# Title: Environment dependence of rhizobial relative fitness in the legume-rhizobia symbiosis

Liana T. Burghardt[a,b], Brendan Epstein[a], Michelle Hoge[a], Diana Trujillo[c], and Peter Tiffin[a]

[a]Department of Plant and Microbial Biology, University of Minnesota, St. Paul, MN 55108
[b]current affiliation Department of Plant Science, The Pennsylvania State University, University Park, Pennsylvania
[c]Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108

*Running Title:* Environmental dependence of rhizobial fitness

*Keywords:* nitrogen addition, inoculation density, community complexity, genotype by environment, legume-rhizobia, rhizobial fitness, nodules, strain competition, host benefit

*Author for correspondence:*
Liana T. Burghardt
Department of Plant Science
109 Tyson Building
University Park, PA 16802
liana.burghardt@gmail.com
1-(814)-863-6168

*Authorship:* LTB designed the experiments, collected and analyzed data, and wrote the manuscript, BE designed the experiments, collected and analyzed data, MH and DT collected data, and PT designed the experiments, collected data, and wrote the manuscript

*Data Accessibility:* The data supporting the results of this publication and code necessary for these analyses will be archived in a Dryad repository (XXXXX) and the raw reads have been deposited in NCBI BioProject PRJNA401437 (SRX4557823–SRX4557965).

*Supplemental Figures and Tables:*

*Abstract:*

Spatial and temporal variation in resource availability, population density, and composition likely affect the ecology and evolution of symbiotic interactions. We examined how host genotype, Nitrogen addition, rhizobial density, and community complexity affected a legume-rhizobia (*Medicago truncatula* - *Ensifer meliloti*) mutualism. Host genotype had the strongest effect on the size, number, and rhizobial composition of root nodules (the symbiotic organ). By contrast, the effect of small changes in N-availability and the complexity of the inoculum community (2, 3, 8, or 68 strains) were minor. Higher inoculum density resulted in a nodule community that was less diverse and more beneficial but only in the more selective host. With the less selective host, higher density resulted in more diverse and less beneficial nodule communities. Density effects on strain composition deserve additional scrutiny as they can create eco-evolutionary feedback and have translational relevance for overcoming establishment barriers in bio-inoculants.

*Short Abstract:*

The environmental context of the nitrogen-fixing mutualism between leguminous plants and rhizobial bacteria varies over space and time. The understudied environmental variable of rhizobial density had a larger effect on the relative fitness of 68 rhizobia (*Ensifer meliloti*) strains in nodules than the addition of low-levels of nitrogen or community complexity.

*Introduction:*

Biotic interactions have important consequences for population dynamics (Morris *et al.* 2019), selection (Caruso *et al.* 2017), and local adaptation (Runquist *et al.* 2020) of interacting species. Of course, these biotic interactions do not occur in isolation and the benefits and costs of the interaction can be modified (Bronstein 1994) by population density, the presence of additional species (Wood *et al.* 2018), genetics (Ford *et al.* 2017) and abiotic factors such as resource availability (Schultz *et al.* 2001; Lau *et al.* 2014; Wurzburger & Ford Miniat 2014; Rivett *et al.* 2016) or moisture levels (Wolinska & King 2009; Louthan *et al.* 2018; Benning & Moeller 2019). These responses (*i.e.* plasticity) can evolve if there is genetic variation in traits that modify the sensitivity of an interaction to additional environmental variables such as immunity, stress tolerance, or phenology (e.g. Ramegowda & Senthil-Kumar 2015; Garrido-Oter *et al.* 2018). Here we examine how three sources of environmental variation, resource availability, rhizobia density, and rhizobial community complexity, affect the rhizobium species (*Ensifer meliloti*) as it engages in symbiosis with its leguminous host plant (*Medicago truncatula*).

Environment dependence is a recurring theme in the study of the symbiosis between legume plants and rhizobial bacteria (Heath & Tiffin 2007; Keller & Lau 2018; Burghardt 2020). In this mutualistic relationship, rhizobia convert atmospheric nitrogen ($N_2$) into a plant-useable form to support host growth and reproduction while rhizobia gain carbon resources from the plant to support the growth and reproduction prior to release back into the soil (Denison & Kiers 2011; Poole *et al.* 2018). While this relationship is commonly beneficial to the plant host (Gibert *et al.* 2018), the magnitude of these fitness benefits to the plant depends on the identity of the rhizobial strain as well as additional environmental parameters including N, P, water- and light-availability, and temperature (Larimer *et al.* 2014; Vuong *et al.* 2017; Keller & Lau 2018; Heath *et al.* 2020). Experiments in which plants are inoculated with a single rhizobial strain, often at very high density, have shown that the benefits rhizobia obtain from symbiosis also can be context dependent (*e.g.* Friel & Friesen 2019; Batstone *et al.* 2020). However, in natural and agricultural populations rhizobia densities are likely to vary and multiple rhizobia strains compete for nodule occupancy and host enrichment (Burghardt 2020). Results from single-strain experiments may not be directly translatable to multi-strain environments because between-strain

85 competition can strongly affect nodulation success (Triplett & Sadowsky 1992). Indeed, strain
86 fitness proxies in single-strain environments are not strongly correlated with strain fitness in
87 multi-strain communities (Burghardt *et al.* 2018).
88
89 Theoretically, resource availability has the potential to shape the evolution of resource-
90 based symbiosis (Akcay *et al.* 2011; Bever 2015). N-availability could affect selection acting on
91 rhizobia and plant hosts via a number of mechanisms. For instance, additional N can reduce the
92 overall frequency of associations with hosts by reducing nodule number or size (Heath & Tiffin
93 2007; Heath *et al.* 2010; Menge *et al.* 2015). While forming fewer nodules in high N
94 environments may have little effect on plant fitness, it certainly reduces the chances of each
95 rhizobia associating with a legume and, if nodules remain small, also reduces the number of
96 rhizobia released from host nodules (a major component of the fitness benefit rhizobia receive
97 from engaging in the symbiosis). Nitrogen can also alter competitiveness among symbionts,
98 perhaps through altering the strength of host preference (Elliott *et al.* 2009) or enrichment via
99 host rewards/sanctions (Kiers *et al.* 2006; Oono *et al.* 2011). Despite the intuitive appeal of
100 theoretical predictions that additional N will reduce host dependence on rhizobia and lead to
101 relaxed selection for rhizobial host benefit, there is limited empirical support for N-mediated
102 shifts in competitive outcomes between rhizobia. Partner choice as measured by nodule
103 occupancy does not shift with N-addition in *Acmispon* (Regus 2014, Wendlandt *et al.* 2019) or
104 *Medicago* (Laguerre *et al.* 2012; Grillo *et al.* 2016) and only weakly shifts in *Mimosa* (Elliott *et*
105 *al.* 2009). However, which strain initiates each nodule only represents the first stage of selection.
106 Once nodules form, differential nodule growth and strain reproduction can allow some strains to
107 increase in frequency relative to others, but again, studies to date suggest that N has only a
108 limited effect on rhizobial fitness (Laguerre *et al.* 2012; Vuong *et al.* 2017).
109
110 Unlike N-addition, the effect of rhizobial population density on the legume benefit and
111 rhizobial fitness has received scant empirical attention. However, population densities can
112 strongly affect the ecology and evolution of biotic interactions including hosts and pathogens
113 (*e.g.* Schuhegger *et al.* 2006), predator and prey (*e.g.* Jaffee 2003), and plants and pollinators
114 (*e.g.* Moeller 2004). The density of rhizobial symbionts varies over space and time. For example,
115 twenty years of cropping system differences resulted in four-orders-of-magnitude differences in

rhizobia population densities: 6.8 x $10^6$ rhizobia $gram^{-1}$in soy/wheat/maize rotations, 4.5 x $10^5$ in continuous soy, and 6.1 x $10^2$ in continuous maize (Yan *et al.* 2014). There are many reasons to suspect that selection could be density dependent; the density of rhizobia in the soil could affect the number of nodules that a host forms (Oldroyd *et al.* 2001), the role of quorum sensing (Gonzalez & Marketon 2003; Sanchez-Contreras *et al.* 2007; Veliz-Vallejos *et al.* 2014), and the relative importance of host-mediated vs. soil-mediated selection (Daubech *et al.* 2017; Westhoek *et al.* 2017). Predicting the outcome of rhizobial density on strain selection is, however, difficult. For instance, when rhizobial population densities are high more nodulation sites (root hairs) will interact with multiple strains and more nodules will be formed and thus, we might expect opportunities for plant-imposed selection on bacterial populations to increase (Westhoek *et al.* 2017). On the other hand, when rhizobial densities are high, plant control may decrease as traits that influence rhizobial competitiveness become more important (Triplett & Sadowsky 1992).

