Toward a trait-based framework for studying mutualism evolution: genotypic variation in benefit and cost traits in the legume-rhizobia mutualism.

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Abstract

Mutualisms exhibit extensive context-dependent genetic variation in fitness outcomes. However, the specific traits that underpin fitness variation in mutualisms remain understudied. Pinpointing fitness-associated traits in mutualisms is critical to understanding how mutualisms evolve, as well as identifying the mechanisms that maintain genetic variation for fitness in interacting populations. In this study, we quantified the contribution of host and symbiont genotypes to variation in resource exchange, use, and production traits measured in the host using the model mutualism between legumes and nitrogen fixing rhizobia as our study system. We predicted that plant genotype x rhizobia genotype (GxG) effects would be common to resource exchange traits because resource exchange is hypothesized to be governed by input from both interacting partners through bargaining. On the other hand, we predicted that plant genotype effects would be common to host resource use and production traits because these traits are indirectly related to the exchange of resources. Consistent with our prediction for resource exchange traits, but not our prediction for resource use and production traits, we found that rhizobia genotype and GxG effects were the most common sources of variation in the traits that we measured. The results of this study complement the commonly observed phenomenon of GxG effects for fitness by showing that numerous mutualism traits also exhibit GxG variation. Furthermore, they highlight the possibility that the exchange of resources as well as how partners use and produce traded resources can influence the evolution of mutualistic interactions. Future studies can now quantify the relationship between resource exchange, use and production traits and fitness (i.e. selection) to test the competing hypotheses proposed to explain the maintenance of fitness variation in mutualisms.

Key words: mutualism evolution, genotype by genotype interaction, resource exchange, resource use, resource production, legume, rhizobia, *Medicago truncatula*, *Enisfer*

Introduction

Almost all multicellular life engages in some form of mutualism. Mutualisms are interspecific interactions in which benefits are reciprocally exchanged. The benefits gained from mutualistic interactions can have strong effects on the fitness of interacting partners, such as size and fecundity (Bronstein 1994, 2015). Contrary to the expectation that natural selection favoring the most beneficial partners would erode genetic variation for fitness in mutualism, there is abundant variation in many mutualisms in the wild (Heath and Stinchcombe 2014). This body of work has generated important insights into the potential evolutionary forces that drive mutualism evolution and maintain genetic variation in populations of interacting species (Thompson 2005; Heath and Stinchcombe 2014). The abundant variation in fitness outcomes in mutualisms raises an important question: what underlying traits give rise to this variation?

Fitness-related traits, as commonly measured in many quantitative genetic studies on mutualisms, have been useful for inferring net interaction outcomes and for identifying putative selective agents. However, the measurement of fitness-related traits also obscures the individual traits that underlie fitness variation. Pinpointing the traits underlying fitness variation in mutualisms is key to understanding how mutualisms evolve, as well as the mechanisms that maintain genetic variation for fitness in interacting populations. Trait-based approaches have been employed to great effect in plant-pollinator mutualisms to test evolutionary hypotheses about the forces acting on the underlying traits. For example, a combination of observation and quantitative genetic experiments in the orchid family has revealed that increased pollen transfer efficiency precipitated the emergence and maintenance of distinct pollination strategies, ecological adaptation, and reproductive isolation (Peakall and Beattie 1996; Scopece et al. 2010, 2013, 2015). These studies along with others on plant-pollinator mutualisms, highlight the power of applying trait-based approaches to studying evolution in mutualisms.

The net benefit of a mutualistic interaction, and by extension, fitness, is determined by the balance between the benefits and costs associated with the interaction. Therefore, genetic variation in net benefit could arise from variation in the benefits alone, the costs alone, or both. Mutualism benefits can take a variety of forms, including transportation (e.g. the movement of gametes in plant-pollinator mutualisms; Albrecht et al. 2012), protection (e.g. defense against herbivory in ant-acacia mutualisms; Janzen 1966), and nutrition (e.g. the exchange of limiting resources between plants and microbes; Friesen et al. 2011). Costs are related to the energetic investment involved in producing benefits that are transferred to the interacting partner (Bronstein 2001, 2015). For example, in plant-microbe mutualisms that involve an exchange of resources, a principal cost for the host is the carbon that is transferred to microbial partners (Akçay and Roughgarden 2007; Clark et al. 2019) .

Beyond the traits involved in the direct exchange of resources, traits related to the use or production of the traded resources can also influence the net fitness consequences of a mutualism (Vidal and Segraves 2021). Contrary to resource exchange traits, which capture the *total* benefit and cost, resource use and production traits influence the *relative* benefits and costs. Traits like resource use efficiency dictate how efficiently the benefits received from an interacting partner are incorporated into physiological processes (e.g., growth, reproduction, defense) that are linked to fitness. Resource production traits, on the other hand, are involved in the creation of the goods and services that are traded to an interacting partner. These traits dictate the total resource pool available to invest in mutualism, and thereby affect the

proportional cost of resource exchange. In plant-microbe interactions, a common resource production trait is photosynthetic rate, because it determines the size of a plant's carbon pool, a portion of which is traded to microbial partners (Grman et al. 2012; Clark et al. 2019).

An individual can achieve a large fitness benefit from mutualism via multiple trait combinations: by maximizing the benefit it receives, minimizing the cost it pays, maximizing its resource use efficiency, or modifying its rate of resource production (Vidal and Segraves 2021). Genetic variation in fitness outcomes in mutualisms could therefore emerge from genotypic differences in any trait involved in resource exchange, use, or production. An added layer of complication is that traits mediating species interactions are often jointly determined by both interacting partners (Moore et al. 1997; O'Brien et al. 2021b; De Lisle et al. 2022). Traits are jointly determined when variation in a trait is determined by contributions from the genomes of both interacting partners. Therefore, genetic variation in traits that mediate a host-microbe mutualism could arise from host genotype effects, microbe genotype effects, or from genotype-by-genotype (GxG) interactions (Heath 2010; Friesen et al. 2011).

Quantifying genotypic differences in mutualism traits represents an important synthesis between the subfields of mutualism physiology, functional ecology, and evolution. The traits involved in resource exchange, use, and production are well defined in many mutualisms, especially in nutritional mutualisms between hosts and symbionts (Udvardi and Poole 2013; Levy et al. 2021). For example, some legume species possess the ability to both "choose" the high quality microbial partners and "sanction" low quality microbial partners (Denison 2000; Kiers et al. 2003, 2011). Furthermore, there is an extensive body of theoretical work on the resource economics of mutualism (Akçay and Roughgarden 2007; Akçay and Simms 2011; Werner et al. 2014). Nevertheless, few empirical studies have investigated whether there is heritable variation in these traits in interacting populations.

