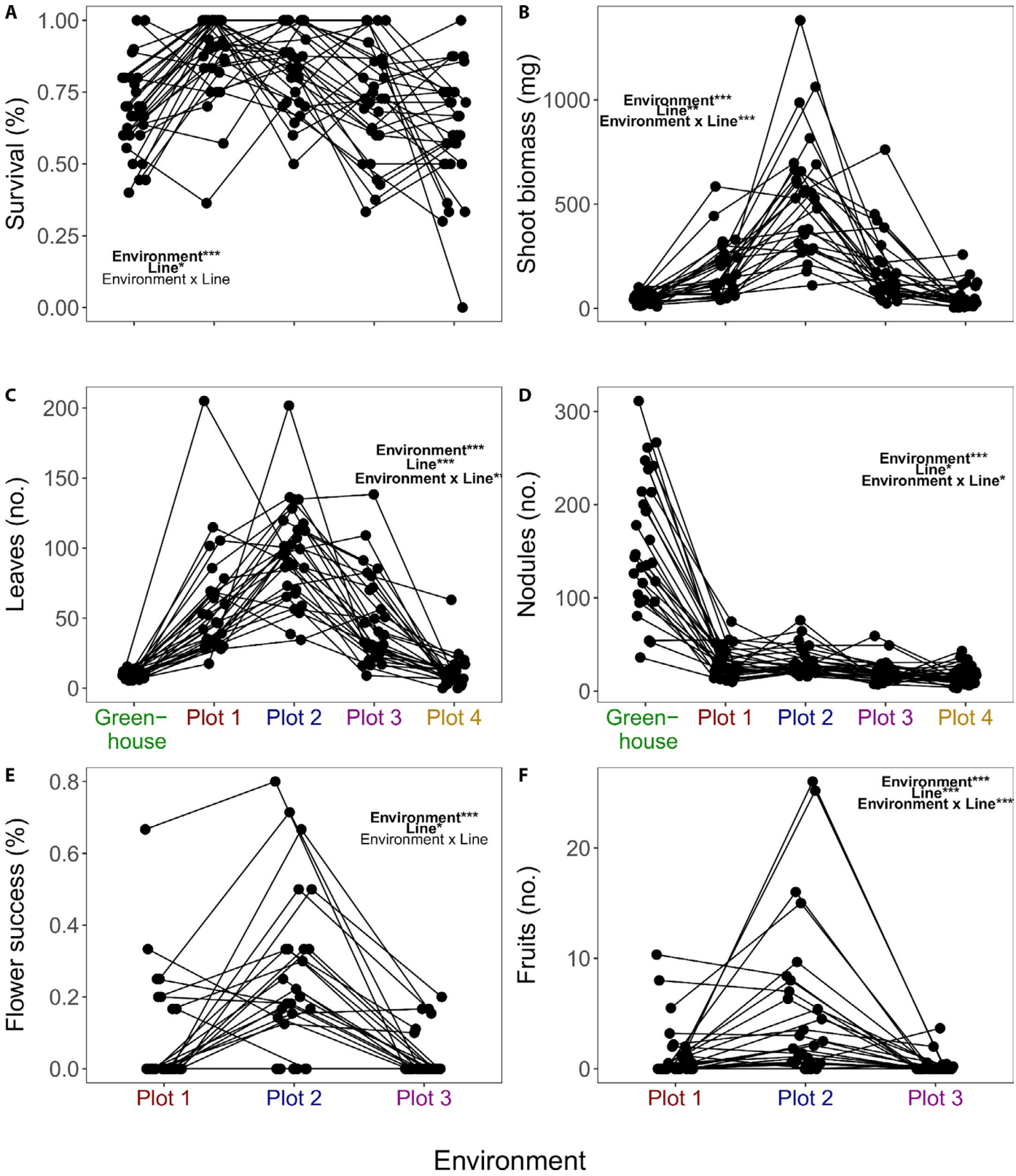

FIGURE 2. Reaction norms of six traits (A–F) measured in the greenhouse and in four field common gardens for the 30 lines of *Medicago lupulina*. Each dot is a line mean calculated within each environment; black lines connect dots for the same plant line. Significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