Much of the empirical work on legume-rhizobia symbiosis relies on single-strain inoculations, however, in nature legume hosts often form nodules with a diverse community of strains (Thies *et al.* 1991; Rangin *et al.* 2008). Microbial community complexity can affect community assembly and interactions (Billick & Case 1994). Synthetic communities are increasingly being used to query the effect of additional community members (Coward *et al.* 2008; Großkopf & Soyer 2014; Widder *et al.* 2016). For example, Freidman *et al.* (2017) showed that competitive outcomes between two bacterial strains living in the guts of *C. elegans* are not affected by the presence of additional strains (Lopez *et al.* 2019). While rhizobial strain frequency in nodules is clearly dependent on the presence of other strains (Triplett & Sadowsky 1992; Laguerre *et al.* 2012; Westhoek *et al.* 2017), experiments have not investigated whether pairwise competitive outcomes are affected by the presence of other strains. In other words, does strain A always beat strain B regardless of which and how many other strains are present in the community?

Here we report on the extent to which strain fitness in nodules and plant traits are affected by each of three environmental factors, N-availability, population density, and the complexity of the rhizobial community to which plants are exposed. Given previous work showing that rhizobial fitness can strongly depend on host genotype (Burghardt *et al.* 2018, 2019b, a; Gano-

Cohen *et al.* 2019) we examine the effect of these treatments on each of two commonly used plant genotypes. Our results indicate that host-genotype has a much greater effect on strain fitness than the environmental manipulations. Among the environmental manipulations, the effects of bacterial density were approximately twice as great as the effects of N-addition. Community complexity had little effect on the relative fitness rankings of rhizobial strains suggesting that higher-order interactions between strains are rare. Our results suggest that variation in population densities could influence ecological and evolutionary dynamics in rhizobial communities and are a factor that should be considered more explicitly in empirical, theoretical, and applied work.

## Material and methods

We measured strain communities inside host nodules using a select-and-resequence approach, a variant of evolve-and-resequence approaches (Burghardt *et al.* 2018). We inoculated plants with a community of 68 rhizobial strains (hereafter referred to as C68). These strains were chosen to capture the majority of genetic variation present among 160 sequenced strains (Epstein *et al.* 2018; Nelson *et al.* 2018). To evaluate whether increasing the amount of N available to plants altered strain composition of the nodule community, we grew plants with a low level of additional N (+100ml 3mMol $KNO_3$ $week^{-1}$). To evaluate the potential for rhizobia density to affect strain fitness, we inoculated plants with two rhizobial densities: low ($5x10^5$ rhizobia $plant^{-1}$) and high ($5x10^7$ rhizobia $plant^{-1}$). To examine the effect of community complexity, we constructed communities of nested subsets of eight, three, and all pairwise competitions of the three. To form the rhizobia communities, we grew each of 68 *E. meliloti* strains in 3ml Tryptone yeast media (6g tryptone, 3g yeast extract, 0.38g $CaCl_2$ per L) for 3 days and then combined an equal volume of each culture to generate a community (C68) with approximately equal representation of each strain (median strain frequency 0.014, range 0.009-0.02). We used the same method to form the eight (C8), three (C3), and three two-strain communities (M:H, M:K, and H:K). All strain names are list in Supplemental Figure S3.

Seeds of two host genotypes A17 var. Varma and R108 (Medicago HapMap accession numbers HM101 and HM340, Stanton-Geddes et al. 2013) were bleached, rinsed, scarified with

a razor blade, stratified on wet filter paper at 4°C in the dark for two days, and then allowed to germinate at room temperature for one day. Twelve germinated seeds were then planted in each of 45 1L pots filled with sterilized Sunshine Mix. When seedlings were three days old 100 ul of each rhizobial community diluted in 9.9 ml 0.85% NaCl w/v solution were used to inoculate each pot (approximately $10^8$ cells, except for the low-density treatment which was inoculated with ~ $10^6$ cells, Figure S1). Plants were fertilized with 150ml of N-free fertilizer (Bucciarelli *et al.* 2006 see Burghardt et. al. 2018 for details) once a week and watered with sterile water as needed. Six weeks after planting, we sampled ~500 (A17) or ~200 (R108) nodules from the plants in each pot (Figure S2), crushed them, and used a series of differential centrifugation steps to enrich for and pellet undifferentiated bacteria (Burghardt et al. 2018). Pellets were stored at -20°C until we extracted DNA using the UltraClean Microbial DNA Isolation Kit (no. 12224; Mo Bio Laboratories). In addition to harvesting the nodules, we measured on a per plant basis: nodule number, nodule fresh weight, and vegetative and root biomass (dried at 60°C for 72 hours).

*Strain frequencies:* We estimated the frequency of each strain in each nodule pool using the method in Burghardt et al. 2018. In brief, DNA isolated from each replicate was sequenced on an Illumina HiSeq 2500 (NexteraXT libraries, 125 bp paired-end reads, 3.6-9.4 million read pairs library$^{-1}$). Reads were trimmed with TrimGalore! (v0.4.1) using default settings, except with minimum read length = 100 bp, quality threshold = 30, and minimum adapter match = 3. We used bwa mem (v0.7.17; Li and Durbin, 2010) with default settings to align reads to the *E. meliloti* USDA1106 genome (Nelson *et al.* 2018; NCBI BioProject: PRJNA388336). We identified SNPs segregating in each of the sequenced communities using FreeBayes (v1.2.0-2-g29c4002; (Garrison & Marth 2012) with a minimum read mapping quality of 30. After cleaning and alignment, median read depth per sample was 65X (range 29X-115X). To estimate strain frequency, we used only SNPs for which every strain had an unambiguous call. We then estimated the frequency of each strain in each sample using HARP (Kessner *et al.* 2013) as described in Burghardt *et al.* (2018).

*Nodule community measurements:* Based on strain frequencies, we calculated three nodule community metrics for each replicate: composition, diversity, and host benefit. We estimated

community composition as the fold change in the frequency of each strain ($q_x$) in a nodule community relative to mean frequency of that strain across four sequencing replicates of the initial community (fitness = $\log_2(q_{x\ \text{selected}} / \bar{q}_{x\ \text{initial}})$). This transformation both normalizes the frequency distribution and controls for small differences in initial strain frequency (median $q_{x\ \text{initial}}$=0.0092, 5%-95% quantile: 0.0054-0.0154). We estimated community diversity as the exponent of Shannon diversity, which we calculated using the 'renyi' function in vegan package of R. We calculated predicted host benefit as the sum of the per strain frequency in nodules multiplied by the dry plant weight from a single-strain inoculation experiment (data from (Burghardt *et al.* 2018):

$$\frac{\sum_{x=1}^{n} q_x * \ dry\ plant\ weight\ in\ single\ strain\ experiment_x}{\sum_{x=1}^{n} q_x}$$

For A17 replicates we used A17 single-strain data and for R108 we used R108 single-strain data. We scaled each host-specific dataset to range from zero (complete occupancy by the least beneficial strain) to one (complete occupancy by the most beneficial strain).