In this study, we quantified the contributions of host and symbiont genotype to host resource exchange, use, and production traits in the model mutualism between legumes and nitrogen-fixing bacteria (rhizobia). The legume-rhizobia mutualism is centered on the exchange of carbon for nitrogen, whereby the legume host trades a portion of its carbon budget to rhizobia in exchange for fixed atmospheric nitrogen (Oldroyd et al. 2011; Udvardi and Poole 2013). This symbiosis is a powerful system to explore the traits underlying fitness variation. Quantitative genetic experiments have established that fitness outcomes in the legume-rhizobia mutualism are highly context-dependent, varying across plant and rhizobia genotypes as well as environmental factors like resource availability (Heath and Tiffin 2007; Heath 2010; Weese et al. 2015; Batstone et al. 2017; Burghardt et al. 2017). However, little is known about how resource exchange, use, and production traits vary in natural populations of legumes and rhizobia and potentially underlie fitness variation.

Here, we performed a factorial experiment in which we inoculated two plant genotypes with two rhizobia genotypes and measured the contributions of plant genotype, rhizobia genotype, and their interaction (GxG) to resource exchange, use, and production traits. Exchange traits are those directly related to the transfer of nitrogen (benefits) to host plant tissue and the transfer of carbon (costs) to rhizobia. Resource use and production traits are those involved in how host plants allocate the nitrogen received from rhizobia and how efficiently they fix (via photosynthesis) the carbon they transfer to rhizobia. Given that the outcomes of resource exchange are hypothesized to be governed by bargaining between both

interacting partners (Sachs et al. 2004; Akçay and Roughgarden 2007; Clark et al. 2019), we predicted that GxG effects would be common to exchange traits. On the other hand, we predicted that only plant genotype effects would be found for resource use and production traits because those traits represent components of plant physiology that should only be indirectly related to the exchange of resources among partners. We found extensive differences between plant and rhizobia genotypes, as well as GxG interactions, for all of the plant traits we measured. Our results suggest that multiple resource-related traits likely contribute to variation in mutualism fitness outcomes, and suggest that mutualism evolution could be driven by underlying change in resource exchange, use, and production traits.

Methods

Study system

The mutualism between *Medicago truncatula* and *Ensifer* spp. is a deeply-studied model system that has been used to uncover the molecular and genetic basis of symbiotic interactions and to explore the evolution of mutualisms (Udvardi and Poole 2013). *M. truncatula* is an annual plant native to the Mediterranean Basin(Urban 1873; Lesins and Lesins 1979). *M. truncatula* forms an endosymbiotic interaction with rhizobia species belonging to the genus *Ensifer* (Andrews and Andrews 2017). This interaction is localized to specialized structures on the host plant's roots called nodules. As with other legume-rhizobia interactions, *M. truncatula* trades photosynthetically-fixed carbon to rhizobia in nodules in exchange for fixed nitrogen. Numerous previous studies involving the *M. truncatula* - *Ensifer* mutualisms have observed genotype-dependent variation in fitness in response to different genotypes of the interacting partner (G x G) (Heath 2010). Past studies using *M. truncatula* have primarily used aboveground biomass as a proxy for fitness.

For this study, we used two inbred lines of *M. truncatula*, HM001 and HM028, sourced from the Medicago HapMap Project (Stanton-Geddes et al. 2013). These accessions are derived from collections made in the natural range of *M. truncatula* in the Mediterranean Basin. We chose these accessions because they, along with many other accessions, display genetic variation for fitness proxies like aboveground biomass (Stanton-Geddes et al. 2013; Wood et al. 2018). We also used two strains of *Ensifer meliloti*, MAG 154 and MAG 282, sourced from a panel of 191 strains isolated from *M. truncatula* sampled from the Mediterranean Basin (Katy Heath, U. Illinois). As with the inbred lines of *M. truncatula,* we chose these strains because they exhibited variable effects on plant fitness proxies like aboveground biomass (Batstone et al. 2022)**.**

Trait measurement

We measured a series of resource exchange, use, and production traits on host plants (Table 1). We also measured two sets of mutualism outcomes: plant fitness and nodulation (Table 1). Plant fitness was estimated by weighing the total dry aboveground biomass for an individual plant. We measured three nodule traits: total nodule number, total nodule mass, and average nodule size (i.e., total nodule number / total nodule mass). By harvesting plants and

Table 1: The mutualism outcome and resource exchange, use, and production traits measured in this study. Brief descriptions of how each trait was measured and calculated are also provided.

Mutualism component		**Trait**	**Measurement / Calculation**
Outcome	**Plant fitness**	Aboveground biomass	Dry weight of aboveground plant tissue
		Aboveground biomass growth rate	Dry weight of aboveground plant tissue measured at two timepoints (see methods)
	Nodulation	Total nodule mass	Dry weight of all nodules from an individual plant's root system
		Nodule number	Count of all nodules on individual plant's root system
		Average nodule mass	Total nodule mass / nodule number
Exchange traits	**Benefit**	Nitrogen composition	Proportion of aboveground plant tissue that is composed of nitrogen (measured via elemental analysis)
		Total nitrogen benefit	Nitrogen composition of aboveground tissue * aboveground biomass
		Total nitrogen benefit per symbiont	Slope of the relationship between total nitrogen benefit and total nodule mass (see "statistical analysis" in methods)
	Cost	Total carbon cost	Carbon composition of nodule tissue * total nodule mass
		Nodule carbon fraction	Total carbon cost / (total carbon cost + (aboveground tissue carbon composition * aboveground biomass)
	Resource exchange ratio	Carbon-for-nitrogen exchange ratio	Total carbon cost / total nitrogen benefit
Resource Use	**Nitrogen use efficiency**	Carbon:nitrogen ratio in aboveground tissue	Nitrogen composition in aboveground plant tissue / carbon composition in aboveground plant tissue
Resource Production		Leaf photosynthetic rate	See Methods and Supplementary Methods

measuring aboveground biomass at two different timepoints, we were also able to infer genotype effects on growth rate. *M. truncatula* populations are thought to be subject to viability selection in their dry, Mediterranean habitat, thus growth rate could be an important fitness component (Urban 1873; Lesins and Lesins 1979).

Unless otherwise noted, all resource exchange, use, and production traits were measured using carbon and nitrogen elemental composition data collected via combustion analysis. To perform combustion analysis, tissue was pulverized using either a mechanical ball grinder or a mortar and pestle. Then, approximately 4 mg of pulverized tissue was weighed using an ultra-micro balance (CP2P Microbalance, Sartorius GmbH, Goettingen, Germany), packaged into tin capsules, and ran on a Costech ECS4010 Elemental Analyzer (Costech Analytical Technologies, Inc. Valencia, CA, USA). The elemental analyzer returns measurements of carbon and nitrogen composition (%) for the tissue sample analyzed. We multiplied carbon and nitrogen composition by the total dry mass that the elemental analysis sample came from to infer the amount of total biomass carbon and nitrogen in a given sample (Table 1). We performed combustion analysis separately on one aboveground plant tissue sample and one nodule tissue from between 6 and 10 individual plants from each genotype combination.