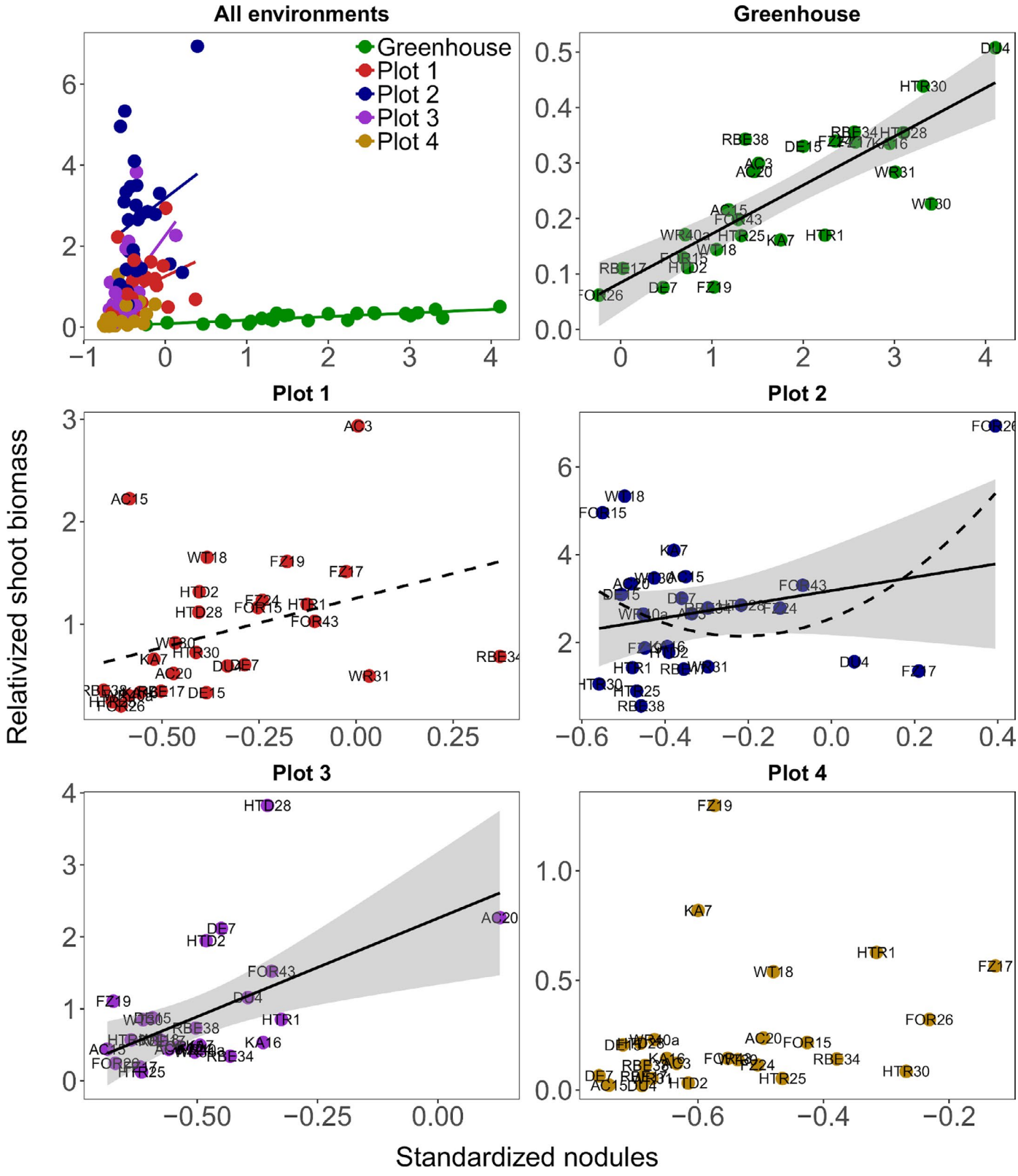

FIGURE 3. Selection on nodule number using shoot biomass as a fitness measure. Solid lines with shading represent significant selection differentials ($p < 0.05$) and corresponding standard errors; dotted lines without shading represent marginally significant selection differentials ($0.05 < p < 1$). The "All environments" panel illustrates the S × E analysis showing significantly different types of selection on nodulation among environments (Table 1). The corresponding selection differentials, intercepts, and quadratic terms calculated within each environment appear in Table 2.