*Statistical analysis of community measures:* We used redundancy analysis (RDA, "rda" in the vegan R package Oskanen et al. 2017) and ANOVA to analyze the effects of Host genotype (H), Density (D), and Nitrogen (N) and their interactions on each of the nodule community measures. To collapse the dimensionality of the strain relative fitness data and analyze shifts in relative fitness across treatments, we used RDA. RDA fits a multivariate linear regression to centered and scaled data and then uses principal component analysis (PCA) to decompose the major axes of variation in the fitted parameters. The adjusted $R^2$ of each RDA model provides an estimate of the proportion of variance in relative fitness explained by the model predictor(s). We permuted the data to determine the probability that fitness differences occurred by chance ('anova' function, 999 permutations). To analyze strain diversity, we used an ANOVA ('lm' and 'anova') to test for differences amongst treatments. To analyze host benefit, we used an ANOVA ('lm' and 'anova'). Because the effects of D and N were host dependent (H x D, H x N interactions), we also analyzed the effects of N, D, and their interaction for each host separately.

*Analysis of rank order and community complexity:* For each pairwise competition of the three strains used to form three-strain community (C3), we identified the strain with higher strain

frequency and asked if the same strain was at higher frequency in the more complex three strain community. We also examined whether strain frequency rankings in the C3 community remained the same in the eight- (C8) and sixty-eight- (C68) strain communities. Likewise, we examined whether the frequency rankings of each of the strains in the C8 community remained the same in the C68 community.

*Statistical analysis of plant phenotypes:* We used an ANOVA ('lm' and 'anova') to test for the effects of host genotype, inoculum density, and nitrogen level and their interactions on six plant traits (nodule number, average nodule weight, nodule weight per, shoot biomass, root biomass, and root to shoot ratio). The first two traits violated the assumption of homogeneity of variance between hosts, so we focus on host-specific analyses. To evaluate the effect of community complexity on plant phenotypes, we ran a model with Host (H) and community complexity (C; 2, 3, 8, and 68 strains) and their interaction as continuous predictors. Although we test for the among-treatment differences in plant phenotypes, the experiments were designed to evaluate the effect of treatments on strain fitness and were not well powered to detect among-treatment differences in plant phenotypes.

**Results:**

*Host genotype has a large effect on strain fitness and nodule phenotypes*

Consistent with previous work, the strain composition of the nodule communities was strongly affected by host genotype. In fact, host identity had a greater effect on strain composition, Shannon's diversity, and predicted benefit of the nodule community (Figure 2, Table 1, Table S1, Table S2), than did N-addition, inoculum density, or the complexity of the inoculum community. Relative to R108, A17 hosts produced ~ 10 times more nodules (Fig 3a) that were approximately one tenth the size (Fig 3b), and harbored nodule communities that were less diverse (Fig 2b) and more beneficial (Fig 2c).

*N-availability weakly affected strain composition, diversity, and benefit*

Because Nitrogen is the primary resource rhizobia provide to their host, researchers have speculated, and modeling has suggested that N-availability could alter the relative fitness of

rhizobia strains. However, we found that N-availability explained only a small portion of the variance in the overall composition of the nodule communities (2.2% of the RDA variance, $p$=0.23; Figure 2), although the effect was slightly greater when hosts were analyzed separately (in A17 $p$=0.027, 6.4% of the variance and in R108 $p$ = 0.46, 4.2% of the variance; Table 1). N-addition had similar magnitude of effect on the diversity (Shannon's) of the nodule community and the predicted benefit of the strain community, with the effects being greater in A17 (diversity: $p$=0.039, 8.7% of variance and strain benefit: $p$ = 0.030, 7.9% of variance) than R108 ($p$=0.85, 0.17% of variance and $p$ = 0.28, 3.2% of variance, respectively). See Table S1 and Table S2 for full results.

*Rhizobia inoculation density had a stronger effect than N-addition*

Inoculating plants with 100-fold less rhizobia cells caused larger changes in strain diversity and predicted benefit of the nodule community than N-addition (Figure 2). Moreover, the effect of density depended on the host. With A17 hosts, density explained 16.1% ($p$=0.001) of variance in community composition vs. 6.4% ($p$=0.027) of the variance explained by N-addition. With R108, density explained 6.1% ($p$=0.053) of the variance vs 4.2 % ($p$=0.46) explained by N-addition. Density affected diversity of the nodule community in opposite directions in the two hosts. Compared to the low-density inoculation, the high-density inoculation resulted in A17 nodule communities being less diverse (based on Shannon's diversity) and more beneficial (both $p < 0.001$). By contrast, with R108 the low-density inoculation resulted in nodule community that was more diverse, although not greater than expected by chance ($p$ = 0.78) and less beneficial ($p < 0.003$). See Table S1 and Table S2 for details.

*Relative fitness rankings are similar across communities of increasing complexity*

In nature, the complexity of rhizobial communities varies in both space and time. We found that although the absolute frequency of each of three focal strains was strongly affected by the presence of additional strains in the inoculum, we found little evidence that additional strains in the inoculum community altered strain-rankings relative to each other. In other words, with minor exceptions, the more frequent strain in pairwise competitions was also the more frequent strain in more complex communities (Table 2). The minor exceptions involved two strains

(KH46c and HM006.1) that had nearly equal frequencies in pairwise inoculations and also nearly equal frequency when part of more strain-rich communities (Figure S6; Table 2). Similarly the relative-frequency ranking of strains when plants were inoculated with three strains (C3) were almost always the same as the relative ranking of those strains when they were part of a 68 strain inoculum community (Figure 4a) and the relative ranking of the strains in C8 treatment were nearly always the same as they were in the C68 treatment (Figure 4b). These results suggest that strain competitiveness is relatively robust to the presence of additional strains and strains with higher frequency in pairwise competitions will also have higher frequencies in more complex communities.

*Effects of N, D, and complexity on nodule and plant traits were host specific*

The effect of N-addition and inoculum density on plant phenotypes were host-specific (Table S3). In A17, increased rhizobial density resulted in plants forming more nodules ($p$=0.004; Fig 3a) that weighed less ($p$<0.001; Fig 3b). In R108, an increase in rhizobia density resulted in plants forming more, but smaller nodules, but only in the N-addition environment (Nitrogen x Density $p$ = 0.036 & $p$ = 0.033 respectively). Neither N-addition or rhizobia density had measurable effects on either above or belowground biomass, although both N ($p$=0.029) and to a lesser extent density ($p$=0.087) decreased the root to shoot ratio in A17 but not in R108 (Fig S7). Complexity of the inoculum community had little effect on nodule number or vegetative biomass (Fig S8; Table S4) although it did have minor effects on nodule weight in A17 but not R108 (Host x Complexity $p$= 0.018), and root weight ($p$ = 0.053) of both hosts.

## Discussion

The symbiosis between legumes and rhizobia plays an important ecological role by contributing nitrogen to natural and agricultural ecosystems and to the success of legumes (Vitousek *et al.* 2013; Harrison *et al.* 2018; Taylor *et al.* 2020). Here we asked how host genotype and each of three environmental factors, that are likely to vary spatially and temporally affect the rhizobia community in nodules. Nitrogen, despite being the key benefit that rhizobia provide plants, had relatively small effects on the strain composition, diversity, and benefit of nodule communities. Similarly, the complexity of the inoculum community (number of strains

used to inoculate a plant) had only small effects on relative strain competitiveness, suggesting that higher-order interactions among strains do not alter the pairwise rankings of strain success. Interestingly, inoculum density had large and host-specific effects on the nodule community—density had a larger effect on strain composition, diversity, and benefit in the host (A17) that imposes greater selection on the rhizobia population.