We measured three benefit-associated exchange traits: nitrogen composition (%) in aboveground tissue (hereafter referred to as nitrogen composition), total nitrogen in aboveground tissue (hereafter referred to as total nitrogen benefit), and total nitrogen in aboveground tissue as a function of total nodule mass (hereafter referred to as total nitrogen benefit per symbiont; see statistical analysis section for more details; Table 1). We measured two cost-associated exchange traits: total carbon in nodule tissue (hereafter referred to as total carbon cost), and the fraction of total carbon in aboveground tissue and nodule tissue that is in nodule tissue (hereafter referred to as nodule carbon fraction). These cost traits incorporate both the carbon cost of constructing nodules as well as the carbon traded to the symbiont. This approach is unable to quantify the portion of carbon transferred to rhizobia that was respired by rhizobia, thus our analysis is making the simplifying assumption that respiration rates are consistent across genotype combinations. We also calculated the ratio of total nitrogen in aboveground tissue to total carbon in nodule tissue as a proxy of the carbon-for-nitrogen exchange ratio.

We measured one resource use trait and one resource production trait. The resource use trait was measured as the ratio of total carbon to total nitrogen in aboveground tissue as a proxy for nitrogen use efficiency (Dovrat et al. 2020). The benefit production trait we measured was leaf photosynthesis rate. Photosynthesis rate measurements were made using a LI-6400 Portable Photosynthesis System (LI-COR, Inc., Lincoln, NE, USA) with an 6400-02B LED Light Source (LI-COR, Inc., Lincoln, NE, USA) attached to the leaf chamber to apply a consistent level of light for all measurements. See the supplementary methods for details on how photosynthesis rate was measured and calculated (Supplementary methods). Table 1 provides short summaries of how each resource exchange, use, and production trait was quantified.

Experimental design

To quantify genotype-dependent variation (i.e., an effect of plant genotype, rhizobia genotype, and GxG interactions) in the traits we measured we grew our two *M. truncatula* accessions in fully factorial design with our two *E. meliloti* strains for a total of four plant-rhizobia genotype combinations. Each of the four genotype combinations was replicated 33 times totalling 132 experimental units.

Seeds from our two plant genotypes were scarified, sterilized, imbibed, and stratified using the methods reported in the “Seed storage and germination” chapter of the Medicago Handbook (Noble Institute; Supplementary methods). Prior to planting, we filled 66 mL Cone-tainer pots (Stuewe & Sons, Inc., Tangent, OR, USA) with a growing media consisting of a 1:4 mix of perlite and sand. We sterilized the filled Cone-tainer by autoclaving them twice. One seedling was placed in each Cone-tainer. One day after planting, seedlings were given 1 mL of 0.625 mM nitrogen Fahraeus fertilizer (Batstone et al. 2017). Afterwards, plants were given 0.0 mM N Fahraeus fertilizer every 2 to 3 days to ensure that rhizobia were the only source of nitrogen (Batstone et al. 2017).

Cone-tainer pots were placed in 98-well racks. To avoid the risk of cross contamination of rhizobia strains, every plant in a rack received the same rhizobia strain. Each rack was enclosed in a semi-permeable tent consisting of plastic wrap and PVC pipe to further reduce the risk of cross contamination. Plants were grown in two growth chambers in a complete block design. The height of light fixtures in both chambers was adjusted so that a photosynthetic active radiation (PAR) reading of ~250 $\mu mol/m^{-2}/s^{-1}$ was consistently measured on the floor of the growth chambers. Chambers were set to 22 °C and a 16:8 light:dark photoperiod. Seedings were maintained in a high humidity environment for about one week, after which they were exposed to ambient humidity, usually 40-50%.

Two weeks after planting, we inoculated plants with rhizobia. Plants received either strain MAG 154 or MAG 282. We grew strains from glycerol stocks in sterile tryptone yeast liquid media in a shaking incubator at 30 °C and 180 rpm. We let the rhizobia cultures grow for approximately two days. We then streaked the media on sterile Petri plates containing tryptone yeast agar under a laminar flow hood. We incubated the plates at 30 °C until colonies started to form. Next, we selected an individual colony from each strain to grow in tryptone yeast liquid media. Again, rhizobia cultures were allowed to grow for approximately two days. We diluted the liquid media to an OD_{600} of 0.1 and inoculated each plant with 1 ml of the diluted culture.

Plants were harvested in three separate cohorts at 9, 10, and 11 weeks post planting. Approximately 20 plants from each treatment group were harvested at 11 weeks. Of those plants harvested after 11 weeks, approximately 10 from each treatment were used to measure the benefit and cost associated traits described above via elemental analysis. Approximately 3 plants from each treatment group were harvested at 10 weeks to measure photosynthesis. Prior to harvest at the 10 week timepoint, photosynthesis rate measurements were taken on 2-3 leaves of each plant. Approximately 10 plants from each treatment group were harvested at 9 weeks. Plants in the 9-week cohort were compared to those in the 11-week cohort to estimate growth rate (see statistical analysis section for more details).

At each harvest, we gently removed plants from their Cone-tainer pots and cut the shoots from the roots. The shoots were placed in brown paper bags and dried at 60 °C for at least a week before being weighed with an analytical scale to measure aboveground biomass. Plants used for benefit and cost trait measurement via elemental analysis were processed as

described in the “Trait measurement” section. The roots were washed before being placed in plastic bags and stored in a freezer at -20 °C.

To collect nodulation data, frozen roots were thawed and nodules were removed under a dissecting microscope. Nodules were counted as they were removed to quantify nodule number. To assess the consistency of nodule count estimates across researchers, we had each research count nodules on the same subset of 10 plants. Nodule counts were highly repeatable across researchers in a linear mixed effects model with nodule number as the response, and plant and researcher as random effects (researcher explained only 0.4% of variation in nodule counts). Removed nodules were placed in coin envelopes, dried at 60 °C for at least a week, and weighed. Nodules from plants that were used for benefit and cost trait measurement via elemental analysis were processed as described in the “Trait measurement” section.

Statistical analysis

We used linear models to test for an effect of plant genotype, rhizobia genotype, and plant genotype by rhizobia genotype (GxG) interactions on the traits we measured. All models used a normal error distribution except for the model with nodule number as the response variable, which used a negative binomial error distribution. Block was included as a fixed effect in all models. Continuous predictors were mean-centered to avoid spurious inference on main effects in the presence of interactions (Schielzeth 2010). For some models, response variables were log-transformed to better conform with the assumption of normality. If a response variable was log transformed, it will be indicated in the figure caption of the associated figure. Models with nodule traits as the response included total root mass as a covariate to account for differences in nodule traits associated with differences in root system size.

To test for genotype effects on growth rate in aboveground biomass, we included the following interaction terms in the model, plant genotype x timepoint, rhizobia genotype x timepoint, and plant genotype x rhizobia genotype x timepoint.

For analysis of elemental composition measurements (e.g. nitrogen composition), arcsine square root transformations were performed to normalize the data prior to running the models.

