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Dysregulation of host-control causes interspecific conflict over host investment into symbiotic organs

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Microbial mutualists provide substantial benefits to hosts that feed back to enhance the fitness of the associated microbes. In many systems, beneficial microbes colonize symbiotic organs, specialized host structures that house symbionts and mediate resources exchanged between parties. Mutualisms are characterized by net benefits exchanged among members of different species, however, inequalities in the magnitude of these exchanges could result in evolutionary conflict, destabilizing the mutualism. We investigated joint fitness effects of root nodule formation, the symbiotic organ of legumes that house nitrogen-fixing rhizobia *in planta*. We quantified host and symbiont fitness parameters dependent on the number of nodules formed using near-isogenic *Lotus japonicus* and *Mesorhizobium loti* mutants, respectively. Empirically estimated fitness functions suggest that legume and rhizobia fitness is aligned as the number of nodules formed increases from zero until the host optimum is reached, a point where aligned fitness interests shift to diverging fitness interests between host and symbiont. However, fitness conflict was only inferred when analyzing wild-type hosts along with their mutants dysregulated for control over nodule formation. These data demonstrate that to avoid conflict, hosts must tightly regulate investment into symbiotic organs maximizing their benefit to cost ratio of associating with microbes.

KEY WORDS: **Conflict, legume-rhizobium, Lotus-Mesorhizobium, microbial symbiosis, mutualism, symbiotic organ.**

Microbial mutualists can dramatically improve the fitness of plant and animal hosts. Associations with microbes can accelerate host growth (Sprent et al. 1987), enhance immune defense (Gerardo and Parker 2014; Pieterse et al. 2014), increase stress tolerance (Schützendübel and Polle 2002; Rubin et al. 2017), and temper fitness costs during interactions with predators, pathogens, and competitors (Friesen et al. 2011). Plant and animal hosts have evolved a diverse array of symbiotic organs, defined as specialized host structures that house microbes and can enhance host benefits from microbial mutualists (Currie et al. 2006; Markmann and Parniske 2009; Ohbayashi et al. 2015; Belcaid et al. 2019). Symbiotic organs include root nodules in plants that house nitrogen-fixing bacteria (Markmann and Parniske 2009; Desbrosses and Stougaard 2011), light organs in bobtail squid that support bioluminescent *Vibrio fischeri* (McFall-Ngai 2014; Belcaid et al. 2019), exoskeletal crypts in Hymenoptera that accommodate antibiotic-producing bacteria (Currie et al. 2006; Kaltenpoth et al. 2014), pit-like mycangia in ambrosia beetles that carry fungal symbionts (Skelton et al. 2019), and symbiont-sorting organs of hemipteran midguts (Ohbayashi et al. 2015). Because both the host and microbe partners contribute to structural and functional variation of symbiotic organs (Desbrosses and Stougaard 2011; Ohbayashi et al. 2015; Skelton et al. 2019), natural selection on each partner could lead to evolutionary conflict, wherein "joint phenotypes" are predicted to be pushed in opposite directions by each party (Queller and Strassmann 2018). Evolutionary conflict is well established in antagonistic interactions between hosts and pathogens

(Decaestecker et al. 2007), and predators and prey (Brodie et al. 2005), but there is controversy over the role of conflict in mutualisms. Some evidence suggests the fitness interests of mutualistic partners should be largely aligned (Friesen 2012; Frederickson 2013, 2017; Friesen and Heath 2013; Kiers et al. 2013), and models have demonstrated the stabilizing role of coevolution in mutualisms when exposed to perturbations (Nuismer et al. 2018). However, the net benefits that define mutualisms are predicted to conceal variation in underlying costs paid by each partner that manifest as context-dependent fitness conflict (Trivers 1971; Axelrod and Hamilton 1981; Bull and Rice 1991).

