Light availability and rhizobium variation interactively mediate the outcomes of legume-rhizobium symbiosis

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Running headline: Light availability and legume-rhizobium mutualism

Abstract

Premise of the study: Nutrients, light, water, and temperature are key factors limiting the growth of individual plants in nature. Mutualistic interactions between plants and microbes often mediate resource limitation for both partners. In the mutualism between legumes and rhizobia, plants provide rhizobia with carbon in exchange for fixed nitrogen. Because partner quality in mutualisms is genotype-dependent, within-species genetic variation is expected to alter the responses of mutualists to changes in the resource environment. Here we ask whether partner quality variation in rhizobia mediates the response of host plants to changing light availability, and conversely, whether light alters the expression of partner quality variation.

Methods: We inoculated clover hosts with 11 rhizobium strains that differed in partner quality, grew plants under either ambient or low light conditions in the greenhouse, and measured plant growth, nodule traits, and foliar nutrient composition.

Key results: Light availability and rhizobium inocula interactively determined plant growth, and rhizobium partner quality variation was more apparent in ambient light.

Conclusions: Our results suggest that variation in the costs and benefits of rhizobium symbionts mediate host responses to light availability, and that rhizobium variation might more important in higher-light environments. Our work adds to a growing appreciation for the role of microbial intraspecific and interspecific diversity in mediating extended phenotypes in their hosts and suggests an important role for light availability in the ecology and evolution of legume-rhizobium symbiosis.

Key words: symbiosis, genetic variation, Fabaceae, *Rhizobium leguminosarum*, plant–microbe interactions, isotopes, resource, trade

Introduction

Resource limitation influences all levels of biological organization, from the vast community of detritivores and saprophytes belowground, to the primary producers, herbivores, and predators aboveground. In addition to underlying major theories in community ecology (Leibig, 1840; Tilman, 1977; 1985; Bloom et al., 1985), the concept of limiting resources underlies much theory in mutualism ecology and evolution (reviewed by Bronstein, 2015). Symbioses that are based on the exchange of resources are beneficial when they alter patterns of resource limitation in ways that increase the fitness of both partners. Heuristic theory (Collins Johnson, 1993; Bronstein, 1994; Collins Johnson et al., 1997; Kiers et al., 2002; O'Brien et al., 2018), mathematical theory (Schwartz and Hoeksema, 1998; Bever, 2015; Christian and Bever, 2018; Clark et al., 2019), and empirical observations (Collins Johnson et al., 2010; 2015; Zheng et al., 2015; Ji and Bever, 2016; Shantz et al., 2016; Ossler and Heath, 2018) all indicate that the ecological outcome of resource mutualisms can shift along the mutualism-parasitism continuum depending on the availability of traded resources. Theory also suggests that resource availability will also influence the evolution of resource mutualisms (West et al., 2002; Thrall et al., 2007; Akçay and Simms, 2011). However, our ability to predict mutualism evolution, and the response of mutualisms to environmental change (Six, 2009; Kiers et al., 2010; Shantz et al., 2016), requires a nuanced understanding of how the resource environment alters both the ecological outcomes of mutualism *and* the quality of different partner genotypes and the expression of genetic variation for mutualism traits.

61 The legume-rhizobium symbiosis is a classic resource mutualism, wherein
62 rhizobial bacteria housed in legume root nodules fix atmospheric dinitrogen (N_2) into
63 plant-available forms and receive fixed carbon (C) generated by photosynthesis in return.
64 As predicted by resource mutualism theory (Schwartz and Hoeksema, 1998; West et al.,
65 2002; Neuhauser and Fargione, 2004; Akçay and Simms, 2011), the fitness outcomes of
66 legume-rhizobium mutualism are known to be sensitive to the external availability of
67 both N (Heath et al., 2010; Barrett et al., 2012; Weese et al., 2015; Regus et al., 2017;
68 Forrester and Ashman, 2018) and C (light) (Sprent, 1973; Murphy, 1986; Hansen et al.,
69 1990; Myster, 2006; Lau et al., 2012; Ballhorn et al., 2016; Taylor and Menge, 2018).
70 First, increased N typically reduces the plant benefit from associating with rhizobia
71 (Regus et al., 2017; Wendlandt et al., 2019), and plants often (but not always) respond by
72 reducing resource allocation to rhizobia (Streeter and Wong, 1988; Heath et al., 2010;
73 Simonsen et al., 2015; Regus et al., 2017; Wendlandt et al., 2019). Second, the net
74 benefits for plant hosts (i.e., growth or fitness increase from associating with rhizobia,
75 which will be a function of the growth benefits resulting from N gained versus the fitness
76 costs of C spent) are expected to decrease as C becomes more limiting relative to N, since
77 mutualism with rhizobia requires that plants possess adequate C stocks to support these
78 costly N-fixing symbionts (Minchin and Witty, 2005; Pringle, 2016). Thus, low light and
79 high N environments are both predicted to reduce mutualism benefits to both plants and
80 rhizobia (Johnson et al. 1997).
81 Not all rhizobium mutualists, however, are equally beneficial. Rhizobium strains
82 are well-known to vary in partner quality, which is most often measured as relative plant
83 growth and fitness (reviewed by Denison, 2000; Simms and Taylor, 2002; Heath and