To test for significant genotype effects on total nitrogen benefit per symbiont, we included the interaction terms plant genotype x total nodule mass, rhizobia genotype x total nodule mass, and plant genotype x rhizobia genotype x total nodule mass terms in our models. Each of these interaction terms estimates differences in the slope of the relationship between total nitrogen in aboveground tissue and nodule mass that are due to genotype effects.

Our model with photosynthesis rate as the response included each individual leaf measurement as an independent replicate. To account for any variation due to measurements made on leaves from the same plant, we included plant ID as a random effect.

The significance of model terms in each model was tested using type III sum of squares. For linear models, significant testing was performed using *F*-statistics, and for generalized linear models, significance testing was performed with Wald χ^2 tests.

All statistics and data visualization were performed in R v4.2.3 (R Core Team 2023). For simple linear models, we used the base function lm(); for linear mixed effects models we used the lmer() function in the lme4 package (Bates et al. 2014), and for generalized linear mixed

effects models we used the glmmtmb() function in the glmmTMB package (Brooks et al. 2017). Model assumptions were evaluated using the DHARMa package (Hartig and Lohse 2022). Significant testing was performed with the Anova() function in the car package (Fox and Weisberg 2018). All plots include estimated marginal means that were calculated using the ggeffects package (Lüdecke 2018). The ggplot2 (Wickham 2016), ghibli (Henderson et al. 2022), and ggpubr (Kassambara 2023) packages were used to make all of the plots presented here. All data and code used in this study can be found here [Dryad repository will be added if/when manuscript is accepted for publication]

Results

Genetic variation in fitness and mutualism outcomes

We found a significant plant genotype x rhizobia genotype (GxG) interaction effect on aboveground biomass, our proxy for plant fitness, but no main effects of either plant or rhizobia genotype (Fig. 1, Table 2). We also found significant plant genotype, rhizobia genotype, and GxG effects on aboveground biomass growth rate (Fig. 1, Table 2). During the analysis of growth rate, we identified an outlier in the HM028 - MAG 282 - Timepoint 1 treatment group. Removal of this outlier does not change the statistical significance of any model terms.

We observed a significant rhizobia genotype effect on total nodule mass, and significant plant genotype effects and rhizobia genotype effects on total nodule number and average nodule size (Fig. 1, Table 2).

Genetic variation in exchange traits

We measured three benefit-associated exchange traits, nitrogen composition, total nitrogen benefit, and total nitrogen benefit per symbiont (Table 1). We observed a significant rhizobia genotype effect and GxG effect for nitrogen composition in aboveground tissue (Fig. 2, Table 3), but no significant genotype effects were observed for total nitrogen biomass in aboveground tissue (Fig. 2, Table 3). For total nitrogen per symbiont, we observed significant plant genotype x total nodule mass, rhizobia genotype x total nodule mass, and G x G x total nodule mass mass effects, indicating that our measure of benefit per symbiont varies in response to plant genotype, rhizobia genotype, and the interaction of plant genotype and rhizobia genotype (Fig. 2, Table 3).

We measured two cost-associated exchange traits, total carbon cost, and the nodule carbon fraction (Table 1). We observed a significant rhizobia genotype and GxG effect for total carbon cost, but no significant genotype effects for nodule carbon fraction (Fig. 3, Table 3).

We also quantified the nitrogen-for-carbon exchange ratio by dividing total nitrogen benefit by the total carbon cost (Table 1). We observed a significant rhizobia genotype effect for the nitrogen-for-carbon exchange (Fig. 4, table 3).

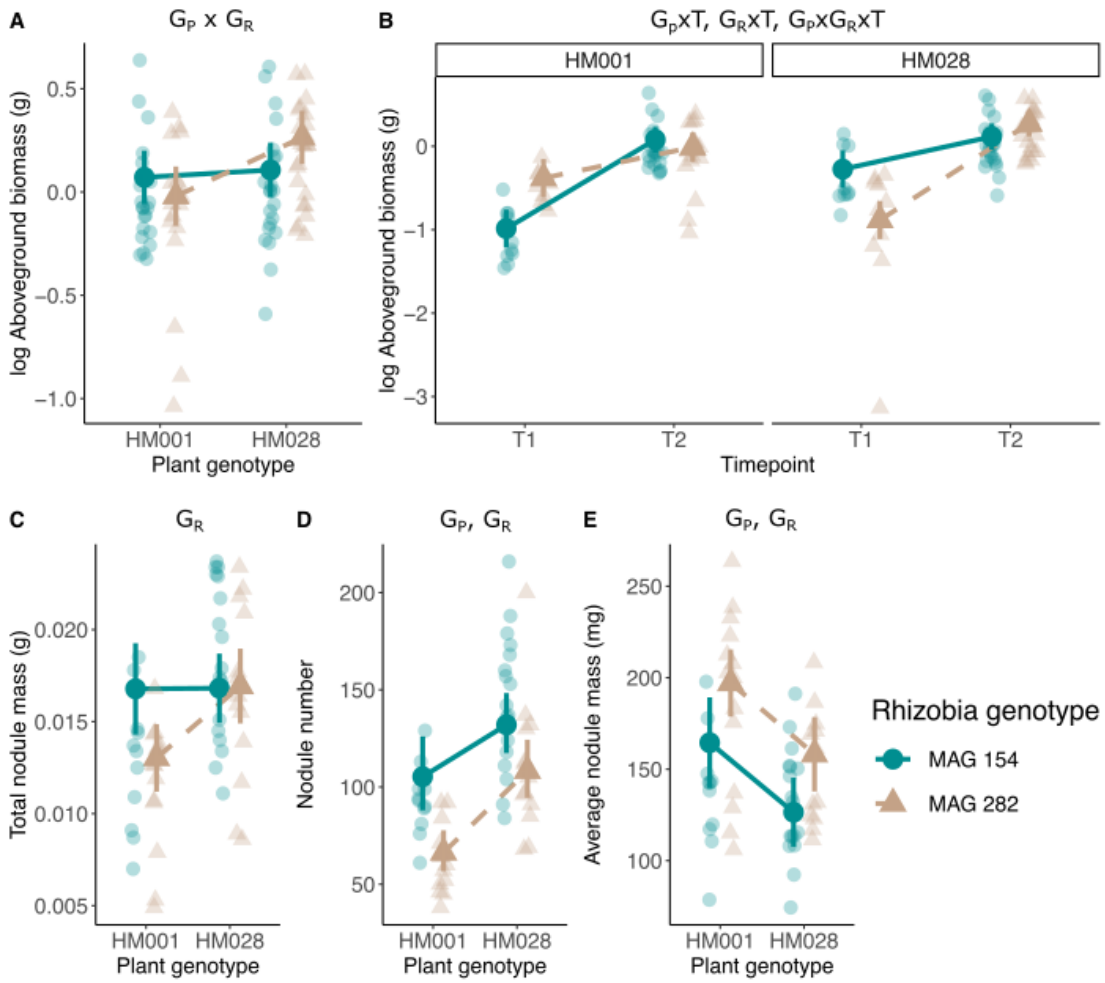

Figure 1: Genetic variation for (A) log transformed aboveground biomass, our proxy for plant fitness, (B) log transformed aboveground biomass growth rate, (C) total nodule mass, (D) nodule number, and (E) average nodule size. In all panels, transparent points are the raw data and opaque points are the estimated marginal means. Error bars are the 95% confidence interval. Significant genotype effects are listed on the figure: G_P = plant genotype effect, G_R = rhizobia genotype effect, G_PxG_R = plant genotype x rhizobia genotype interaction, G_P x T = plant genotype x timepoint interaction, G_RxT = rhizobia genotype x timepoint interaction, and G_PxG_RxT = plant genotype x rhizobia genotype x timepoint interaction. Model term statistical significance did not change when the outlier in the HM028-MAG282-Timepoint 1 treatment group in panel B was removed.