Theoretically, the availability of alternative sources of the resources provided by symbiosis can alter selection on beneficial symbionts (West *et al.* 2002; Akcay *et al.* 2011; Bever 2015). When mutualistic relationships are considered in a market framework, external N improves the bargaining power of the host and increases the relative fitness of less beneficial strains (Akcay *et al.* 2011). Similarly, relaxed selection for symbiont benefit could occur because it is less costly for the host to allocate resources to a less beneficial partner if it can obtain the resource from an alternative source (Bever 2015). Despite the potential, we found the addition of small amounts of N had little effect on rhizobial strain composition, diversity, and the host benefits of the nodule community. Indeed, in one host genotype (A17), selection for beneficial strains was stronger, not weaker, in the N-addition environment. Our findings of subtle effects of N-addition are consistent with several other studies of nodule occupancy (Kosslak & Bohlool 1985; Laguerre *et al.* 2012; Regus *et al.* 2014; Grillo *et al.* 2016; Wendlandt *et al.* 2019). Similarly, our results align with two previous studies that assessed composite strain fitness across all nodules formed by a plant. Nitrogen addition did not affect rhizobial composition in pools of 4-week-old *Medicago* nodules inoculated with a mixture of two *Ensifer* species or a mixture of Fix- and Fix+ strains (Laguerre *et al.* 2012) and had weak effects on strain fitness two species of *Acacia*—drought and phosphorous addition treatments had larger effects (Vuong *et al.* 2017).

Although our results are consistent with these short-term (single growing season) studies, it is clear that addition of N over longer periods of time can affect the symbiosis. Many legumes regulate nodule formation and symbiotic nitrogen fixation when high levels of abiotic N are available, presumably reflecting a cost of forming and maintaining the symbiosis—(Menge *et al.* 2015). For instance, Weese *et al.* found rhizobia in a grassland community to be less beneficial to hosts after 22 years of N addition (Weese *et al.* 2015; Klinger *et al.* 2016). One way to reconcile discrepancies between short and long-term experiments is to note that even if relative

363 fitness is not altered by N- addition other parameters that influence absolute fitness could
364 change. For instance, reductions in host population sizes, nodule numbers, or nodule size could
365 all increase the importance of rhizobial adaptation to environments external to the host and result
366 in a population of strains with reduced host benefit via selective tradeoffs or drift (Denison &
367 Kiers 2004; Heath & Stinchcombe 2014; Hollowell *et al.* 2016; Burghardt *et al.* 2018; Burghardt
368 2020). Experiments that hold relative fitness constant while varying absolute fitness and vice
369 versa will clarify the key drivers of evolutionary trajectories in response to N.
370
371 Whereas the effects of N on the legume-rhizobial symbiosis has been the focus of many
372 studies, the potential for density-dependent selection shaping legume-rhizobia symbiosis has
373 received scant attention. Nevertheless, rhizobial density varies widely in nature (e.g. $10^2$ -$10^6$ per
374 gram Yan *et al.* 2014). The 100-fold difference in inoculum density we examined resulted in
375 host-dependent shifts in the composition of the nodule community, with increased density
376 resulting in more beneficial strains being favored in one host and less beneficial strains being
377 favored in the other host. The results raise questions about the generality of laboratory
378 experiments that rely on extremely high densities of rhizobia—presumably to ensure that hosts
379 will not be rhizobia limited. Interestingly, while the majority of nodules screened in the field
380 tend to have a single occupant (Kosslak & Bohlool 1985; Moawad & Schmidt 1987; Ndungu *et*
381 *al.* 2018), the probability of one nodule being infected by multiple strains increases with
382 rhizobial density (Daubech *et al.* 2017; Westhoek *et al.* 2017). Whether strain competition occurs
383 via direct interactions within a nodule vs. competition between nodules inhabited by different
384 strains can affect mechanisms of rewards/sanction and the scale at which selection occurs
385 (Akçay 2015).
386
387 The two host genotypes differed markedly in the characteristics of the nodules they
388 produced, A17 made many small nodules while R108 made a few large nodules, a pattern found
389 in all nitrogen, density, and inoculum environments. Interestingly, overall investment in nodule
390 tissue per plant was very similar for both hosts, and decreases in nodule numbers at low
391 inoculum densities were offset by increases in nodule size (a pattern previously observed by
392 Singleton & Tavares 1986). In R108 plants, the combination of lower nodule number and low
393 selectivity resulted in increased variability in strain fitness amongst replicates. Increased

394 stochasticity in strain fitness could influence rhizobial evolution—not by directly determining
395 rhizobial fitness—but because it determines the strength of drift.
396
397 *Conclusions:*
398 Using a high-throughput methodology, we evaluated the context-dependence of host-
399 dependent strain fitness in legume-rhizobia symbiosis. Our finding that host genetic variation is
400 a consistent driver of rhizobial relative fitness across environmental perturbations suggests it is
401 possible to identify the genomic basis of strain x host interactions (Wang *et al.* 2012, 2018;
402 Gourion & Alunni 2018) and use that information to breed legume crops that associate with
403 beneficial rhizobial strains even when environmental conditions change across years or locations.
404 Our results also suggest that host-imposed selection might depend on the density of the rhizobia
405 population in the soil. From a translational perspective, understanding density-dependent
406 selection could aid in the developing of beneficial inoculants that overcome establishment
407 barriers (Kaminsky *et al.* 2019).
408
409
410 **Acknowledgements:** We thank Roxanne Denny for help with seeds. Computational resources
411 were provided by the Minnesota Supercomputing Institute (MSI) at the University of
412 Minnesota. This work was supported by the National Science Foundation (NSF) awards IOS-
413 1724993 and IOS-1856744. Any opinions, findings, conclusions, or recommendations expressed
414 in this material are those of the authors and do not necessarily reflect the views of the NSF.

415 **References:**

416 Akçay, E. (2015). Evolutionary models of mutualism. In: *Mutualism* (ed. Bronstein, J.L.).
417 Oxford University Press, New York, NY, pp. 57–76.
418 Akcay, E., Simms, E.L., Akçay, E., Simms, E.L., Akcay, E. & Simms, E.L. (2011). Negotiation,
419 sanctions, and context dependency in the legume-rhizobium mutualism. *Am. Nat.*, 178, 1–
420 14.
421 Batstone, R.T., Peters, M.A.E., Simonsen, A.K., Stinchcombe, J.R. & Frederickson, M.E.
422 (2020). Environmental variation impacts trait expression and selection in the legume–
423 rhizobium symbiosis. *Am. J. Bot.*, 107, 195–208.
424 Benning, J.W. & Moeller, D.A. (2019). Maladaptation beyond a geographic range limit driven
425 by antagonistic and mutualistic biotic interactions across an abiotic gradient. *Evolution (N.*
426 *Y).*, 73, 2044–2059.
427 Bever, J.D. (2015). Preferential allocation, physio-evolutionary feedbacks, and the stability and
428 environmental patterns of mutualism between plants and their root symbionts. *New Phytol.*,
429 205, 1503–1514.
430 Billick, I. & Case, T.J. (1994). Higher order interactions in ecological communities: What are
431 they and how can they be detected? *Ecology*, 75, 1529–1543.
432 Bronstein, J.L. (1994). Conditional outcomes in mutualistic interactions. *Trends Ecol. Evol.*, 9,
433 214–217.
434 Bucciarelli, B., Hanan, J., Palmquist, D. & Vance, C. (2006). A standardized method for analysis
435 of *Medicago truncatula* phenotypic development. *Plant Physiol.*, 142, 207–219.
436 Burghardt, L.T. (2020). Evolving together, evolving apart: measuring the fitness of rhizobial
437 bacteria in and out of symbiosis with leguminous plants. *New Phytol.*, 228, 28–34.
438 Burghardt, L.T., Epstein, B., Guhlin, J., Nelson, M.S., Taylor, M.R., Young, N.D., *et al.* (2018).
439 Select and resequence reveals relative fitness of bacteria in symbiotic and free-living
440 environments. *Proc. Natl. Acad. Sci.*, 115, 2425–2430.
441 Burghardt, L.T., Epstein, B. & Tiffin, P. (2019a). Legacy of prior host and soil selection on
442 rhizobial fitness in planta. *Evolution (N. Y).*, 73, 2013–2023.
443 Burghardt, L.T., Trujillo, D.I., Epstein, B., Tiffin, P. & Young, N.D. (2019b). A "Select and
444 Resequence" approach reveals strain-specific effects of *Medicago* nodule-specific PLAT-
445 domain genes. *Plant Physiol.*, 182, 463–471.