84 Stinchcombe, 2014; Sachs et al., 2018). These growth and fitness benefits to the plant
85 depend on both the benefits and costs of symbiosis, i.e., the benefits of fixed N received
86 minus the C costs including nodule formation, nodule respiration, and production of the
87 bacterial storage compound poly-3-hydroxybutyrate or PHB (Tjepkema and Winship,
88 1980; Minchin and Witty, 2005; Ratcliff et al., 2008; Ruess et al., 2013). Plant nutrient
89 status and light interactively determine plant growth (reviewed by Elser et al., 2010);
90 therefore, genetic variation in N-fixing rhizobium symbionts may interact with light
91 availability to influence the outcome of mutualism for plant hosts. Likewise the relative
92 partner quality of different nutritional symbionts like rhizobia should depend on the
93 resource stoichiometry of the hosts, and thus we might expect that the relative fitness
94 benefits of interacting with different rhizobium inocula might shift as hosts encounter
95 different light environments. For example, variation in rhizobium partner quality might
96 be reduced in low light environments if plants are so C-limited that additional N provides
97 little growth benefit.
98 Thus, on the one hand, a plant's ability to respond to a favorable shift in the
99 resource environment, like increased light availability, might depend on its limitation by
100 other resources (like N) and, therefore, the quality of its mutualist partner. On the other
101 hand, the relative quality of different mutualist partners might depend on the resource
102 environment. Here we investigate how the net growth benefits to plants from rhizobia
103 respond to light availability and vary across substantial rhizobium genetic variation. We
104 also measure plant traits associated with the benefits they receive from rhizobia through
105 N fixation (foliar N, C:N ratio, and $\delta^{15}N$), and with some of the costs associated with
106 nodulation (nodule number and per-nodule weight) to better understand how light

availability interacts with rhizobium strain variation to shift the costs and benefits of symbiosis. We grew *Trifolium hybridum* (hybrid clover) hosts with one of 11 strains of *Rhizobium leguminosarum* in either ambient light or shade in the greenhouse to ask how rhizobium strain and the light environment interact to affect plant growth. These experiments shed light on how genetic variation in rhizobial mutualists mediates the response of plant hosts to different light environments, and reciprocally how the light environment alters the relative benefits of different rhizobia or the expression of rhizobium partner quality variation.

Materials and Methods

Study system: We studied the effects of 11 *R. leguminosarum* (hereafter rhizobium) strains on *T. hybridum* growth in two different greenhouse light treatments (ambient or shade). These strains were a subset of those studied previously (Weese et al., 2015), and full methods for rhizobium strain isolations and partner quality assessments may be found there. Previously-assessed strain partner quality may also be found in Appendix S1 (see the Supplementary Data with this article). Briefly, rhizobia isolated from soils at the Kellogg Biological Station Long Term Ecological Research Site (KBS LTER; http://lter.kbs.msu.edu/) by isolating them from nodules of three *Trifolium* species (*T. hybridum, T. repens, and T. pratense*) in a large common garden experiment (Weese et al., 2015). Subsequently a single strain common garden experiment (Weese et al., 2015) was used to assess the effects of individual strains on plant growth and chlorophyll content (a proxy for plant N status; Swiader and Moore, 2002). The 11 strains used to

129 inoculate the current experiment were selected to represent a range of partner quality (see
130 Appendix S1).
131
132 *Greenhouse experiment*: To study how rhizobium genetic variation influences plant
133 responses to light, *T. hybridium* plants were grown with one of 12 rhizobium treatments
134 (11 strains plus an uninoculated control) in either ambient light or under 50% shade cloth
135 (open on sides to minimize effects on humidity). The split-plot design included two light
136 treatments (ambient or shade) that were applied to whole plots (2 plots per light
137 treatment), and the twelve inoculation treatments were randomly assigned to individual
138 plants within each plot (10 replicates per inoculation treatment per plot, for 480 plants
139 total). We purchased *T. hybridium* seeds from a local seed supply company (Illini FS,
140 Urbana, IL, USA). Seeds were washed extensively and then surface-sterilized for one
141 minute in ethanol followed by 10 minutes in a 5-6% sodium hypochlorite solution before
142 planting into 107 mL SC7 Cone-tainers (Steuwe and Sons Inc., Tangent, OR, USA)
143 containing root wash mix (1:1:1 soil: calcined clay: torpedo sand). Plants were inoculated
144 at eight days post-planting with the appropriate rhizobium strain (OD_{600} = 0.1 or ~10^5
145 cells). Plants were grown under 14 hour days in the greenhouse, provided with
146 supplemental light to reach a maximum 600 W/m^2, given adequate water throughout the
147 experiment, and fertilized with N-free Fahraeus solution (Somasegaran and Hoben, 1994)
148 every four days.

149 *Data:* At harvest, we gathered data on three main types of symbiotically-relevant
150 phenotypes: 1) Data on aboveground and belowground biomass and root:shoot ratio
151 provide information on plant growth responses (i.e., the net benefit of associating with

rhizobia), 2) nodule number and nodule weight (mean individual weight of a nodule) as a proxy for host costs of nodulation, allowing us to calculate how the net benefits to plants per infection (per nodule) change across treatment combinations, and finally 3) plant foliar C and N data (C, N, C:N ratio, and $\delta^{15}N$), which provide more direct information on how the balance of C and N shifts across inoculum and light treatments. Together these growth and functional phenotypes provide a more mechanistic understanding of how rhizobium partner quality affects plant nutrient status and in turn mediates the response of plants to light availability, though we note that some costs and benefits of the symbiosis were not measured directly (e.g., nodule respiration, N acquired from symbiosis, PHB production, any non-C costs of nodulation).