Genetic variation in resource use and production traits

We measured variation in two traits associated with resource use and production, the carbon:nitrogen ratio in aboveground tissue (a proxy for nitrogen use efficiency) and leaf photosynthetic rate (Table 1). The carbon:nitrogen ratio in aboveground tissue was used to infer how efficiently host plants are able to use nitrogen received through trade to incorporate carbon in aboveground tissue (Dovrat et al. 2020). Leaf photosynthetic rate to infer the efficiency of the production of carbon, some of which is ultimately traded away. We found a significant effect of

Table 2: Genotype effects on mutualism outcome traits. Significant model terms are highlighted in gray.

	Outcomes									
	Plant Fitness				Nodulation					
	Above-ground biomass		Above-ground biomass growth rate		Total nodule mass		Nodule number		Average nodule mass	
	$F_{1,81}$	P	$F_{1,117}$	P	$F_{1,50}$	P	$\chi^2_{df=1}$	P	$F_{1,50}$	P
Rhizobia genotype (G_R)	1.09	0.3	**15.0**	**0.0002**	**7.17**	**0.004**	**16.74**	**<0.0001**	**5.41**	**0.024**
Plant genotype (G_P)	0.18	0.7	**20.76**	**<0.0001**	0.0004	0.98	**3.97**	**0.046**	**5.62**	**0.02**
G_R x G_P	**4.25**	**0.04**	**30.21**	**<0.0001**	3.96	0.05	3.25	0.071	0.002	0.96
Timepoint (T)	-	-	**64.9**	**<0.0001**	-	-	-	-	-	-
T x G_R	-	-	**13.28**	**0.0005**	-	-	-	-	-	-
T x G_P	-	-	**12.96**	**0.0004**	-	-	-	-	-	-
T x G_R x G_P	-	-	**29.81**	**0.0001**	-	-	-	-	-	-

rhizobia genotype and the interaction between rhizobia genotype and plant genotype on **Table 1:** Genotype effects on mutualism outcome traits. Significant model terms are highlighted in gray.

carbon:nitrogen ratio (Fig. 5, Table 3). We observed a significant plant genotype x rhizobia genotype interaction on photosynthesis rate (Fig. 5, Table 3).

Discussion

In this study, we found that variation in mutualism resource exchange, use, and production traits are genotype-dependent. Consistent with our expectations, we observed rhizobia genotype and GxG effects on both benefit-associated and cost-associated exchange traits. However, we only observed differences between rhizobia genotypes for the nitrogen-for-carbon exchange ratio. Contrary to our expectation, rhizobia genotype affected plant resource use and production traits as much or more than plant genotype. Our results suggest that rhizobia can contribute to the evolution of resource use and production traits in their hosts. Furthermore, our work indicates that the evolution of mutualisms may not be limited to resource exchange traits. Rather, how efficiently mutualistic partners can integrate the benefits of trade (i.e. resource use efficiency) and/or how efficiently they can produce the resource traded away may be just as important for determining the evolution of mutualisms. The ubiquity of concurrent

Table 3: Genotype effects on resource exchange, use, and production traits. Significant model terms are highlighted in gray.

	Exchange traits												Resource use		Resource production	
	Benefits						Costs				Benefit: cost					
	Nitrogen composition		Total nitrogen benefit		Total nitrogen benefit per symbiont		Total carbon cost		Nodule carbon fraction		Nitrogen-for-carbon exchange ratio		Nitrogen use efficiency		Leaf photosynthesis rate	
	$F_{1,36}$	P	$F_{1,36}$	P	$F_{1,29}$	P	$F_{1,26}$	P	$F_{1,35}$	P	$F_{1,30}$	P	$F_{1,36}$	P	$\square^2_{df=1}$	P
Rhizobia genotype (G_R)	**11.48**	**0.002**	0.29	0.6	**7.54**	**0.01**	**5.04**	**0.03**	1.53	0.223	**5.6**	**0.02**	**6.72**	**0.01**	1.73	0.19
Plant genotype (G_P)	1.12	0.3	0.03	0.85	0.35	0.56	0.006	0.94	1.83	0.19	0.005	0.95	1.46	0.23	0.74	0.39
G_R x G_P	**6.3**	**0.02**	0.31	0.58	2.58	0.12	**4.19**	**0.049**	0.0003	0.99	1.88	0.18	**5.78**	**0.02**	**4.62**	**0.03**
Centered total nodule mass (tnm)	-	-	-	-	1.64	0.21	-	-	-	-	-	-	-	-	-	-
Tnm x G_R	-	-	-	-	**9.68**	**0.004**	-	-	-	-	-	-	-	-	-	-
Tnm x G_P	-	-	-	-	**4.63**	**0.04**	-	-	-	-	-	-	-	-	-	-
Tnm x G_P x G_R	-	-	-	-	**8.69**	**0.006**	-	-	-	-	-	-	-	-	-	-