TABLE 1. Analysis of the covariance (ANCOVA) tables for linear models that test for selection × environment (S × E) interactions. The response variables are line means for relativized fitness proxies (survival, shoot biomass, leaf number, flower success, fruit number, fruit success); the covariate is standardized nodule number (i.e., sNods). *F*-values are based on type III sum of squares. Bold and italicized terms are significant ($p < 0.05$) and marginally significant ($p = 0.05$–0.1), respectively.

Response variable	Term	Sum of squares	Df	*F*-value
Traits measured in all five environments (greenhouse and four field plots)				
Survival	**(Intercept)**	23.396	1	**508.434*****
	sNods	0.035	1	0.758
	Environment	1.181	4	**6.415*****
	sNods:Environment	0.102	4	0.554
	Residuals	5.982	130	NA
Shoot biomass	**(Intercept)**	13.565	1	**23.389*****
	sNods	0.466	1	0.803
	$sNods^2$	0.170	1	0.293
	Environment	16.433	4	**7.084*****
	sNods:Environment	8.437	4	**3.637****
	$sNods^2$:Environment	8.843	4	**3.812****
	Residuals	66.696	115	NA
Leaves	**(Intercept)**	49.238	1	**158.572*****
	sNods	8.686	1	**27.972*****
	Environment	31.847	4	**25.640*****
	sNods:Environment	10.880	4	**8.759*****
	Residuals	40.366	130	NA
Traits measured in the field only				
Flower success	**(Intercept)**	23.761	1	**11.730*****
	sNods	0.856	1	0.423
	Environment	16.757	2	**4.136***
	sNods:Environment	0.985	2	0.243
	Residuals	158.000	78	NA
Fruits	**(Intercept)**	19.555	1	**5.259***
	sNods	0.009	1	0.002
	Environment	10.783	2	1.450
	sNods:Environment	3.030	2	0.407
	Residuals	290.055	78	NA
Fruit success	**(Intercept)**	30.195	1	**20.748*****
	sNods	2.969	1	2.040
	Environment	9.122	2	**3.134***
	sNods:Environment	2.129	2	0.732
	Residuals	113.515	78	NA

Significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

between the number of red (i.e., effective N-fixing) nodules (measured in the greenhouse only) and total nodule number (measured in all environments) (Fig. 4), indicating that lines that form more nodules with effective N-fixers form more nodules overall. We also found that trait correlations tended to be stronger within rather than between environments (Fig. 5).

DISCUSSION

We paired a previous greenhouse experiment with a field experiment using the same 30 lines of *M. lupulina* to test whether important traits underlying the legume–rhizobium symbiosis showed significant G × E or S × E interactions. We found that environmental variation affected trait expression, which plant genotypes were favored by selection, and the type of selection acting on nodulation. Legumes made many more nodules under greenhouse than field conditions, whereas plants grew much larger in the field. The most-fit genotypes tended to vary across environments, potentially promoting the maintenance of genetic variation within field populations. Finally, differences in mean fitness among field plots resulted in environmental heterogeneity in selection. Taken together, our results emphasize the importance of environmental variation in shaping both the expression of genetic variation and selection on key traits in the legume–rhizobium symbiosis.