At week seven, 48 plants (one from each plot from each treatment combination) were randomly selected and harvested early for preliminary analysis and to ensure that plants were nodulated. At week nine, we counted the number of leaflets for all remaining plants. The remainder of the experiment was harvested at week 15. At harvest, above- and belowground plant tissue were separated and, for half of the plants in each treatment combination (5 replicates per plot), nodule number was counted and 10 haphazardly-chosen nodules were removed, dried at 60^{o} C, and weighed to estimate mean per-nodule weight for each plant (hereafter nodule weight). Plant tissue was dried at 60^{o} C for at least 48 hours prior to weighing. We calculated per-nodule plant biomass for each plant in the experiment as belowground biomass + aboveground biomass and divided by the total number of nodules on the root system. We calculated root:shoot ratio for each plant by dividing belowground biomass by aboveground biomass.

After harvest, dried leaf tissue from the subset of 5 replicate plants per treatment and block combination used to estimate nodule number and nodule biomass was submitted to the University of Wyoming's Stable Isotope Facility (Laramie, WY, USA) for grinding and estimation of C and N content as well as $\delta^{15}N$ using a Costech 4010 elemental analyzer coupled to a Thermo Delta Plus XP IRMS (Thermo Fisher, Waltham, MA, USA). Without non-symbiotic controls, it is not possible to say with certainty how much plant $\delta^{15}N$ was derived from symbiotic N-fixation (Shearer and Kohl, 1986); therefore, all variation in $\delta^{15}N$ levels is relative to other treatment combinations. Because plant N derived from symbiotic N-fixation is more similar to atmospheric N in isotope composition (versus soil N), field-grown plants with higher rates of N-fixation generally have decreased $\delta^{15}N$ values, relative to those with lower fixation rates (Shearer and Kohl, 1986; Handley and Raven, 1992), though these dynamics are more difficult to predict in pot experiments where many drivers of soil N isotope ratios from the field (Craine et al., 2015) may be missing.

While plants were initially inoculated with isogenic populations of a single strain, the fact that all uninoculated plants formed nodules revealed cross-contamination, which generally occurs when bacteria move among neighboring pots (K.D. Heath, personal observation). Control plants had 50% fewer nodules, compared to inoculated plants (72.7 vs. 139.5 nodules, respectively; $p = 0.0098$). Given the randomized experimental design, this cross-contamination was random with respect to treatment and thus should reduce the likelihood of detecting treatment effects, making tests for genetic differences conservative. The highly significant variation among the 11 inoculum treatments for all measured variables (see results; Table 1) indicated that these treatments differed even in

197 the face of contamination. A cautious interpretation, therefore, is that plants in different
198 inoculation treatments formed symbiosis with genetically distinct, but not necessarily
199 isogenic, populations of rhizobia. Uninoculated plants were not included in further
200 analyses.
201 *Analyses:* All analyses were implemented in SAS (version 9.2, SAS Institute, Cary NC).
202 Phenotypic correlations (calculated using PROC CORR) among all measured variables,
203 in both ambient and shade environments, are presented in Appendix S2. We used mixed
204 model ANOVA (PROC MIXED) specifying the Satterthwaite approximation for the
205 denominator degrees of freedom (DDFM=SATTERTHWAITE) to test for the fixed
206 effects of light treatment, rhizobium inoculum (11 inocula), light × inoculum interaction,
207 and blocking variables (random effect of greenhouse plot nested within light treatment
208 and fixed effect of early vs. late harvest date) on measures of plant growth and
209 nodulation. Random effects were tested using the log-likelihood ratio of nested models as
210 described elsewhere in detail (Littell et al., 1996; Heath, 2010). Because we were
211 interested in proportional rather than absolute changes in most traits across treatment
212 combinations, variables were natural log-transformed before analysis (Wootton, 1994;
213 Hamback and Beckerman, 2003), with the exception of foliar %C and %N (arcsine
214 square root transformation) and $\delta^{15}N$ (not transformed). Qualitatively, results did not
215 depend on the choice of data transformation. In addition, we used separate MANOVA of
216 plant growth traits (early leaflets, aboveground biomass, belowground biomass,
217 root:shoot ratio, per-nodule plant biomass) and foliar nutrient traits (%C, %N, C:N ratio,
218 and $\delta^{15}N$) to test for the overall effects of experimental treatments on these suites of
219 traits.