Mutualistic interactions are predicted to be vulnerable to evolutionary conflict whenever one or both partners provide a costly service to the other (Sachs et al. 2004). For example, rhizobial symbionts must spend 16 ATP to reduce one dinitrogen molecule for a host plant (Dixon and Kahn 2004), and insect endosymbionts use up to 10 ATP to synthesize essential amino acids for insect hosts (Douglas 2016). In both cases, these energetic costs are ultimately subsidized by the hosts (White et al. 2007; Ankrah et al. 2017), leaving hosts susceptible to symbionts that limit their own costs in the association while still extracting benefits from hosts. Either partner can drive evolutionary conflict, but microbes exhibit a substantial evolutionary advantage over hosts due to greater population sizes and faster reproduction rates (Sachs et al. 2018). Thus, the rapid mutation rates of microbial populations allow for selection to efficiently favor mutants with traits that downregulate or arrest energetically costly services to hosts, gaining a fitness advantage over beneficial genotypes (Sachs et al. 2004; Foster and Wenseleers 2006).

The mutualism between legumes and rhizobia provides a powerful system to investigate interspecific conflict (West et al. 2002b, 2002a; Denison 2000; Sachs and Simms 2008; Heath and Tiffin 2009; Sachs et al. 2018). Diverse legumes form root nodules, symbiotic organs that develop in response to rhizobial infection and that house dense intracellular populations of differentiated nitrogen-fixing symbionts (Markmann and Parniske 2009). To initiate this interaction, legume seedlings release flavonoids into the soil, and in response, the rhizobia secrete nod factors that cause a cascade of transcriptional changes on compatible host roots that initiate nodule organogenesis (Liu and Murray 2016). The rhizobia then enter root cells, where they differentiate into bacteroids and can fix nitrogen within the root nodule in exchange for photosynthates. For legumes, a predominant cost of interacting with rhizobia is attributed to the energy expended during the formation and maintenance of root nodules (Krusell et al. 2002; Nishimura et al. 2002), which includes forgoing some lateral root formation (Wopereis et al. 2000) and supplying energy to drive nitrogen fixation (White et al. 2007). Subsequently, rhizobia predominantly pay a cost attributed to energetically demanding nitrogen fixation (Dixon and Kahn 2004; Trainer and Charles 2006). The high cost paid by each partner to participate in the mutualism introduces a potential conflict, as each partner can be selected to minimize their individual costs of reciprocation.

The root nodule is an ideal symbiotic organ to examine the joint fitness effects of variable host investment into a mutualism. Root nodule formation fulfills the definition of a joint phenotype – predicted to be susceptible to conflict (Queller and Strassmann 2018) – because the genotypes of both the legume and rhizobia partners contribute to nodule formation (Heath and Tiffin 2007). Legumes regulate the number of nodules formed through a mechanism termed autoregulation of nodulation (Reid et al. 2011) and mutations to host genes associated with this pathway result in hypernodulated hosts that have reduced growth (Krusell et al. 2002; Nishimura et al. 2002). This balance between the dramatic fitness benefits received from moderate nodulation and the detrimental effects of hypernodulation is consistent with host mechanisms that regulate nodule formation, and predicts that legumes experience stabilizing selection on the number of nodules formed (Sachs et al. 2018). Rhizobial genotype also influences nodule formation (Sachs et al. 2010b; Heath and Tiffin 2007; Porter and Simms 2014), including strains that produce copious numbers of nodules on legumes such as *Medicago truncatula* (Crook et al. 2012; Price et al. 2015) and soybeans (Faruque et al. 2015; Yasuda et al. 2016). Modeling fitness outcomes suggests that legume and rhizobia fitness is aligned as the number of nodules formed increases from zero until the host optimum is reached, suggesting a zone of cooperation, and thereafter symbiont fitness continues to increase with the addition of more nodules (Sachs et al. 2010a; Kiers et al. 2003; Ratcliff et al. 2011; Quides et al. 2017), but host fitness begins to decrease, suggesting a zone of conflict (Fig. 1; Sachs et al. 2018). However, there remains to be a clear empirical test of this model predicting fitness conflict between legumes and rhizobia over nodule formation.