Differences in mean nodulation across environments

The level of legume investment into the symbiosis, captured by nodule number, differed not only between the greenhouse and field, but also among field plots (Fig. 2D). The power of greenhouse experiments is that they can be stripped down to isolate particular variables of interest, but as a result, they also simplify the ecological conditions plants experience. To ensure nodulation and reduce contamination in greenhouse experiments, legumes are often inoculated with high densities of rhizobia, upward of 10^6 cells/mL (e.g., Simonsen and Stinchcombe, 2014b; Batstone et al., 2017), potentially orders of magnitude higher than rhizobia densities in natural soils that also contain thousands of other microbial species. Additionally, legumes are often supplied with N-free fertilizer, facilitating measures of rhizobia quality, or the fitness effect rhizobia have on hosts. Thus, it is not surprising that legumes formed many more nodules

TABLE 2. Models testing for linear and nonlinear selection on standardized nodules (i.e., sNods) within each environment. ANOVA *F*-values (type II sum of squares), *t*-values and standard errors (SE) associated with model estimates are presented for the linear selection term in every model, and additionally for the quadratic term ($sNods^2$) when this term approached significance. Bold and italicized terms are significant ($p < 0.05$) and marginally significant ($p = 0.05$–0.1), respectively.

		Shoot biomass (g)			Leaves (no.)		
Environment	**Term**	**Sum of squares**	**df**	***F***	**Sum of squares**	**df**	***F***
Greenhouse	sNods	**0.242**	1	50.96***	**0.052**	1	37.17***
	Residuals	0.114	24	NA	0.037	26	NA
Plot 2	sNods	**10.334**	1	5.40*	**2.446**	1	4.37*
	$sNods^2$	*7.657*	*1*	*4.00*			
	Residuals	44.044	23	NA	14.539	26	NA
Plot 3	sNods	**5.128**	1	10.11**	**4.334**	1	11.64**
	Residuals	12.170	24	NA	9.681	26	NA
Plot 4	sNods	0.173	1	2.06	0.091	1	1.26
	Residuals	2.011	24	NA	1.868	26	NA
		S	**SE**	**t-value**	***S***	**SE**	**t-value**
Greenhouse	(Intercept)	**0.085**	**0.026**	3.31**	**0.155**	**0.013**	12.14***
	sNods	**0.088**	**0.012**	7.14***	**0.039**	**0.006**	6.10***
Plot 1	(Intercept)	**1.254**	**0.211**	5.96***	**1.921**	**0.231**	8.30***
	sNods	*0.964*	*0.509*	*1.89*	**1.845**	**0.564**	3.27**
Plot 2	(Intercept)	**2.552**	**0.558**	4.57***	**2.544**	**0.238**	10.67***
	sNods	**3.778**	**1.626**	2.32*	**1.290**	**0.617**	2.09*
	$sNods^2$	*8.726*	*4.364*	*2.00*			
Plot 3	(Intercept)	**2.257**	**0.448**	5.04***	**2.117**	**0.327**	6.47***
	sNods	**2.735**	**0.860**	3.18**	**2.150**	**0.630**	3.41**
Plot 4	(Intercept)	**0.521**	**0.195**	2.67*	**0.438**	**0.179**	2.46*
	sNods	0.492	0.343	1.43	0.350	0.311	1.12

Significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

in the greenhouse than in the field (our study Fig. 2D; Ossler et al., 2015; terHorst et al., 2018). However, even across field plots, we found variation in mean nodulation, being higher at plots 1 and 2 and lower at 3 and 4 (Fig. 2).

In the absence of abiotic N in the soil or biotic N provided by other symbionts such as arbuscular mycorrhizal fungi, legumes may rely exclusively on N fixed by rhizobia and as a result, invest more heavily into the symbiosis. Yet, low abiotic N and high rhizobia densities do not guarantee high legume investment. For example, effective N-fixing rhizobia could be abundantly available, but associating with such strains could lead to greater herbivory because they make leaves more N-rich and palatable to herbivores (Simonsen and Stinchcombe, 2014a). Additionally, low light levels could increase the cost of associating with rhizobia (Lau et al., 2012; Heath et al., in press in this issue). Field studies that simultaneously measure nodulation and multiple environmental factors including soil characteristics (e.g., Regus et al., 2017), plant competition (e.g., terHorst et al., 2018), and soil community composition would be powerful for identifying the most important factors explaining variation in symbiotic investment.