446 Caruso, C.M., Martin, R.A., Sletvold, N., Morrissey, M.B., Wade, M.J., Augustine, K.E., *et al.*
447 (2017). What are the environmental determinants of phenotypic selection? A meta-analysis
448 of experimental studies. *Am. Nat.*, 190, 363–376.
449 Coward, C., Van Diemen, P.M., Conlan, A.J.K., Gog, J.R., Stevens, M.P., Jones, M.A., *et al.*
450 (2008). Competing isogenic *Campylobacter* strains exhibit variable population structures in
451 vivo. *Appl. Environ. Microbiol.*, 74, 3857–3867.
452 Daubech, B., Remigi, P., Doin de Moura, G., Marchetti, M., Pouzet, C., Auriac, M.-C., *et al.*
453 (2017). Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen
454 fixation. *Elife*, 6, 1–21.
455 Denison, R.F. & Kiers, E.T. (2004). Lifestyle alternatives for rhizobia: Mutualism, parasitism,
456 and forgoing symbiosis. *FEMS Microbiol. Lett.*, 237, 187–193.
457 Denison, R.F. & Kiers, E.T. (2011). Life histories of symbiotic rhizobia and mycorrhizal fungi.
458 *Curr. Biol.*, 21, R775–R785.
459 Elliott, G.N., Chou, J.H., Chen, W.M., Bloemberg, G. V., Bontemps, C., Martínez-Romero, E.,
460 *et al.* (2009). *Burkholderia* spp. are the most competitive symbionts of *Mimosa*, particularly
461 under N-limited conditions. *Environ. Microbiol.*, 11, 762–778.
462 Epstein, B., Abou-Shanab, R.A.I., Shamseldin, A., Taylor, M.R., Guhlin, J., Burghardt, L.T., *et*
463 *al.* (2018). Genome-wide association analyses in the model rhizobium *Ensifer meliloti*.
464 *mSphere*, 3, 1–15.
465 Ford, S.A., Williams, D., Paterson, S. & King, K.C. (2017). Co-evolutionary dynamics between
466 a defensive microbe and a pathogen driven by fluctuating selection. *Mol. Ecol.*, 26, 1778–
467 1789.
468 Friedman, J., Higgins, L.M. & Gore, J. (2017). Community structure follows simple assembly
469 rules in microbial microcosms. *Nat. Ecol. Evol.*, 1, 1–7.
470 Friel, C.A. & Friesen, M.L. (2019). Legumes Modulate Allocation to Rhizobial Nitrogen
471 Fixation in Response to Factorial Light and Nitrogen Manipulation. *Front. Plant Sci.*, 10, 1–
472 9.
473 Gano-Cohen, K.A., Wendlandt, C.E., Stokes, P.J., Blanton, M.A., Quides, K.W., Zomorrodian,
474 A., *et al.* (2019). Interspecific conflict and the evolution of ineffective rhizobia. *Ecol. Lett.*,
475 22, 914–924.
476 Garrido-Oter, R., Nakano, R.T., Dombrowski, N., Ma, K.W., McHardy, A.C. & Schulze-Lefert,

P. (2018). Modular traits of the *Rhizobiales* root microbiota and their evolutionary relationship with symbiotic rhizobia. *Cell Host Microbe*, 24, 155-167.e5.

Garrison, E. & Marth, G. (2012). Haplotype-based variant detection from short-read sequencing. *arXiv:1207.3907*, 1–9.

Gibert, A., Tozer, W. & Westoby, M. (2018). Plant performance response to eight different types of symbiosis. *New Phytol.*, 222, 526–542.

Gonzalez, J.E. & Marketon, M.M. (2003). Quorum sensing in nitrogen-fixing rhizobia. *Microbiol. Mol. Biol. Rev.*, 67, 574–592.

Gourion, B. & Alunni, B. (2018). Strain-specific symbiotic genes: A new level of control in the intracellular accommodation of rhizobia within legume nodule cells. *Mol. Plant-Microbe Interact.*, 31, MPMI-01-18-0010.

Grillo, M.A., Stinchcombe, J.R. & Heath, K.D. (2016). Nitrogen addition does not influence pre-infection partner choice in the legume-rhizobium symbiosis. *Am. J. Bot.*, 103, 1763–1770.

Großkopf, T. & Soyer, O.S. (2014). Synthetic microbial communities. *Curr. Opin. Microbiol.*, 18, 72–77.

Harrison, T.L., Simonsen, A.K., Stinchcombe, J.R. & Frederickson, E. (2018). More partners , more ranges : generalist legumes spread more easily around the globe.

Heath, K.D., Podowski, J.C., Heniff, S., Klinger, C.R., Burke, P. V., Weese, D.J., *et al.* (2020). Light availability and rhizobium variation interactively mediate the outcomes of legume–rhizobium symbiosis. *Am. J. Bot.*, 107, 229–238.

Heath, K.D. & Stinchcombe, J.R. (2014). Explaining mutualism variation: A new evolutionary paradox? *Evolution (N. Y).*, 68, 309–317.

Heath, K.D., Stock, A.J. & Stinchcombe, J.R. (2010). Mutualism variation in the nodulation response to nitrate. *J. Evol. Biol.*, 23, 2494–2500.

Heath, K.D. & Tiffin, P. (2007). Context dependence in the coevolution of plant and rhizobial mutualists. *Proc. R. Soc. London B Biol. Sci.*, 274, 1905–1912.

Hollowell, A.C., Regus, J.U., Turissini, D., Gano-Cohen, K.A., Bantay, R., Bernardo, A., *et al.* (2016). Metapopulation dominance and genomic-island acquisition of *Bradyrhizobium* with superior catabolic capabilities. *Proc. R. Soc. London B Biol. Sci.*, 283.

Jaffee, B.A. (2003). Correlations between most probable number and activity of nematode-trapping fungi. *Phytopathology*, 93, 1599–1605.