Here, we investigated conflict over nodulation in the model *Lotus-Mesorhizobium* mutualism. We employed four *Lotus japonicus* genotypes that vary in their regulation of nodule formation, including the related ecotypes *L. japonicus* Gifu B-129 (Gifu) and *L. japonicus* "Miyakojima" MG-20 (MG-20; Kawaguchi 2000) and two near-isogenic hypernodulating mutants of MG-20, *plenty*, and *har1*, that formed 250% and 500% nodules respectively compared to MG-20 in previous experiments (Krusell et al. 2002; Nishimura et al. 2002; Yoshida et al. 2010). Hosts were inoculated with the compatible nitrogen-fixing rhizobia *M. loti* MAFF303099, with and without a near-isogenic mutant lacking in nitrogen fixation function (mlr5906; strain ID 17T02d02; Quides et al. 2017). For host plants, we measured proxies of physiological investment (i.e., the number of nodules formed) and net benefit from the association (i.e., shoot mass and stable isotope analysis of nitrogen fixation, $\delta^{15}N$). For

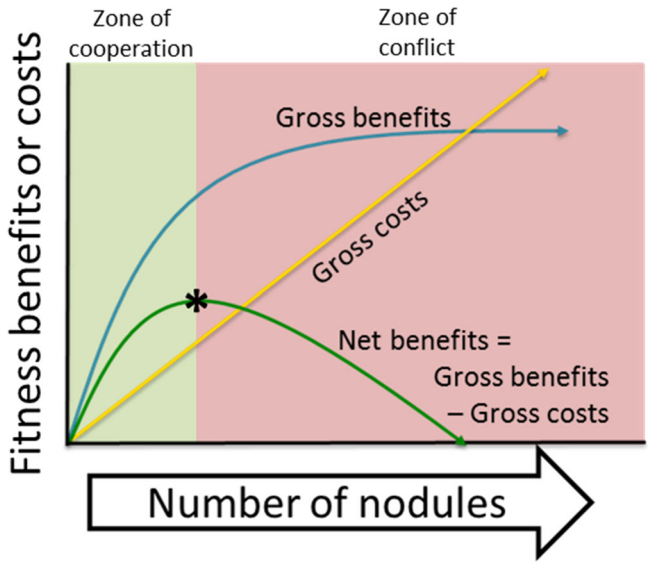

Figure 1. **Model of legume fitness benefits and costs of nodulation. Gross costs of nodulation for the legume (yellow line) are linear as the carbon cost to form additional nodules is predicted to be constant. Gross benefits of nodulation for the host (blue line) diminish as additional nodules are formed and hosts become satiated for nitrogen. The net benefits that results from subtracting costs from benefits (green line) are predicted to represent a unimodal function wherein hosts fitness is maximized at an optimal, intermediate, number of nodules formed (asterisk). Rhizobia are assumed to exhibit an increasing fitness function (Kiers et al. 2003; Ratcliff et al. 2011; Quides et al. 2017), and the symbiont fitness function is not indicated here. Thus, the fitness interests of the host and symbiont are predicted to be aligned within the zone of cooperation (green) and contrast within the zone of conflict (red). Figure modified from (Sachs et al. 2018).**

rhizobia, we measured proxies of fitness, including *in planta* rhizobial population size (via quantitative culturing), and histological measures of *in planta* rhizobial proliferation. Our goals were to (i) investigate how host and symbiont traits varied with host genotype under controlled conditions, (ii) examine fitness effects of increased nodule number on both host and symbiont partners, and (iii) test for fitness alignment or conflict over nodule number by modeling empirical data for host and symbiont fitness proxies over the phenotypic range of nodulation.