Environmental heterogeneity in selection driven by differences in mean fitness

Based on within-environment selection analyses, we found that the type of selection acting on nodulation differed across environments, ranging from no selection (e.g., plot 4, Fig. 3), to directional selection, where plants that formed the most nodules gained a selective advantage (e.g., plots 1 and 3, Fig. 3; Table 2), to disruptive selection, where plants that formed very few or many nodules gained a selective advantage (e.g., plot 2, Fig. 3; Table 2). Different forms of selection on nodulation suggest that the benefit of nodulation is largely environment-dependent. Theoretically, the carbon cost of nodulation may begin to exceed the benefits of N fixation at a critical nodulation threshold (Sachs et al., 2018), and thus, could lead to stabilizing selection on nodulation. However, this critical nodulation threshold is likely contingent on factors that differ among environments, including the availability of N in the soil, the availability of rhizobia and other microbial partners, as well as the other plant species present.

The analyses based on both absolute and globally relativized fitness proxies (i.e., shoot biomass and leaf number) revealed significant S × E interactions, while local models, where traits were standardized and relativized within each environment, did not (Table 1, Appendix S3). These S × E interactions thus appear to be mostly driven by differences in mean fitness across environments (which are eliminated mathematically when fitness is relativized within each environment), rather than by differences in the covariance between nodulation and fitness. Whether differences in mean fitness generate S × E interactions depends critically on whether the legume populations we studied were regulated locally and under soft selection, or globally and under hard selection (Gomulkiewicz and Kirkpatrick, 1992; Kelley et al., 2005; De Lisle and Svensson, 2017). Under hard selection, the contribution of a patch or habitat to the gamete pool depends on the mean fitness of the patch, while the contributions of each patch to the gamete pool are fixed under soft selection and independent of the mean fitness of the individuals in it (Levene, 1953; Gomulkiewicz and Kirkpatrick, 1992). We only found appreciable flower and fruit production at field plots 1 and 2 (Fig. 2), suggesting that genotypes at those sites contribute more to the global gamete pool than the sites with little to no flower and fruit success. Additionally, if N availability and rhizobial