508 Kaminsky, L.M., Trexler, R. V., Malik, R.J., Hockett, K.L. & Bell, T.H. (2019). The inherent
509 conflicts in developing soil microbial inoculants. *Trends Biotechnol.*, 37, 140–151.
510 Keller, K.R. & Lau, J.A. (2018). When mutualisms matter: Rhizobia effects on plant
511 communities depend on host plant population and soil nitrogen availability. *J. Ecol.*, 106,
512 1046–1056.
513 Kessner, D., Turner, T.L. & Novembre, J. (2013). Maximum likelihood estimation of
514 frequencies of known haplotypes from pooled sequence data. *Mol. Biol. Evol.*, 30, 1145–
515 1158.
516 Kiers, E.T., Rousseau, R.A. & Denison, R.F. (2006). Measured sanctions: Legume hosts detect
517 quantitative variation in rhizobium cooperation and punish accordingly. *Evol. Ecol. Res.*, 8,
518 1077–1086.
519 Klinger, C.R., Lau, J.A. & Heath, K.D. (2016). Ecological genomics of mutualism decline in
520 nitrogen-fixing bacteria. *Proc. R. Soc. B Biol. Sci.*, 283, 20152563.
521 Kosslak, R.M. & Bohlool, B. Ben. (1985). Influence of environmental factors on interstrain
522 competition in <i>Rhizobium japonicum. *Appl. Environ. Microbiol.*, 49, 1128–1133.
523 Laguerre, G., Heulin-Gotty, K., Brunel, B., Klonowska, A., Le Quéré, A., Tillard, P., *et al.*
524 (2012). Local and systemic N signaling are involved in *Medicago truncatula* preference for
525 the most efficient *Sinorhizobium* symbiotic partners. *New Phytol.*, 195, 437–449.
526 Larimer, A.L., Clay, K. & Bever, J.D. (2014). Synergism and context dependency of interactions
527 between arbuscular mycorrhizal fungi and rhizobia with a prairie legume. *Ecology*, 95,
528 1045–1054.
529 Lau, J.A., Shaw, R.G., Reich, P.B. & Tiffin, P. (2014). Indirect effects drive evolutionary
530 responses to global change. *New Phytol.*, 201, 335–343.
531 Li, H. & Durbin, R. (2009). Fast and accurate long-read alignment with Burrows-Wheeler
532 transform. *Bioinformatics*, 25, 1754–1760.
533 Lopez, A.O., Vega, N.M. & Gore, J. (2019). Interspecies bacterial competition determines
534 community assembly in the *C. elegans* intestine. *bioRxiv*, 535633.
535 Louthan, A.M., Pringle, R.M., Goheen, J.R., Palmer, T.M., Morris, W.F. & Doak, D.F. (2018).
536 Aridity weakens population-level effects of multiple species interactions on Hibiscus
537 meyeri. *Proc. Natl. Acad. Sci. U. S. A.*, 115, 543–548.
538 Menge, D.N.L., Wolf, A. a. & Funk, J.L. (2015). Diversity of nitrogen fixation strategies in

539 Mediterranean legumes. *Nat. Plants*, 1, 15064.
540 Moawad, M. & Schmidt, E.L. (1987). Occurrence and nature of mixed infections in nodules of
541 field-grown soybeans (*Glycine max*). *Biol. Fertil. Soils*, 5, 112–114.
542 Moeller, D.A. (2004). Facilitative interactions among plants via shared pollinators. *Ecology*, 85,
543 3289–3301.
544 Morris, W., Ehrlén, J., Dahlgren, J.P., Loomis, A.K. & Louthan, A.M. (2019). Biotic and
545 anthropogenic forces rival climatic/abiotic factors in determining global plant population
546 growth and fitness. *Proc. Natl. Acad. Sci.*, 1–6.
547 Ndungu, S.M., Messmer, M.M., Ziegler, D., Thuita, M., Vanlauwe, B., Frossard, E., *et al.*
548 (2018). Evaluation of MALDI-TOF mass spectrometry for the competitiveness analysis of
549 selected indigenous cowpea (*Vigna unguiculata L. Walp.*) Bradyrhizobium strains from
550 Kenya. *Appl. Microbiol. Biotechnol.*, 102, 5265–5278.
551 Nelson, M., Guhlin, J., Epstein, B., Tiffin, P. & Sadowsky, M.J. (2018). The complete replicons
552 of 16 *Ensifer meliloti* strains offer insights into intra- and inter-replicon gene transfer,
553 transposon-associated loci, and repeat elements. *Microb. Genomics*, 4, 1–11.
554 Oldroyd, G.E.D., Mitra, R.M., Wais, R.J. & Long, S.R. (2001). Evidence for structurally specific
555 negative feedback in the nod factor signal transduction pathway. *Plant J.*, 28, 191–199.
556 Oono, R., Anderson, C.G. & Denison, R.F. (2011). Failure to fix nitrogen by non-reproductive
557 symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive
558 clonemates. *Proc. Biol. Sci.*, 278, 2698–703.
559 Oskanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., *et al.* (2017).
560 vegan: Community Ecology Package.
561 Poole, P., Ramachandran, V. & Terpolilli, J. (2018). Rhizobia: from saprophytes to
562 endosymbionts. *Nat. Rev. Microbiol.*, 16, 291–303.
563 Ramegowda, V. & Senthil-Kumar, M. (2015). The interactive effects of simultaneous biotic and
564 abiotic stresses on plants: Mechanistic understanding from drought and pathogen
565 combination. *J. Plant Physiol.*, 176, 47–54.
566 Rangin, C., Brunel, B., Cleyet-Marel, J.C., Perrineau, M.M. & Béna, G. (2008). Effects of
567 Medicago truncatula genetic diversity, rhizobial competition, and strain effectiveness on the
568 diversity of a natural *Sinorhizobium* species community. *Appl. Environ. Microbiol.*, 74,
569 5653–5661.

570 Regus, J.U., Gano, K.A., Hollowell, A.C. & Sachs, J.L. (2014). Efficiency of partner choice and
571 sanctions in *Lotus* is not altered by nitrogen fertilization. *Proc. R. Soc. B Biol. Sci.*, 281, 1–
572 8.
573 Rivett, D.W., Scheuerl, T., Culbert, C.T., Mombrikotb, S.B., Johnstone, E., Barraclough, T.G., *et*
574 *al.* (2016). Resource-dependent attenuation of species interactions during bacterial
575 succession. *ISME J.*, 10, 2259–2268.
576 Runquist, R.D.B., Gorton, A.J., Yoder, J.B., Deacon, N.J., Grossman, J.J., Kothari, S., *et al.*
577 (2020). Context dependence of local adaptation to abiotic and biotic environments: A
578 quantitative and qualitative synthesis. *Am. Nat.*, 195, 412–431.
579 Sanchez-Contreras, M., Bauer, W.D., Gao, M., Robinson, J.B. & Downie, J.A. (2007). Quorum-
580 sensing regulation in rhizobia and its role in symbiotic interactions with legumes. *Philos.*
581 *Trans. R. Soc. B Biol. Sci.*, 362, 1149–1163.
582 Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., *et al.* (2006).
583 Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing
584 rhizosphere bacteria. *Plant, Cell Environ.*, 29, 909–918.
585 Schultz, P.A., Michael Miller, R., Jastrow, J.D., Rivetta, C. V. & Bever, J.D. (2001). Evidence of
586 a mycorrhizal mechanism for the adaptation of *Andropogon gerardii (Poaceae)* to high- and
587 low-nutrient prairies. *Am. J. Bot.*, 88, 1650–1656.
588 Singleton, P.W. & Tavares, J.W. (1986). Inoculation response of legumes in relation to the
589 number and effectiveness of indigenous rhizobium populations. *Appl. Environ. Microbiol.*,
590 51, 1013–1018.
591 Taylor, B.N., Simms, E.L. & Komatsu, K.J. (2020). More than a functional group: Diversity
592 within the legume-rhizobia mutualism and its relationship with ecosystem function.
593 *Diversity*, 12.
594 Thies, J.E., Singleton, P.W. & Bohlool, B.B. (1991). Influence of the size of indigenous rhizobial
595 populations on establishment and symbiotic performance of introduced rhizobia on field-
596 grown legumes. *Appl. Environ. Microbiol.*, 57, 19–28.
597 Triplett, E.W. & Sadowsky, M. (1992). Genetics of competition for nodulation of legumes.
598 *Annu. Rev. Microbiol.*, 46, 399–428.
599 Veliz-Vallejos, D.F., Van Noorden, G.E., Yuan, M. & Mathesius, U. (2014). A *Sinorhizobium*
600 *meliloti*-specific N-acyl homoserine lactone quorum-sensing signal increases nodule