220 To investigate how changes in plant biomass were related to nodulation traits and
221 foliar nutrient levels, we calculated correlations between all measured traits using
222 Pearson correlations (PROC CORR) of inoculum trait means (11 inocula in each of two
223 light environments). We used Spearman rank correlations between the 11 inoculum
224 means in ambient versus shade environments to test whether significant interactions of
225 light treatment and rhizobium inoculum (see Results) were driven by changes in rank
226 versus changes in variance. Finally, to explore whether the variation in plant growth
227 caused by rhizobium inocula of varying quality was magnified in the ambient light
228 environment, we used Levene's tests for homogeneity of variances (implemented in
229 PROC GLM) to test whether the among-inoculum variance in traits differed between
230 light environments.
231
232 **Results**
233 MANOVA indicated strong effects of all model terms on plant growth traits
234 (Table 1A). With few exceptions, the effects of genetically-variable rhizobium inocula
235 greatly exceeded the effects of light on plant growth, nodulation, and foliar C and N
236 (Table 1A-C; Figure 1). For example, inoculation with the highest quality rhizobium
237 strain resulted in ~15X more aboveground biomass on average, compared to the lowest
238 quality strain (493: 1.33g ± 0.49 versus 498: 0.09g ± 0.04). For comparison, plants in the
239 ambient light treatment produced just ~1.3X more aboveground biomass than plants in
240 the shade treatment. However, we also detected evidence that the response of plant hosts
241 to the light environment depended on rhizobium inoculum (significant light × inoculum
242 interactions, Table 1A-C). Plants inoculated with some strains exhibited large biomass

243 increases in ambient light compared to shade (e.g., strain 262: 78% and over 300% for
244 above- and belowground biomass respectively). In contrast, plants inoculated with other
245 strains did not respond much to increased light availability, or even had slightly
246 decreased growth in ambient light (see reaction norms in Figure 1A-B, Appendix S3).
247 Compared to the interactive effects with rhizobium inoculum, the main effect of shade on
248 plant traits was less dramatic, with marginal reductions in belowground biomass,
249 significant reductions in root:shoot ratio, per-nodule plant biomass, and C:N ratio (34%,
250 33%, and 23% decrease in shade, respectively; Table 1A,C). We included harvest date as
251 a blocking factor, and its significant effect on nearly all traits was consistent with plants
252 harvested later being larger (e.g., significant effects on biomass and nodule number;
253 Table 1A-C).
254 Like plant growth, MANOVA for foliar nutrients indicated strong effects of all
255 model terms on plant growth traits (Table 1C). Percent N & C, C:N ratio, and $\delta^{15}N$ varied
256 widely among inocula (Table 1C; Figure 1), although the magnitude of the observed
257 strain differences in $\delta^{15}N$ varied across light environments (significant inoculum x light
258 interaction on $\delta^{15}N$, Table 1C). Moreover genetic correlations indicate that N content and
259 $\delta^{15}N$ strongly predicted aboveground biomass in both light environments (Table 2),
260 which together suggest that the availability of fixed N increased plant biomass. For
261 example, plants inoculated with strains 498 and 699 had extremely high C:N ratios and
262 large, positive $\delta^{15}N$ values, suggesting little biologically fixed N in both light
263 environments (Figure 1E-F). These plants made little biomass even in the ambient light
264 environment (Figure 1A-B). On the other hand, inocula generating the most negative

265 $\delta^{15}N$ values (*e.g.*, 209, 627), suggesting more biologically fixed N, resulted in large gains
266 in plant biomass when light became less limiting.

267 Overall we found a tradeoff between nodule number and nodule weight, i.e.,
268 inocula producing more nodules tended to produce smaller nodules (Table 2). Unlike
269 plant growth, nodule number and nodule weight differed among inocula but did not
270 respond to light (no significant effects of light or light x inoculum interactions; Table 1;
271 Figure 1C). However, the relationship between these nodulation traits (number and
272 weight) and plant growth did depend on the light environment. In ambient light, neither
273 nodule number nor nodule weight predicted shoot biomass (Table 2). In the shade,
274 however, inocula producing abundant nodules resulted in host plants with fewer leaflets
275 and less above- and belowground biomass (Table 2), suggesting the formation of
276 numerous nodules was costly in low light environments. Indeed plant biomass expressed
277 on a per-nodule basis decreased by 34% on average in the shade and depended on
278 inoculum (Table 1; Figure 1D). Moreover per-nodule plant biomass was positively
279 correlated with nodule size and negatively correlated with both C:N ratio and $\delta^{15}N$ in the
280 shade (Table 2) – suggesting that inocula producing fewer, larger nodules were more
281 beneficial for shaded hosts.

282 Together, our trait data suggest that one inoculum (strain 262) was particularly
283 interesting in the context of net nodulation benefits. Inoculation with 262 resulted in
284 plants that had negative $\delta^{15}N$ values and low C:N ratios, similar to other highly beneficial
285 inocula (Figure 1E,F), yet produced only moderate per-nodule biomass and responded
286 with very large increases in both nodulation and plant biomass in ambient light (Figure
287 1A-D). Together these observations suggest that, unlike low-fixing, low-biomass

inoculum treatments (strains 498 and 699), an inoculum dominated by strain 262 might result in a high-benefit, high cost symbiosis – fixing adequate N, but also requiring abundant plant C.