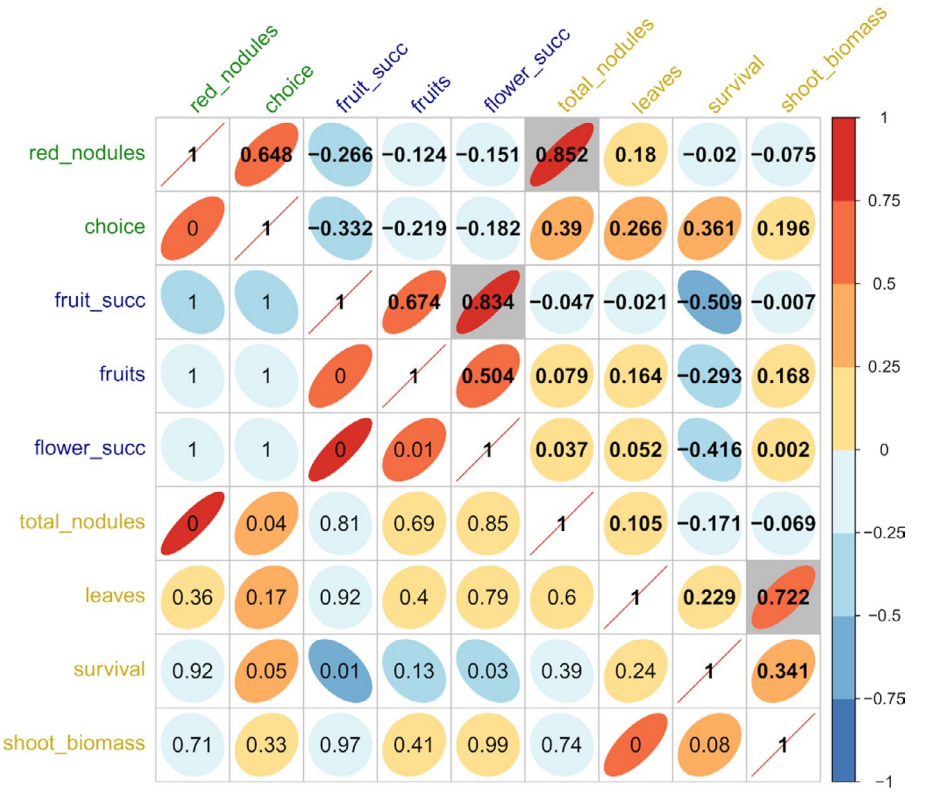

FIGURE 4. Correlation plot for line means across environments. Numbers above the diagonal indicate correlation coefficients, which are also represented in the color key on the right; numbers below the diagonal are *p*-values. A gray background indicates correlations that are significant after correcting for multiple tests. Traits in yellow were measured in all environments; green or blue traits were measured in the greenhouse only or in the field only, respectively.

densities differ across environments, viability selection on genotypes dispersed to these environments (e.g., due to differences in the production of nodules) could lead to different numbers of adults in these locations, thus supporting hard selection (cf. Gomulkiewicz and Kirkpatrick, 1992). Overall, our results overwhelmingly suggest that nodulation, which reflects legume investment into the symbiosis, is under positive directional selection in both the greenhouse as well as natural field populations, and that differences in mean fitness among field plots likely drives environmental heterogeneity in selection.

G × E interactions for fitness

Our GLMs fit to the whole data set, including all five environments, revealed a significant main effect of plant line for every trait we measured (Appendix S1). When we calculated within each environment separately, however, trait heritabilities were comparable between the field and greenhouse (Appendix S2), but were generally quite low (<0.10) and often not significantly different from zero (Table S2). Conner et al. (2003) also compared trait heritabilities in the field versus greenhouse for the wild radish *Raphanus raphanistrum* and found that both a reduction in additive genetic variance (V_g) and an increase in environmental variance (V_p) contributed to heritabilities being lower in the field compared to the greenhouse. We found that both V_p and V_g varied considerably depending on the trait and the environment from which these terms were calculated (Appendix S2). Overall, we found clear evidence that lines differed significantly, but because we only examined 30 plant lines, we potentially lack the power to detect differences among lines within each environment.

Our GLMs also revealed significant G × E interactions for shoot biomass, as well as leaf, nodule and fruit number (Appendix S1). Finding significant G × E interactions among the greenhouse and field is not all that surprising, given the multitude of factors that differ between these environments. Yet, G × E interactions have been detected even when comparing the same genotypes of *Arabidopsis thaliana* across highly controlled laboratories; slight differences in light quality were enough to produce significant G × E interactions for plant growth phenotypes and metabolic profiles (Massonnet et al., 2010). Light intensity differed among our field plots: 2 and 3 occurred in open fields, whereas 1 and 4 were surrounded by tall grasses or trees (respectively), and thus, experienced greater shading. Concordant with other experiments that manipulated light intensity (e.g., Heath et al., in press in this special issue), we found a much reduced expression of genetic variance for plant growth and fitness traits in plots that experienced greater shading (i.e., plots 1 and 4 in Fig. 2).

We further partitioned G × E interactions into changes in scale versus rank. Differences in scale for fitness-related traits (i.e., fruit number) mean that genetic variance in these traits differs among environments (i.e., vertical distribution of line means within each environment in Fig. 2), whereas differences in rank mean that the identities of the most-fit genotypes differ among environments (Wade, 2007) (i.e., crossing lines between environments in Fig. 2). Greater genetic variance in fitness-related traits permits a more rapid response to selection (Fisher, 1958), which would be most prevalent at field plot 2 for fruit number (Fig. 2F). Rank-order changes are likely if the expression of a particular trait that makes a plant line competitively superior in one environment trades off with its performance in another environment (Des Marais et al., 2013). For example, lines that form more effective N-fixing nodules in the greenhouse could be at a disadvantage in the field if herbivores prefer N-rich leaves (Simonsen and Stinchcombe, 2014a). Although rank-order changes for fitness-related traits were not as high compared to performance-related traits (i.e., 30–40% versus 50–95%, respectively), we did not find any significant genetic correlations among field plots (Fig. 5), whereas fitness traits measured within the same environment were always significantly correlated (Fig. 5). Significant rank-order changes for fitness could help maintain genetic variation in natural populations, even in the face of strong directional selection, because different genotypes are selectively favored across environments (Curtsinger et al., 1994; Wade, 2007).