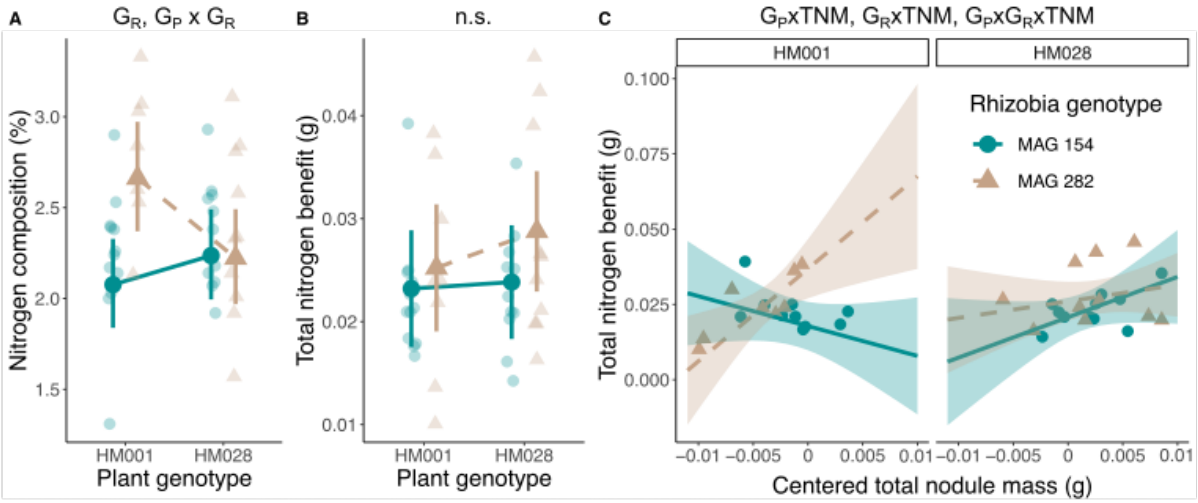

Figure 2: Genetic variation for three benefit-associated exchange traits: (A) nitrogen composition (%), (B) total nitrogen benefit (g), and (C) total nitrogen benefit per symbiont. In panels A and B, transparent points are the raw data, opaque points are the estimated marginal means, and Error bars are 95% confidence intervals. For panel C, transparent points are the raw data, lines represent the estimated marginal slopes derived from the model, and ribbons are the 95% confidence interval for the slope. Significant genotype effects are listed on the figure: G_P = planet genotype effect, G_R = rhizobia genotype effect, G_PxG_R = plant genotype x rhizobia genotype interaction, G_PxTNM, G_RxTNM, G_PxG_RxTNM .

plant genotype and rhizobia genotype effects as well as GxG effects that we observed on resource exchange, use, and production traits complements previous research that has found similar effects on plant fitness components and nodulation traits. Together, these results further emphasize an important role for jointly determined traits in mediating mutualism evolution.

Genotype effects on resource exchange traits

We predicted that GxG effects, which occur when trait variation across genotypes in one interacting partner is dependent on the genotype of the other partner, would be common to exchange traits, because the exchange of resources involves cooperation between the plant and rhizobia (Sachs et al. 2004). Consistent with this prediction, we observed GxG effects for two benefit-associated traits: nitrogen composition and total nitrogen benefit per symbiont. For cost-associated traits we found GxG effects for total carbon cost. However, we did not observe a GxG effect for the carbon-for-nitrogen exchange ratio. Similarly, among our cost-associated traits, we observed a GxG effect for total carbon cost. Total carbon cost is a metric of cumulative cost because it is a representation of the gross amount of carbon that hosts provide to their rhizobia (and to nodule formation).

Our prediction that GxG effects should be common to exchange traits is based on the hypothesis that the outcomes of trade in resource mutualisms are governed by bargaining between partners (Sachs et al. 2004; Akçay and Roughgarden 2007). There are numerous proposed mechanisms by which interacting partners may strengthen their bargaining positions. For example, legumes are able to “sanction” poor quality partners resulting in beneficial trading outcomes (Kiers et al. 2003; Westhoek et al. 2021). Thus, the GxG effects on exchange traits

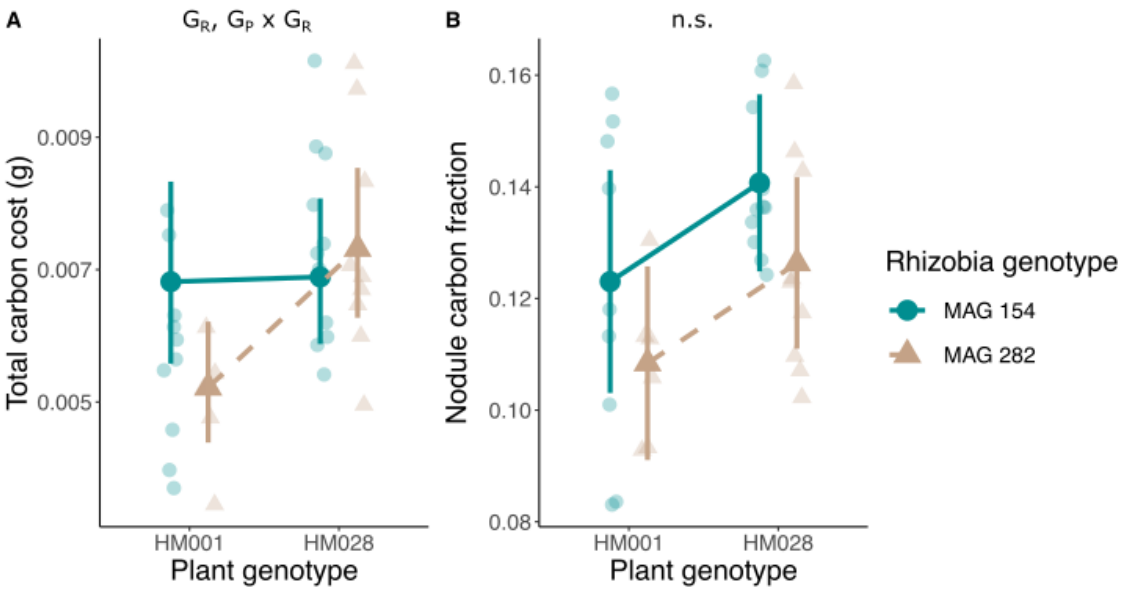

Figure 3: Genetic variation for two cost-associated exchange traits: (A) total carbon cost and (B) the nodule carbon fraction. Transparent points are the raw data and opaque points are the estimated marginal means. Error bars are the 95% confidence interval. Significant genotype effects are listed on the figure: G_P = plant genotype effect, G_R = rhizobia genotype effect, and G_P X G_R = plant genotype X rhizobia genotype interaction.

that we observed here could be mediated by the numerous bargaining strategies that hosts and microbes employ. Alternatively, GxG effects on exchange traits may also emerge via a mismatch between partners (Barrett et al. 2012; Wendlandt et al. 2019). Partner mismatches occur when some rhizobia are more effective partners for some plant genotypes compared to others. Partner mismatches can emerge from a variety of evolutionary mechanisms such as rhizobia adaptation to different hosts (Batstone et al. 2020), coevolution between spatially separated populations of legumes and rhizobia (Gomulkiewicz et al. 2003), and/or isolation by distance.

To our surprise, however, the carbon-for-nitrogen exchange rate was only affected by rhizobia genotype, not by both partners. This result suggests that rhizobia may exert more control over the overall outcome of trade than their host. Rhizobia control conflicts with theoretical and empirical work that suggests that, in general, host plants exert more power in determining the outcomes of trade (Daubech et al. 2017; Clark et al. 2019). Of course, a primary reason that host plants are expected to exert more control over the trade is their ability to “bargain” with multiple different microbial strains simultaneously (Akçay and Simms 2011; Westhoek et al. 2021); a scenario that was unable to occur in this study due to our single strain inoculation design.