While the net growth effects of different rhizobium inocula changed across light environments (light × inoculum interactions; Table 1), this interaction was largely driven by changes in variance rather than rank shifts among different inocula. Spearman rank correlations indicated that the highest quality inocula in ambient light environments were also the most beneficial in low light environments (e.g., early leaflet count, $r_{11} = 0.95$, $p < 0.0001$; aboveground biomass, $r_{11} = 0.85$, $p = 0.0010$; belowground biomass, $r_{11} = 0.83$, $p = 0.0017$; C:N ratio, $r_{11} = 0.59$, $p = 0.0560$; %N, $r_{11} = 0.89$, $p = 0.0002$; $\delta^{15}N$, $r_{11} = 0.71$, $p = 0.0146$). Larger variance among inocula in ambient light for all biomass traits, combined with significant Levene's tests for early leaflet count ($F_{1,20} = 4.75$, $p = 0.0414$) and belowground biomass ($F_{1,20} = 6.98$, $p = 0.0156$), further indicate that the expression of genetic variation in rhizobium quality was magnified when light was more available.

Discussion

Our results indicate that: 1) variation in rhizobium partner quality is substantial and can mediate plant responses to the light environment, and reciprocally, 2) variation in rhizobium partner quality depends on the light environment. Plant biomass responses, together with data on nodule number and nodule weight as well as foliar C:N ratios and $\delta^{15}N$, suggest that these findings are underpinned by variation in both C costs and N benefits among different rhizobium inocula.

311 *Rhizobium variation mediates host plant responses to light:* The effects of rhizobium
312 inoculum on plant growth in our experiment were large and dwarfed the main effects of
313 the light environment (see results), though we note that plants in nature likely have more
314 access to soil N, and there we might expect that rhizobia would have weaker effects
315 compared to light limitation or other environmental effects. Nevertheless, in this
316 experiment, the variation among rhizobium inocula was large and interacted with light to
317 determine plant growth. This finding adds to a growing appreciation for the role of
318 microbial intraspecific and interspecific diversity in mediating ecologically-important
319 host traits: endosymbiont genetic variation confers variation in insect defense, and
320 metabolism (Douglas, 2009; Oliver et al., 2010; Russell et al., 2013; Oliver and Higashi,
321 2019), leaf fungal endophytes contribute to variation in plant defense (Arnold et al.,
322 2003; Busby et al., 2015; Christian et al., 2017), and plants with different mycorrhizal
323 strategies (arbuscular mycorrhizae, ectomycorrhizae, or nonmycorrhizal) exhibit different
324 leaf nutrient compositions (Shi et al., 2013). How much macrobial trait variation,
325 traditionally the focus of evolutionary biologists, will ultimately be attributable to
326 symbiotic microbiota remains to be seen as more research at the interface of evolutionary
327 biology and host-microbiome interactions accumulates.
328 While we did not measure C costs or N fixation rates *per se*, when taken together,
329 our dataset combining plant growth, nodulation, and foliar nutrient composition is
330 consistent with the idea that N benefits and C costs (both in terms of nodule formation
331 and per-nodule costs) vary independently in *R. leguminosarum*, and that together these
332 costs and benefits mediate the responses of host plants to changes in light availability.
333 The costs and benefits of mutualism represent a traditionally intractable, but important,

aspect of understanding the evolution of symbiotic mutualisms (Jones et al., 2015). Typically researchers have studied how rhizobia vary in terms of their effects on whole plant traits (Burdon et al., 1999; Heath, 2010; Barrett et al., 2012; Porter and Simms, 2014) or the instantaneous rate of N-fixation via acetylene reduction assays (McNeil, 1982; Minchin et al., 1983; Tan and Tan, 1986). Ecosystem ecologists and ecophysiologists have long used isotope abundances (natural or enriched) to study biological N fixation in the field (Shearer and Kohl, 1986; Mead and Preston, 2011; Yelenik et al., 2013; Craine et al., 2015) or greenhouse (Menge et al., 2015; Taylor and Menge, 2018), but mutualism research increasingly features the use of isotope abundance (natural or enriched) to study the trade of benefits in resource mutualisms (Ruess et al., 2013; Regus et al., 2017; Schmidt et al., 2017; Taylor and Menge, 2018).

Ruess et al. (2013) estimated nodule respiration, N-fixation, and *Frankia* strain identity in a field survey of *Alnus tenuifolia* and found that *Frankia* vary in terms of both N fixation and respiratory cost. Some inocula in our study seem to be low quality in terms of N-fixation ability (based on C:N and $\delta^{15}N$, relative to other inocula), resulting in N-limited plants independent of C availability, while others appeared to fix more N and thus allow hosts to respond positively to increased C availability, though for some (262) only in ambient light, potentially because of substantial C costs. More physiological measurements would provide additional resolution of the various C costs of symbiosis, as well as the benefits in terms of absolute N fixed.