601 numbers in *Medicago truncatula* independent of autoregulation. *Front. Plant Sci.*, 5, 1–13.
602 Vitousek, P.M., Menge, D.N.L., Reed, S.C., Cleveland, C.C. & Vitousek, P.M. (2013).
603 Biological nitrogen fixation : rates , patterns and ecological controls in terrestrial
604 ecosystems.
605 Vuong, H.B., Thrall, P.H. & Barrett, L.G. (2017). Host species and environmental variation can
606 influence rhizobial community composition. *J. Ecol.*, 105, 540–548.
607 Wang, D., Yang, S., Tang, F. & Zhu, H. (2012). Symbiosis specificity in the legume – rhizobial
608 mutualism, 14, 334–342.
609 Wang, Q., Liu, J., Li, H., Yang, S., Körmöczi, P., Kereszt, A., *et al.* (2018). Nodule-specific
610 cysteine-rich peptides negatively regulate nitrogen-fixing symbiosis in a strain-specific
611 manner in *Medicago truncatula*. *Mol. Plant-Microbe Interact.*, 31, 240–248.
612 Weese, D.J., Heath, K.D., Dentinger, B.T.M. & Lau, J.A. (2015). Long-term nitrogen addition
613 causes the evolution of less-cooperative mutualists. *Evolution (N. Y).*, 69, 631–642.
614 Wendlandt, C.E., Regus, J.U., Gano-Cohen, K.A., Hollowell, A.C., Quides, K.W., Lyu, J.Y., *et*
615 *al.* (2019). Host investment into symbiosis varies among genotypes of the legume
616 Acmispon strigosus , but host sanctions are uniform. *New Phytol.*, 221, 446–458.
617 West, S.A., Toby Kiers, E., Pen, I. & Denison, R.F. (2002). Sanctions and mutualism stability:
618 When should less beneficial mutualists be tolerated? *J. Evol. Biol.*, 15, 830–837.
619 Westhoek, A., Field, E., Rehling, F., Mulley, G., Webb, I., Poole, P.S., *et al.* (2017). Policing the
620 legume-rhizobium symbiosis: A critical test of partner choice. *Sci. Rep.*, 7, 1–10.
621 Widder, S., Allen, R.J., Pfeiffer, T., Curtis, T.P., Wiuf, C., Sloan, W.T., *et al.* (2016). Challenges
622 in microbial ecology: Building predictive understanding of community function and
623 dynamics. *ISME J.*
624 Wolinska, J. & King, K.C. (2009). Environment can alter selection in host-parasite interactions.
625 *Trends Parasitol.*, 25, 236–244.
626 Wood, C.W., Pilkington, B.L., Vaidya, P., Biel, C. & Stinchcombe, J.R. (2018). Genetic conflict
627 with a parasitic nematode disrupts the legume-rhizobia mutualism. *Evol. Lett.*, 2, 233–245.
628 Wurzburger, N. & Ford Miniat, C. (2014). Drought enhances symbiotic dinitrogen fixation and
629 competitive ability of a temperate forest tree. *Oecologia*, 174, 1117–1126.
630 Yan, J., Han, X.Z., Ji, Z.J., Li, Y., Wang, E.T., Xie, Z.H., *et al.* (2014). Abundance and diversity
631 of soybean-nodulating rhizobia in black soil are impacted by land use and crop

632 management. *Appl. Environ. Microbiol.*, 80, 5394–5402.

633

634 Table 1: PERMANOVA on RDA of strain relative fitness shows effects of plant host and
635 inoculum density are far stronger than effects of nitrogen (top). Analyses of each host separately
636 (bottom) revealed that density and nitrogen both had greater effects with A17 than R108 hosts
637

| **Model and Terms** | **DF** | **Prop. Var** | **F** | ***P*** |
|---|---|---|---|---|
| **Both Hosts** (adj. $r^2$= 0.29) | | | | |
| Host | 1 | 0.24 | 16.06 | **< 0.001** |
| Density | 1 | 0.047 | 3.1 | **0.004** |
| Nitrogen | 1 | 0.022 | 1.44 | 0.12 |
| Host x Density | 1 | 0.033 | 2.19 | **0.022** |
| Host x Nitrogen | 1 | 0.02 | 1.33 | 0.18 |
| Density x Nitrogen | 1 | 0.017 | 1.12 | 0.28 |
| Host x Density x Nitrogen | 1 | 0.015 | 0.96 | 0.41 |
| Residual | 40 | 0.60 | | |
| **A17 only** (adj. $r^2$= 0.14) | | | | |
| Density | 1 | 0.16 | 4.3 | **< 0.001** |
| Nitrogen | 1 | 0.064 | 1.72 | **0.027** |
| Density x Nitrogen | 1 | 0.027 | 0.71 | 0.86 |
| Residual | 20 | 0.75 | | |
| **R108 only** (adj. $r^2$= 0.024) | | | | |
| Density | 1 | 0.061 | 1.43 | 0.055 |
| Nitrogen | 1 | 0.042 | 0.98 | 0.46 |
| Density x Nitrogen | 1 | 0.049 | 1.14 | 0.26 |
| Residual | 20 | 0.85 | | |

638 Note: Nitrogen was retained as a term in the sub-models because host values and variances were
639 so divergent from each other.

640 Table 2: Rankings of strain frequencies in two strain communities were consistant across
641 increasingly complex communities: three strains (C3), eight strains (C8), and 68 strains (C68).
642 The higher frequency strain is shown in bold followed by the proportion of replicates in which
643 the higher frequency strain also had higher relative frequency in the more complex communites.
644

| Host | Pairwise Community | Pairwise strain frequency | C3 (5 reps) | C8 (5 reps) | C68 (6 reps) |
|---|---|---|---|---|---|
| A17 | **HM006.1**: KH46c | **0.79** : 0.21 | 1 | 1 | 1 |
| | M162 : **HM006.1** | 0.01 : **0.99** | 1 | 1 | 1 |
| | M162 : **KH46c** | 0.01 : **0.99** | 1 | 1 | 1 |
| R108 | **HM006.1**: KH46c | **0.56** : 0.44 | 0.83 | 0.66 | 1 |
| | M162 : **HM006.1** | 0.01 : **0.99** | 1 | 1 | 1 |
| | M162 : **KH46c** | 0.01 : **0.99** | 1 | 1 | 1 |

645
646

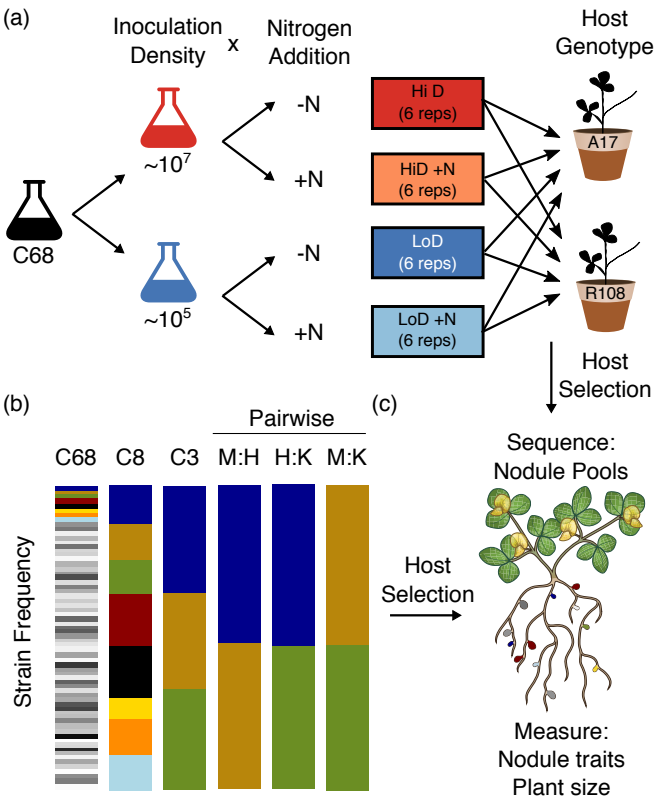


Figure 1: Design of the Nitrogen x Density (a) and Community complexity (b) experiments and traits measured on strains or plants in both experiments (c). All plants were harvested ~6 weeks after planting. Inoculation density is given per plant (~10 plants per pot). Colored stacked bars represent strain frequencies in each of six initial communities of 68 strains (C68- six replicate pots for each N*D treatment), 8 strains (C8- five reps), 3 strains (C3- five reps), and 2 strain pairwise competitions (M:H, H:K, M:K-four reps each).