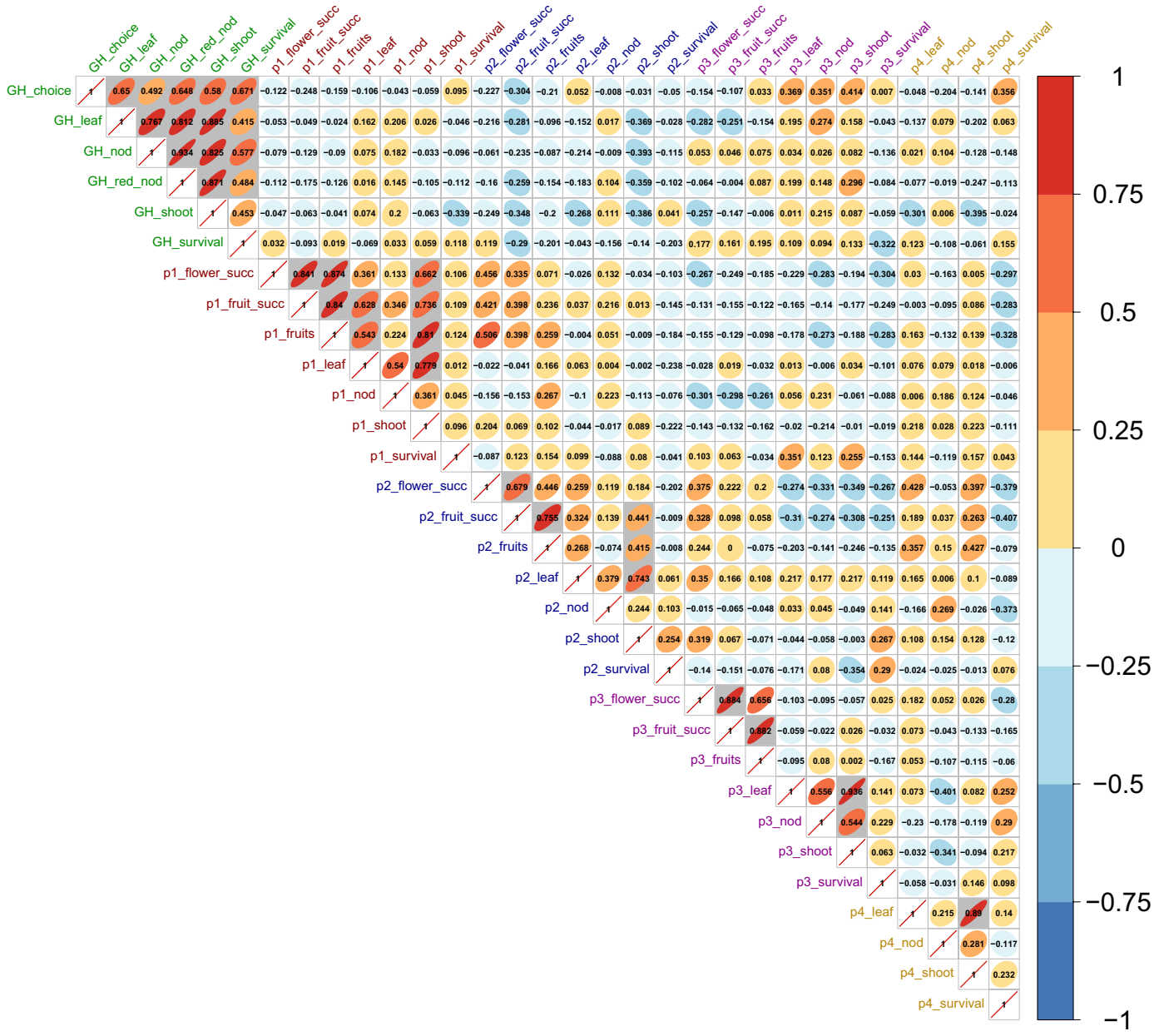

FIGURE 5. Correlation plot for lines means within each environment. Numbers indicate correlation coefficients, which are also represented in the color key on the right. A gray background indicates correlations that are significant after correcting for multiple tests. GH indicates traits measured in the greenhouse, p1 in field plot 1, p2 field plot 2, p3 field plot 3, and p4 field plot 4.

CONCLUSIONS

The net ecological outcome of mutualism often depends on the abiotic or biotic environment in which the interaction is studied (Chamberlain et al., 2014; Hoeksema and Bruna, 2015), and greenhouse experiments can be used to pinpoint the factors that largely determine the outcome. However, greenhouse conditions may not be reflective of field conditions, especially when the most-fit genotypes in the greenhouse are not predictive of those in the field. By pairing both greenhouse and field experiments and measuring trait expression and selection on key traits on the same genotypes, we can better understand how plant–microbe symbioses evolve in the wild. Our comparison of the same 30 *M. lupulina* genotypes across greenhouse and different field conditions shows how profoundly the environment impacts the expression of genetic variance in fitness, and selection on nodulation, a key symbiosis trait. Rather than a single genotype being selectively favored across all environments, we found that the identity of the most-fit genotype depended on the environment, potentially promoting genetic variation in field populations. Future studies could take a similar approach, but compare environments with known differences (such as the level

of N addition apparent at agricultural sites versus old fields), to better understand the evolutionary trajectories of plant–microbe symbioses under changing environmental conditions.