Ecological and evolutionary effects of light on the legume-rhizobium mutualism: The importance of the light environment on the ecology and evolution of plant-symbiont

357 resource mutualisms has not received much theoretical attention, despite the fact that
358 light controls the availability of an essential traded commodity (plant C). In contrast to
359 other recent studies (Lau et al., 2012; Taylor and Menge, 2018), shaded plants did not
360 significantly reduce allocation to rhizobia (i.e., no significant effects of light or light ×
361 inoculum interactions on nodule number or nodule weight), though the observed trends
362 (~20% reductions in nodule number and nodule weight in shade) were consistent with
363 previous findings. In addition, our light reduction was less severe (50% here, compared to
364 80% in Lau et al., 2012 and 92% in Taylor and Menge, 2018), and our split-plot design
365 resulted in less power to detect light main effects.
366 We do find that ambient light environments tend to increase the magnitude of
367 variation among rhizobium inocula, in terms of plant growth (though not nodule traits).
368 This represents a genetic extension of resource mutualism theory showing that the costs
369 and benefits of mutualism change depending on the external availability of traded
370 resources such as C, N, and phosphorus (Collins Johnson et al., 1997; Schwartz and
371 Hoeksema, 1998; Neuhauser and Fargione, 2004; Collins Johnson et al., 2010). In our
372 study, rhizobium inocula did not change rank across light environments, suggesting that
373 selection on plants to interact with different strains would not depend on the light
374 environment. In contrast, in the mycorrhizal mutualism, decreasing light availability
375 through shading has been shown to alter the relative allocation to different fungal species
376 on host roots (Zheng et al., 2015; Knegt et al., 2016).
377 Nevertheless our findings suggest that the light environment could be just as
378 important to rhizobium evolution as the more commonly studied N availability (Akçay
379 and Simms, 2011; Regus et al., 2014; Weese et al., 2015; Klinger et al., 2016; Regus et

al., 2017). Environmental-dependence of rhizobium partner quality variation might suggest that the plant-mediated feedbacks that select for increased rhizobium partner quality (Kiers et al., 2003; Simms et al., 2006; Heath and Tiffin, 2009; Oono et al., 2011; Regus et al., 2014; Batstone et al., 2017) should be strongest in high light situations, as should selection on plants to evolve such mechanisms (Foster and Kokko, 2006; Steidinger and Bever, 2014; Heath and Stinchcombe, 2014; Bever, 2015; Christian and Bever, 2018). Additional experiments will be necessary to test these hypotheses.

Batstone et al. (this issue) found that the expression of plant genetic variation for nodule number depended on the environment, whereas we find that rhizobium variation contributing to plant growth benefits differed across light environments. Thus, while we arrive at similar broad-scale conclusions about the importance of context-dependent genetic variation to mutualism evolution, the particulars of which partner (host vs. symbiont) and traits were different. More studies quantifying genetic variation in mutualism traits across environments will be required before we arrive at a predictive synthesis for which traits and environmental variables are likely the most important for context-dependent evolutionary outcomes.

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Supporting information

Additional Supporting Information may be found online in the supporting information section at the end of the article. Appendix S1: Partner quality information for *Rhizobium* strains used. Appendix S2: Table of phenotypic trait correlations. Appendix S3: Reaction norm plot for total plant biomass.

Data accessibility

All data presented in this study are available on DRYAD (https://doi.org/10.5061/dryad.hx3ffbg9s).

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644

645 **Table 1** Mixed model ANOVA and MANOVA for the effects of light treatment (ambient or shade), rhizobium strain, the light ×
646 strain interaction, and blocking variables (models in A include harvest date) on hybrid clover traits, nodule traits, and foliar nutrient
647 composition. For fixed effects, F is shown; for random effects, chi-square (log likelihood ratio) test statistic is shown. All variables are
648 fixed in MANOVA.

A. Plant traits *Fixed effects*	*N df*	Above-ground biomass	Below-ground biomass	Plant biomass per nodule	Root: Shoot	Leaflet number (wk. 9)	MANOVA N *df* / *D df*	MANOVA Wilks Lambda
Light	1	4.39	16.69^{+}	**3.94***	**60.95******	1.92	4 / 279	**53.74******
Inoculum	10	**87.83******	**55.66******	**6.92******	1.61	**85.32******	40 / 1059.8	**13.24******
Light × Inoculum	10	**3.00****	**6.28******	0.77	1.68^{+}	**4.82******	40 / 1059.8	**2.49******
Harvest Date	1	**313.44** ****	**205.17******	**13.61*****	**5.30***		4 / 279	**75.69******
Random effects								
Plot (Light)		**10.2*****	**9.7*****	0	**4.0***	**48.1******	8 / 558	**3.15****

649

B. Nodule traits *Fixed effects*	*N df*	Nodule number	Nodule weight
Light	1	2.51	0.3
Inoculum	10	**18.21******	**11.04******

Light × Inoculum	10	0.42	0.37
Harvest Date	1	**27.91******	2.29
Random effects			
Plot (Light)		0	**3.9***

650

C. Foliar nutrients *Fixed effects*	*N df*	Foliar Percent C	Foliar Percent N	Foliar C:N ratio	Foliar $\delta^{15}N$	MANOVA N *df* / *D df*	MANOVA Wilks Lambda
Light	1	8.26	6.06	**52.59******	0.35	4 / 187	**21.56******
Inoculum	10	**5.77******	**62.67******	**88.62******	**66.23******	40 / 710.94	**21.07******
Light × Inoculum	10	1.46	0.59	0.32	**2.55****	40 / 710.94	**2.84******
Random effects							
Plot (Light)		**8.0****	**15.9******	**14.5******	**4.5***	8 / 374	**5.44******

651

652 Mixed model denominator degrees of freedom were 282 and 293 for plant biomass per nodule and nodule number (respectively) or
653 between 1.92 and 3.99 (all other variables) for the effect of light, and ranged from 175-405 for other fixed effects. $^{+}P\leq0.1$; $^{*}P\leq0.05$;
654 $^{**}P\leq0.01$; $^{***}P\leq0.001$; $^{****}P\leq0.0001$.