Methods

BIOLOGICAL MATERIALS

Lotus japonicus ecotypes MG-20 and Gifu seeds were acquired from LegumeBase (University of Miyazaki, Japan). The nearly isogenic MG-20 mutants, *plenty*, and *har1*, were acquired from Masayoshi Kawaguchi (National Institute for Basic Biology, Okazaki, Aichi, Japan). The *PLENTY* gene (located on chromosome 2) controls nodulation via expression in root tissue (Yoshida et al. 2010; Yoro et al. 2019). The *HAR1* gene (located on chromosome 3) is part of the autoregulation of nodulation pathway expressed in shoot tissue (Krusell et al. 2002; Nishimura et al. 2002). *Lotus japonicus* lines were grown in the greenhouse to generate seeds following published protocols (Quides et al. 2017).

We used the nitrogen fixing symbiont of *L. japonicus* MAFF303099 (i.e., Fix+) expressing DsRed integrated into the genome (a red fluorescent protein visible under natural light; Maekawa et al. 2009), and a near-isogenic non-nitrogen fixing mutant with a transposon inserted in the *NifD* gene, mlr5906 (strain ID 17T02d02; Fix-). The Fix- strain is easily distinguished from Fix+ on plates by colony color (Quides et al. 2017). The Fix+ and Fix- *M. loti* strains were grown on a solid medium of Modified Arabinose Gluconate (MAG, 1.8% agar w/v, 29°C; Sachs et al. 2009) and exhibit no differences in *in vitro* growth rate on MAG (Quides et al. 2017).

INOCULATION EXPERIMENTS

Seeds were germinated in sterile reverse-osmosis filtered water (ROH_2O) in the dark at 20°C from March 20, 2017 to April 3, 2017. Seedlings were planted in sterilized Conetainers (SC10; Steuwe and Sons, Tangent, OR, USA) filled with sterilized, calcined clay that is inert and offers negligible nutrients (Turface® Pro League®, Turface Athletics, Buffalo Grove, Illinois, USA). Seedlings were grown in a controlled growth facility with daily mist-watering until true leaves emerged, then were fertilized weekly with 5 mL of N-free Jensens for the duration of the experiment. After two weeks in the controlled growth facility, seedlings were transferred to the greenhouse to harden behind 50% shade cloth from April 24 to 27, 2017.

In the greenhouse, plants were arranged into size-matched groups of 16 (four per genotype, by leaf count) and were inoculated with one of four treatments: Fix+, Fix-, Fix+:Fix- (i.e., mixed strain co-inoculation; 1:1 ratio), or sterile ROH_2O (negative control). For each treatment, either 5 mL of water or 5mL of washed rhizobial cells were drip inoculated directly onto the soil surface at a density of 10^8 cells/mL (5×10^8 total cells). Inoculum concentrations and ratios were empirically confirmed by serial dilutions, spread plating (10^{-6} dilution), and counting colonies. The experimental plants were organized into a randomized block design with a total of 20 replicates for each host by inoculum combination with one replicate per block. There were 20 blocks, 10 for each harvest at 3.5 and 5 weeks post-infection (wpi). Three blocks were selected randomly for nodule culturing at each harvest and one block was selected for light microscopy at 5 wpi. At harvest, the shoots and roots were separated and photographed. Nodules were dissected from roots, counted, and photographed. Plant shoots, roots, and nodules were dried separately at 60°C $\geq$3 days prior to weighing dry biomass. During an early

spring heatwave, 10 plants died within a few days after inoculation (April 28, 2017 to May 4, 2017; Table S1).

IN VITRO ESTIMATION OF RHIZOBIAL FITNESS

The *in planta* fitness of rhizobia was estimated by quantitative culturing of nodules (Quides et al. 2017). Three nodules from singly inoculated hosts and five nodules from co-inoculated hosts were randomly selected from each of three plants for culturing. Surface sterilized nodules were crushed and spread plated on MAG (10^{-3}, 10^{-5} dilutions). Colonies were counted to estimate rhizobial population sizes within each nodule. Nodules from co-inoculated plants were cultured as above and were used to estimate population sizes of each symbiont within a nodule (i.e., Fix+ formed red colonies, Fix- formed off-white colonies).