Finally, there were a few exchange traits for which we observed no genotype effects. Among benefit-associated traits, total nitrogen benefit, our metric of absolute benefit, did not differ between plant or rhizobia genotypes. The same was true for nodule carbon fraction, our metric of relative cost. The fact that we observed genotype differences in the relative but not absolute benefit—and the absolute but not relative cost—suggests that absolute and relative costs (and benefits) of mutualism may exhibit different evolutionary dynamics.

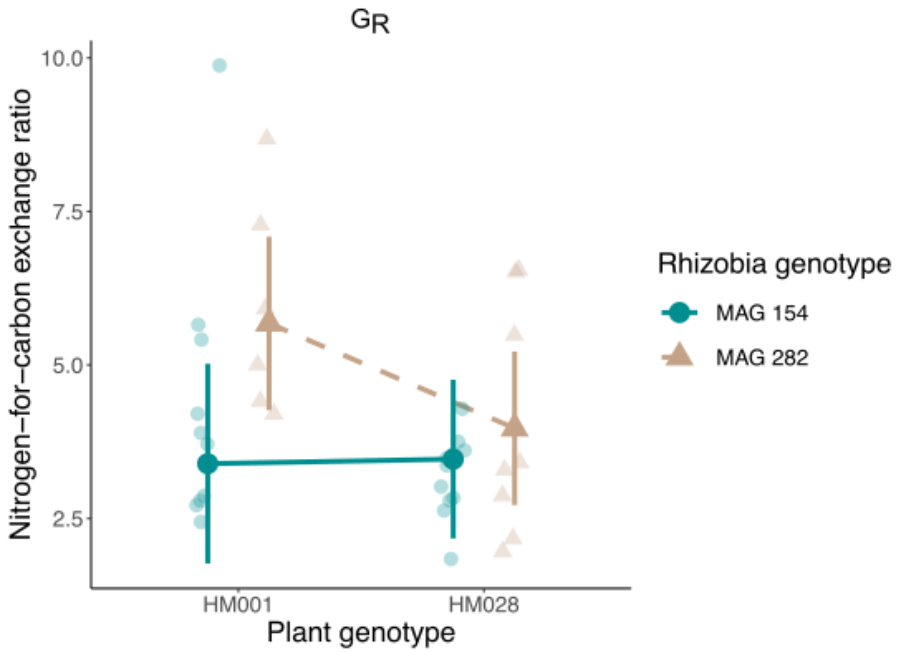

Figure 4: **Genetic variation for the nitrogen-for-carbon exchange ratio. Transparent points are the raw data and** opaque points are the estimated marginal means. Error bars are the 95% confidence interval. Significant genotype effects are listed on the figure: G_P = planet genotype effect, G_R = rhizobia genotype effect, and G_P X G_R = plant genotype X rhizobia genotype interaction.

Genotype effects on resource use and production traits

We predicted that plant genotype would be the major contributor to differences in plant resource use and production traits. Contrary to this prediction, we observed GxG effects for both of the resource use and production traits that we measured: nitrogen use efficiency (our metric of resource use) and leaf photosynthetic rate (our metric of resource production).

We predicted that host resource use and production traits would respond primarily to plant genotype because these traits are indirectly related to resource exchange and represent components of plant physiology. However, rhizobia effects on these host traits are perhaps not surprising if hosts change how they allocate benefits or invest in the production of resources in response to trade. Thus, rhizobia and GxG effects on resource use and production traits may arise from the GxG effects for exchange traits that we observed.

Resource use traits, like nitrogen use efficiency, have previously been shown to be microbially mediated (Dovrat et al. 2020; Zhang et al. 2020). For instance, legumes modify their nitrogen use efficiency in response to both soil nitrogen availability and the presence of rhizobia (Dovrat et al. 2020). Our study raises the important question of why nitrogen use efficiency could vary across combinations of plant and rhizobia genotypes. A GxG effect on nitrogen use efficiency could be due to differences in the efficiency of symbiotic nitrogen fixation across plant

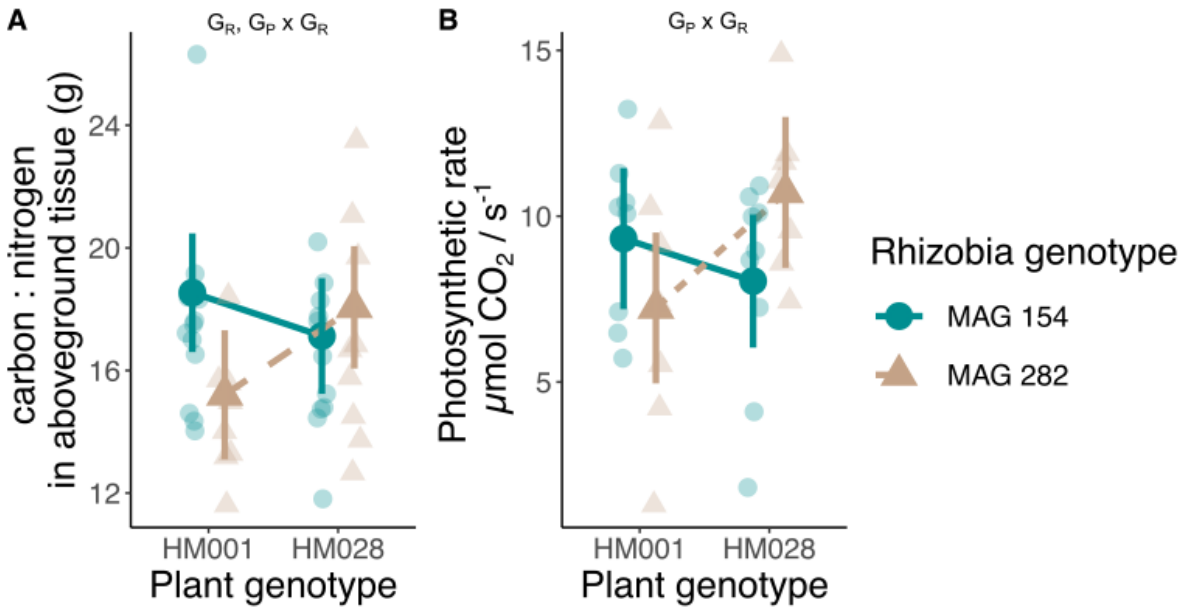

Figure 5: Genetic variation for (A) carbon : nitrogen ratio in aboveground tissue and (B) leaf photosynthetic rate. Transparent points are the raw data and opaque points are the estimated marginal means. Error bars are the 95% confidence interval. Significant genotype effects are listed on the figure: G_P = planet genotype effect, G_R = rhizobia genotype effect, and G_P X G_R = plant genotype X rhizobia genotype interaction.

and rhizobia combinations. Legume species vary in their strategies of regulation of nitrogen fixation. Some, like *M. truncatula,* are obligate fixers and will attempt to maintain a constant nitrogen fixation effort regardless of the activity of their rhizobia partners or external supplies of nitrogen, while others are facultative fixers and can down- or upregulate nitrogen fixation in response to environmental conditions (Hedin et al. 2009). Consequently, variation in nitrogen use efficiency in obligate fixers like *M. truncatula* could simply be a function of the variation in the efficiency of nitrogen fixation across plant and rhizobia genotype combinations. Alternatively, it is also plausible that legumes may have evolved strategies to adjust how they use and allocate the benefits from trade in order to buffer against the consequences of interacting with poor quality partners.