655 **Table 2** Genotypic correlations among all dependent variables in ambient light (above the diagonal) or shade (below the diagonal)

656 treatments. Pearson correlation coefficients (N = 11) are shown, with significant correlations (P < 0.05) indicated in bold.

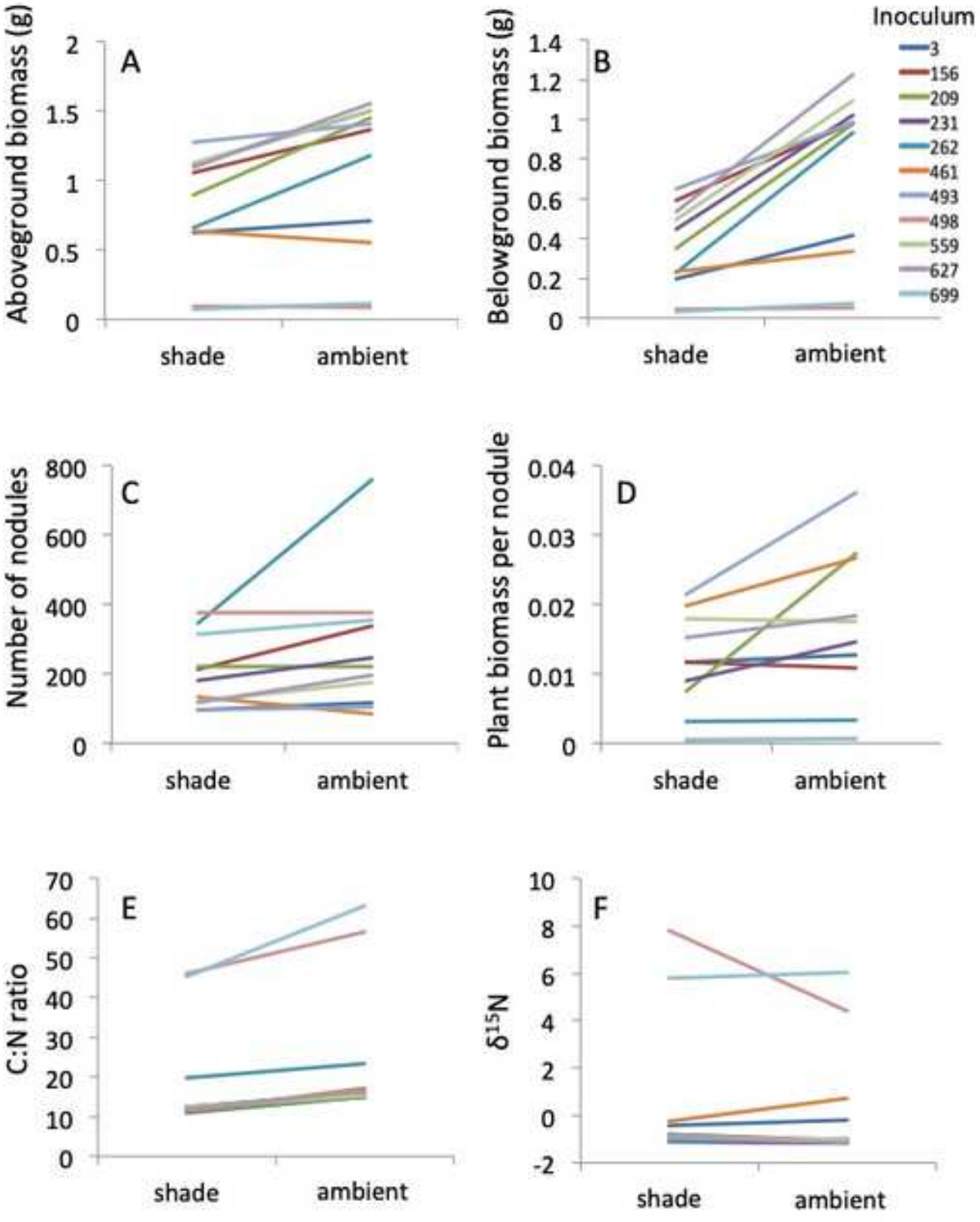
	Leaflet number	Above-ground biomass	Below-ground biomass	Plant biomass per nodule	Root: shoot	Nodule number	Nodule weight	Foliar % C	Foliar % N	Foliar C:N ratio	Foliar $\delta^{15}N$
Leaflet number		**0.981**	**0.989**	0.472	0.238	-.025	-0.214	**0.774**	**0.596**	**-0.723**	**-0.845**
Above-ground biomass	**0.983**		**0.990**	0.528	0.344	-0.115	-0.062	**0.808**	**0.705**	**-0.818**	**-0.908**
Below-ground biomass	**0.976**	**0.960**		0.461	0.327	-0.031	-0.130	**0.805**	**0.641**	**-0.767**	**-0.879**
Plant biomass per nodule	**0.713**	**0.727**	**0.705**		0.354	**-0.692**	0.508	0.309	**0.707**	**-0.678**	-0.586
Root:shoot	-0.192	-0.176	-0.137	0.056		-0.386	0.497	0.445	0.572	-0.532	-0.430
Nodule number	**-0.691**	**-0.704**	**-0.638**	**-0.899**	-0.263		-0.589	-0.014	-0.463	0.356	0.165
Nodule weight	0.075	0.161	0.076	**0.659**	0.518	**-0.678**		0.125	**0.610**	-0.474	-0.255
Foliar %C	**0.771**	**0.823**	**0.762**	**0.826**	0.212	**-0.833**	**0.611**		**0.771**	**-0.808**	**-0.827**
Foliar %N	**0.706**	**0.784**	**0.665**	**0.764**	0.174	**-0.827**	**0.637**	**0.965**		**-0.979**	**-0.892**
Foliar C:N ratio	**-0.767**	**-0.852**	**-0.726**	**-0.744**	-0.068	**0.784**	-0.536	**-0.937**	**-0.973**		**0.965**
Foliar $\delta^{15}N$	**-0.767**	**-0.857**	**-0.727**	**-0.648**	0.044	**0.674**	-0.394	**-0.837**	**-0.882**	**0.964**	