LEAF TISSUE ANALYSIS

We analyzed $\delta^{15}N$ content for all hosts by inoculum treatment combinations at 5 wpi. When plants incorporate symbiotic nitrogen fixed by rhizobia, the leaf tissue exhibits reduced $\delta^{15}N$ relative to uninfected plants because of isotopic fractionation by rhizobia (Regus et al. 2014). Dry leaves were removed from stems and powdered using a bead beater for 10 s at 4 m/s with a 5 mm steel bead. Samples were analyzed at UC Santa Cruz Stable Isotope Laboratory. In many cases one plant did not provide enough leaf tissue for analysis, thus we pooled leaf tissue from up to four plants in a treatment. Due to pooling, each treatment had two to eight replicates.

LIGHT MICROSCOPY

Nodules were randomly selected for light microscopy from all four host genotypes inoculated with Fix+ at 5wpi. Fixation, infiltration, and embedding of nodules in JB-4 Plus methacrylate (Poly-sciences, Warrington, Pennsylvania, USA) followed published protocol (Regus et al. 2017). Briefly, nodules were fixed in 4% v/v paraformaldehyde and 2.5% v/v glutaraldehyde solution for 3 days, and dehydrated in a graded alcohol series to 100% EtOH. Nodules were infiltrated with increasing concentrations of JB-4 Plus methacrylate catalyzed Solution A in 100% EtOH up to 100% catalyzed Solution A. Finally, nodules were embedded in film caps with polymerized JB-4 plus methacrylate and sealed with Parafilm.

Nodules were prepared for imaging as previously described (Regus et al. 2017). Two to four micrometer nodule sections were stained with 0.1% w/v aqueous toluidine Blue O to identify plant cells that are infected with rhizobia. Sections were viewed with a Meiji Techo MT4000L Biological Microscope (Meiji Techo CO., LTD.; Miyoshi machi, Iruma-gun, Japan) and images were acquired with a Nikon D80 DSLR (Nikon Corporations; Minato, Tokyo, Japan) attached to the trinocular tube using Meiji specific adapters and ControlMyNikon tethering software (Tetherscript Technology Corporation; Vancouver, British Colombia, Canada).

An average of 11 sections per nodule was analyzed (range 7–17) from each of three to four nodules per plant. Mean infected cell size was estimated by measuring the total area of all infected plant cells in a nodule section divided by the number of infected cells counted. Infected and uninfected cells were differentiated via observation of toluidine staining, which occurs on all cell walls but only in the cytoplasm of infected cells (from dense rhizobial colonization). Mean uninfected cortex cell size was estimated using a subset of cortex cells since it is difficult to preserve the entire cortex (mean = ca. 53; range = 8–110). The cortex was defined as the plant cells one cell layer away from the central infection region.

DATA ANALYSIS

We characterized variation among host genotypes using ANOVAs and post-hoc Tukey HSD tests. We first established that our four hosts varied in the number of nodules that they formed with all inoculum treatments. To investigate pleiotropic effects of the *plenty* and *har1* mutations on plant physiology traits we analyzed shoot-growth benefits from nodulation, relative $\delta^{15}N$ content, nodule histology, and *in planta* host control over Fix- rhizobia (i.e., host sanctions; Quides et al. 2017; Regus et al. 2017). Shoot-growth benefits from nodulation was used as a proxy for net benefits received from nodulation and was calculated as the difference in dry shoot biomass between inoculated hosts and uninoculated controls ($Host_{inoculum}(g) - Host_{control}(g)$). Relative $\delta^{15}N$ content was used to estimate symbiotic nitrogen fixation. Histological nodule trait data were collected from light micrographs and used to identify microstructural differences including infected cell size (Regus et al. 2017) and uninfected cortex cell size (Guerra et al. 2010). For both cell size measures, means were calculated per nodule and this mean value was used in the ANOVA to avoid pseudoreplication.