Resource production traits, like leaf photosynthetic rate, also have been shown to respond to microbes (Adams et al. 2016). Legumes in particular exhibit significantly higher photosynthetic rates in the presence versus the absence of rhizobia (Clark et al. 2019; Dovrat et al. 2020). Photosynthesis is fundamental to the exchange of resources between legumes and rhizobia because it provides the carbon necessary to support rhizobia and it also requires a large amount of nitrogen, which is provided by the rhizobia. A heightened photosynthetic rate may equate to a larger pool of carbon that can be devoted to the mutualism, and by extension, a large supply of nitrogen that can be returned to the host plant. Variation in photosynthetic rate may also simply be an indication of nitrogen limitation and the outcomes of trade. Photosynthesis involves nitrogen-rich machinery and therefore, genotype combinations with the

highest photosynthetic rates may simply be the genotype combinations in which the largest amount of nitrogen is being transferred to the host plant. Regardless of the mechanisms responsible for GxG effects on photosynthetic rate, the fact that a portion of the genetic variation for leaf photosynthetic rate resides in rhizobia emphasizes the far-reaching effect that this mutualism has on host plant physiology.

The findings of this study underscore the possibility that the evolution of mutualisms may involve traits related to how interacting partners can integrate the benefits of trade (i.e. resource use efficiency) and how efficiently they can produce the resources traded away. This suggestion is corroborated by a recent study involving a synthetic mutualism between engineered strains of brewer's yeast (Vidal and Segraves 2021). The authors observed strong fluctuations in both resource use efficiency and resource production of both strains as they evolved in response to the availability of the limiting resources produced by their partners. Resource use and production traits may represent key components of mutualisms that allow interacting partners the ability to adjust their physiology in response to the outcomes of trade. Future studies should carefully consider selection on all mutualism traits, not just those related to the exchange of resources, as well as covariation among resource exchange, use and production traits.

The importance of jointly determined traits to predicting evolution in mutualism

The ubiquity of rhizobia and GxG effects on mutualism traits has three important implications for the evolution of mutualisms. First, this finding indicates that traits involved in resource exchange, use, and production may be jointly determined by the genomes of both interacting partners, just as is the case with fitness outcomes (Heath 2010). Jointly determined traits, here defined as traits that exhibit variation in response to more than one genome as either two independent main effects or a GxG effect, can have different evolutionary trajectories than those determined by the genome of just one or the other interacting partner (O'Brien et al. 2021b). The evolution of jointly determined traits is a function of the optimal trait values for the different interacting species, the strengths of selection, and fitness feedbacks (O'Brien et al. 2021b). Thus, a trait-based framework, wherein quantitative genetic approaches can be leveraged to identify the sources of variation in mutualism traits, is necessary to develop a better understanding of mutualism evolution.

Second, these results also show that microbial mutualists play an important role in determining the expression of host traits like resource use and production. Microbes are known to mediate host nutrient acquisition (Hestrin et al. 2019; Harbort et al. 2020), life history transitions (Wagner et al. 2014; O'Brien et al. 2021a), defense against antagonists (Johnson et al. 1987; Berg and Koskella 2018), stress tolerance (Lau and Lennon 2012), and morphology (Ortíz-Castro et al. 2008; Batstone et al. 2017). The great majority of the research on microbial mediation of plant traits involves observation and manipulative studies of either individual species of microbes or whole microbiomes. Such a framework allows for questions about how variation in community properties (e.g. relative abundance and composition of specific microbial species) affects plant functional traits. Our work builds on this knowledge by demonstrating that intraspecific variation, in addition to interspecific variation, can also influence the expression of plant functional traits. This finding complements two recent studies, O'Brien et al. (2021b) and De Lisle et al. (2022), which have highlighted the potential evolutionary significance of the effect

of intraspecific partner variation in the expression of mutualism traits. An important take away from those studies and ours is that evolution of rhizobia populations can drive changes in host plant traits. Furthermore, intraspecific microbial variation is invisible to the 16S sequencing that is commonly applied to characterize community composition in microbiome studies because strains belonging to the same species will have identical 16S sequences. Thus, substantial cryptic variation in microbe mediated plant traits measured in microbiome manipulations may go unnoticed.

Third, variation in resource trade, use, and production traits across different combinations of plant and rhizobia genotypes highlights the potential importance of the non-random association of partner genotypes for mutualism evolution. If a mutualism trait responds to a GxG effect and is a target of selection, then the plant genotypes favored by selection will be dependent on which rhizobia genotype they are paired with. If associations between specific plant and rhizobia genotypes are random, then the response to selection will be smaller than expected because most genetic variation is non-additive and a function of which genotypes are paired together. On the other hand, if associations between specific plant and rhizobia genotypes are non-random, then the response to selection could be either larger (if associations between plant and rhizobia genotypes inflate genetic variation for the trait) or smaller (if associations between plant and rhizobia genotypes constrain genetic variation for the trait) than expected. While host plants have a clear influence on their rhizosphere microbes (Miranda-Sánchez et al. 2016; Greenlon et al. 2019) and can vary in their ability to select beneficial microbial genotypes (Burghardt et al. 2018, 2019), the frequency of non-random associations between specific plant and rhizobia genotypes in nature has been rarely measured. Given the apparent importance of rhizobia genotypes in influencing variation in mutualism traits, more studies are needed to address the degrees of non-random associations between interacting genotypes in order to develop a predictive framework for understanding mutualism evolution.

Conclusions and future directions

Our study has identified a number of mutualism traits that could underlie variation in fitness outcomes in mutualisms and are jointly determined by the genotypes of both interacting partners. More studies are now needed to quantify (1) the relationship between mutualism traits measured here and the fitness of both interacting partners, (2) the variation and covariation among these traits, and (3) the non-random association among the genotypes of interacting species. Each of these objectives offers different, but important, extensions of the work presented here to advance our understanding of mutualism evolution. Measuring the relationship between mutualism traits and fitness (i.e. selection) is necessary to both identify the traits that could underlie fitness variation and to identify the evolutionary forces that maintain fitness variation. Quantifying variation and covariation among mutualism traits is necessary to evaluate the physiological interdependence of mutualism traits. For example, if traits like resource use help host plants buffer the outcomes of trade, then we might expect to see high covariation among exchange and resource use traits. Finally, the broad prevalence of rhizobia and GxG effects highlights the potential importance of non-random associations among genotypes in mutualism evolution.

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