Figure legend

Figure 1 Reaction norms of hybrid clover growth, nodule number, and foliar nutrient composition across two light treatments in symbiosis with 11 N-fixing rhizobium inocula. Raw means are shown.

Figure

Click here to access/download;Figure;Figure1.jpg



Appendix S1 Information for 11 rhizobium strains used in the current experiment including partner quality phenotypes estimated from common garden experiments (described in Weese et al. 2015) and host species of origin and field treatment (nitrogen-fertilized, versus control) from which each strain was originally isolated (see Weese et al. 2015 for details). Trait values are back-transformed LS means.

Strain	Field N treatment	Shoot Mass (g) Bktrnsfmd LS mean	Leaf Number Bktrnsfmd LS mean	Stolon Number Bktrnsfmd LS mean	Chlor. Content LS mean	*Trifolium* spp. of origin
498	N	0.1	7.37	0.98	18.87	*T. hybridum*
699	N	0.13	6.54	1.09	20.84	*T. repens*
262	N	0.14	8.98	1.46	33.35	*T. repens*
3	C	0.2	11.15	1.78	38	*T. repens*
493	C	0.27	14.02	1.75	45.47	*T. pratense*
559	C	0.28	14.94	1.92	37.6	*T. pratense*
231	N	0.65	21.78	3.02	43.91	*T. repens*
156	N	0.66	22.97	2.63	45.73	*T. hybridum*
627	C	0.66	22.72	2.49	46.35	*T. repens*
209	N	0.69	23.36	2.65	43.31	*T. hybridum*
461	C	0.72	27.63	2.96	47.21	*T. hybridum*

Appendix S2 Phenotypic correlations among all dependent variables in ambient light (above the diagonal) or shade (below the diagonal) light treatments. Correlation coefficients ($96 < N < 191$) are shown; significant correlations ($P < 0.05$) are indicated in bold.

	Leaflet number	Above-ground biomass	Below-ground biomass	Plant biomass per nodule	Root: shoot	Nodule number	Nodule weight	Foliar % C	Foliar % N	Foliar C:N ratio	Foliar $\delta^{15}N$
Leaflet number		**0.899**	**0.866**	**0.264**	0.086	0.124	**-0.240**	**0.382**	**0.572**	**-0.613**	**-0.761**
Above-ground biomass	**0.853**		**0.962**	**0.185**	0.036	**0.269**	-0.122	**0.416**	**0.666**	**-0.703**	**-0.861**
Below-ground biomass	**0.874**	**0.941**		**0.191**	**0.199**	**0.255**	-0.126	**0.411**	**0.629**	**-0.667**	**-0.832**
Plant biomass per nodule	**0.474**	**0.526**	**0.518**		**0.196**	**-0.622**	**0.231**	0.118	**0.295**	**-0.297**	-0.143
Root:shoot	-0.032	-0.006	**0.186**	**0.176**		**-0.298**	0.149	0.014	0.153	-0.169	-0.187
Nodule number	-0.070	0.083	0.067	**-0.632**	**-0.270**		**-0.475**	0.064	**-0.206**	**0.205**	-0.052
Nodule weight	0.074	0.107	0.098	**0.239**	-0.084	**-0.321**		0.059	**0.317**	**-0.294**	0.064
Foliar %C	**0.362**	**0.308**	**0.216**	**0.204**	-0.102	**-0.305**	**0.250**		**0.517**	**-0.455**	**-0.407**
Foliar %N	**0.495**	**0.552**	**0.420**	**0.397**	-0.065	**-0.492**	**0.309**	**0.763**		**-0.992**	**-0.796**
Foliar C:N ratio	**-0.534**	**-0.600**	**-0.475**	**-0.417**	0.046	**0.490**	**-0.295**	**-0.716**	**-0.994**		**0.827**
Foliar $\delta^{15}N$	**-0.672**	**-0.748**	**-0.688**	**-0.292**	-0.060	**0.239**	0.014	**-0.290**	**-0.655**	**0.712**	

Appendix S3: Reaction norms of total plant biomass for hybrid clover grown across two light treatments in symbiosis with 11 N-fixing rhizobium inocula. Raw, untransformed means are shown.