Figure 2: Host genotype and inoculum density affected rhizobia strain community characteristics. a) Host genotype, density, and their interaction all contributed to variation in strain composition in nodules (percent contribution is in parenthesis, Table 1, Figure S4 for RDA analysis by host genotype). b) Shannon's diversity decreased (i.e. stronger selection) at high densities ($p$<.001) and high nitrogen ($p$=0.039) in A17. R108 nodules were always more diverse than A17 ($p$<0.001) regardless of density or nitrogen (Table S2 for full model) (c) N-addition had little effect on the predicted benefit of nodule communities. However, predicted benefit significantly increased at high densities in A17 ($p$<0.001) and significantly decreased in R108 ($p$=0.003, full results in Table S3).

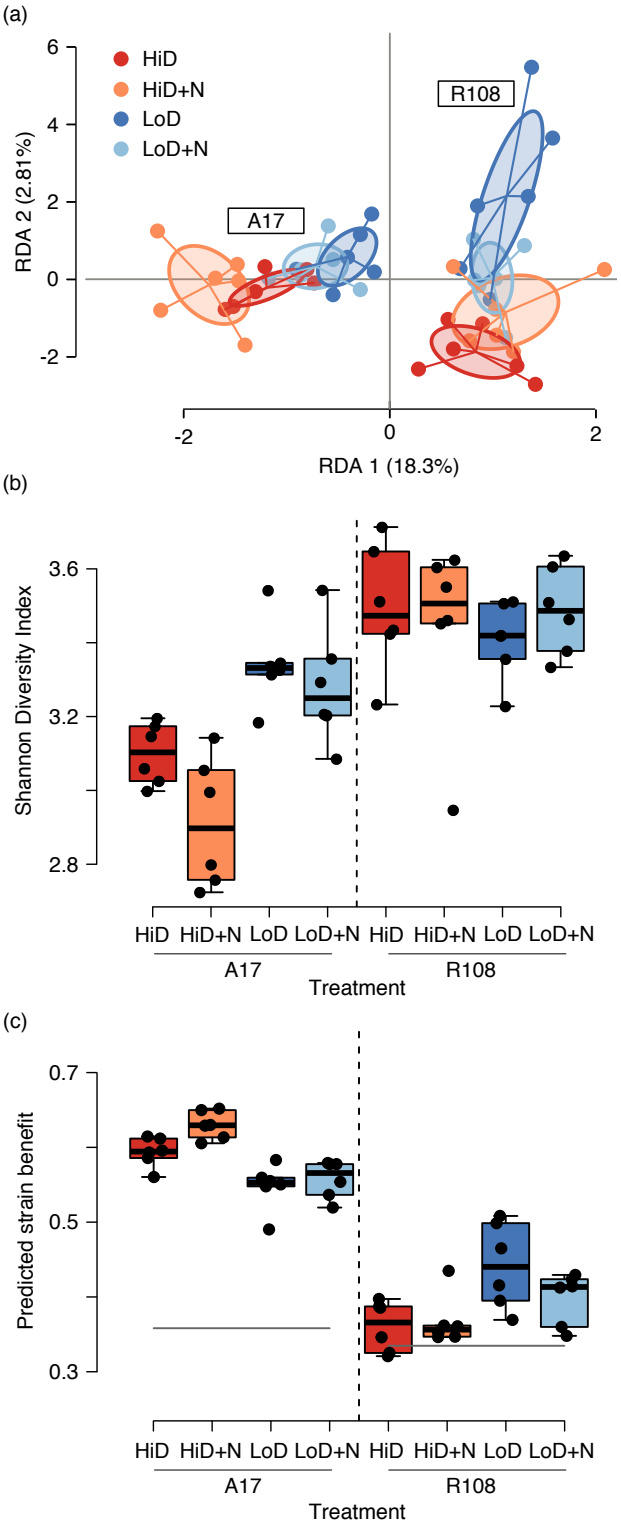

Figure 3: Nodule traits in Density and Nitrogen treatments. Hosts produced very different numbers (a) and sizes (b) of nodules but a similar overall weight (c) across treatments. At low inoculation densities, nodule number decreased and nodule weight increased (full results in Table S2).

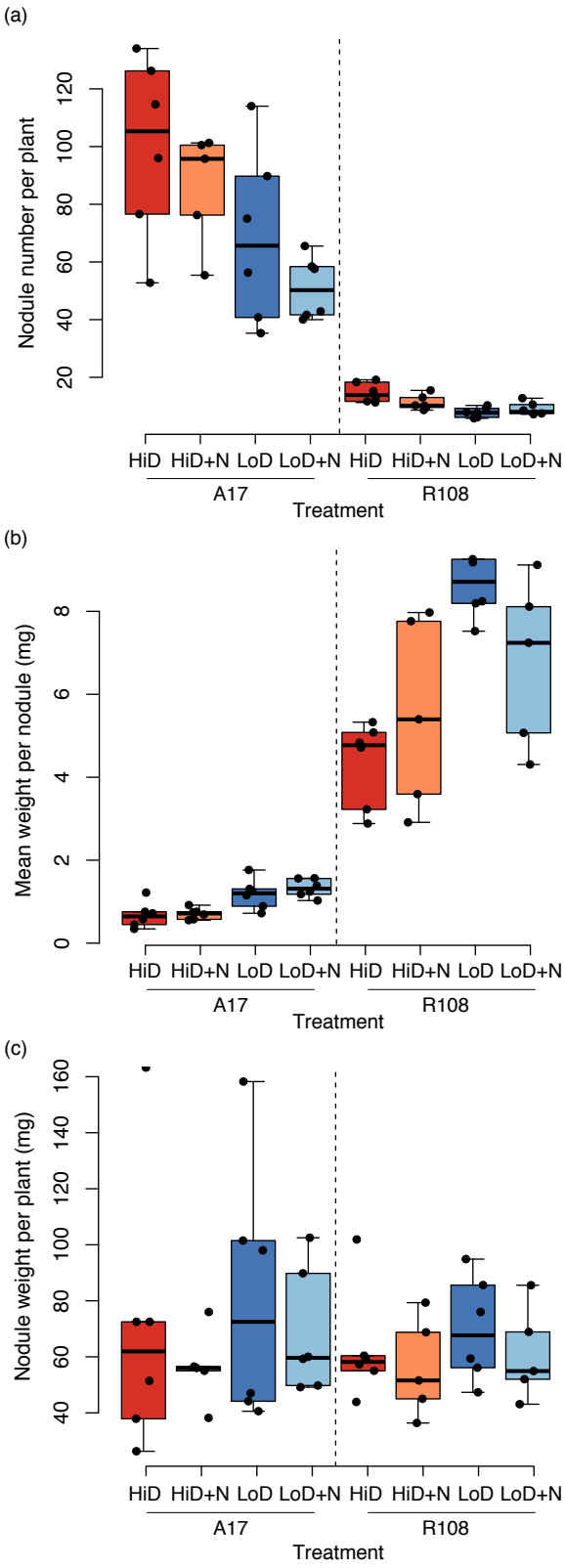

Figure 4: Rank order of relative strain frequencies in the nodule communities were a) identical when plants were inoculated with either the 3-strain (C3) or 68-strain (C68) community, and b) b) nearly identical when inoculated with either the 8-strain (C8) or C68 community. Ranks were determined based on mean strain frequency across 5 (C3 and C8) or 6 (C68) replicates for A17 (triangles) and R108 (circles).