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for their insightful feedback on the manuscript. We also thank the army of undergrads who made this large-scale experiment possible, including Daniel Li, Donglin Wang, Steffi-Anne Alveza, Grant de Jong, Samriti Chandi, Jingqi Hao, Boyang Qi, Shree Senthivasan, Junielle Graham, Simran Dhunna, Monika Ashwin, Jessica Santilli, Andrea Sarbu, and Yumeng Fu. We also thank members of the Frederickson Lab who helped with the field plot setup, planting, and harvesting, including: Emily Dutton, Adam Cembrowski, and Eric Youngerman. The Koffler Scientific Reserve Manager, Steph Schneider, was a lifesaver, bailing us out of the mud and assisting with field plot maintenance and equipment needed throughout the experiment. The EEB horticulturists Bruce Hall and Andrew Petrie very helpfully delivered the many hundreds of kilograms of sand used in the experiment. Finally, we acknowledge funding from NSERC Discovery Grants (M.E.F., J.R.S.), a Zimmerman–Weis Scholarship in Field Biology (R.T.B.), and the University of Toronto (M.E.F. and R.T.B.).

AUTHOR CONTRIBUTIONS

R.T.B., A.K.S., J.R.S., and M.E.F. conceived the study and designed the field experiment; A.K.S. provided seeds; R.T.B. conducted the experiments; R.T.B. and M.A.E.P. collected and analyzed the data; R.T.B. and M.E.F. wrote the manuscript; M.E.F., M.A.E.P., J.R.S., and A.K.S. edited the manuscript and provided extensive feedback on the statistical analyses.

DATA AVAILABILITY

All data and code for analyses are available on GitHub: https://github.com/rtbatstone/Batstone_et_al_2019_AJB. The greenhouse data set previously published by Simonsen and Stinchcombe (2014b) was accessed from the Dryad Digital Repository: https://doi.org/10.5061/dryad.j2063.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. ANODEV tables for fixed effects of GLMs testing for G × E.

APPENDIX S2. Broad-sense heritabilities (H2) and significance tests for genetic variance.

APPENDIX S3. ANCOVA outputs from S × E models that compare different scaling methods.

APPENDIX S4. S × E for nodule number, comparing different scaling methods.

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