In planta host control over Fix- rhizobia was examined in two ways. First, to measure host control over nodule growth, Fix+ and Fix- rhizobial population sizes within nodules of singly inoculated hosts were compared within a host genotype with a Welch's two-sample t-test to account for unequal variances. Second, to examine host sanctions in co-inoculated hosts we compared measures of infection to our expected infection proportion of 0.5 (i.e., Fix+ and Fix- inoculated at equal ratios, confirmed via plating; Quides et al. 2017; Regus et al. 2017). The proportion of cultured nodules with Fix+ present were compared to our expected proportion with a chi-squared goodness of fit test, and the mean observed per plant population proportion of Fix+ per plant was compared to our expected proportion with a one-sample *t*-test. Proportion of cultured nodules with the Fix+ symbiont present was calculated as the number of nodules

Table 1. List of functions used for AICc comparisons.

Function name	Equation
linear	$k1 * x + b$
square root	$(k1 * \sqrt{x}) - (k2 * x) + b$
Log_{10}	$k1 * \log_{10}(x + 1) - (k2 * x) + b$
Negative exponential	$(k1 * (1 - e^{-k2 * x})) - (k3 * x) + b$

x, number of nodules; ki, parameter; b is the y-intercept parameter used for the host fitness functions.

identified to contain the Fix+ symbiont divided by the total number of nodules cultured for a given host genotype. Per plant population proportions of Fix+ rhizobia were extrapolated based on estimated population sizes from a subset of cultured nodules on an individual plant.

We empirically modeled the relationship between the number of nodules formed and host and symbiont net fitness benefits. We used an approach that combined the wildtype host genotype (MG-20) and its two near-isogenic mutants into a single model (*plenty*, *har1*), thus most fitness variation should be accounted by genetic differences in host nodulation control, in addition to within host genotype variance, caused by environmental noise and experimenter error. The relative fit of four different functions were compared, including negative exponential, Log_{10}, square root (i.e., all consistent with fixed costs and varied degrees of diminishing benefits of nodulation; Sachs et al. 2018), as well as a linear function (i.e., fixed benefits and costs). We used dry shoot biomass as a proxy of net host benefits, rhizobial cells per plant to estimate net symbiont benefits (dependent variables), and the number of nodules formed was used as the independent variable (Table 1). Rhizobial cells per plant were calculated as the product of mean nodule population size of a plant and the number of nodules formed by that plant, using only plants that nodules were cultured from. This extrapolation is robust because the rhizobial population size within a nodule might be affected by the total number of nodules formed, and thus an increase in number of nodules formed does not guarantee increased per plant population. Using this whole plant measure is also more meaningful when considering fitness conflict because we need to consider all rhizobia progeny from the interaction, and not just the progeny from individual nodules.

For each host and symbiont inoculum combination and for each harvest we independently fit each basic fitness function to our proxies of host or symbiont fitness using the function nls() within R set to a maximum of 10^7 iterations to converge on parameter values that best fit our dataset. For host fitness functions, the y-intercept was considered a variable when generating functions. For symbiont fitness functions, we set the y-intercept at zero because no nodules would equate to no *in planta* rhizobia. Based on the generated functions, we calculated mean squared error, the standard error of the regression, and optimal nodule number based on the maximum fitness proxy measure for host or symbiont. The observed and expected values were analyzed using a paired t-test to assess the fit of these functions to our data. We also assessed goodness of fit for each function by calculating the corrected Akaike information criterion (AICc) to account for small sample sizes using the function AICc() in the 'MuMIn' package v. 1.43.10 in R (Burnham and Anderson 2002; Burnham et al. 2011). AICc values were used to calculate ΔAICc and AICc weights to further compare the fit of our generated functions (Wagenmakers and Farrell 2004).

Results

HOST GENOTYPE VARIATION IN SYMBIOSIS TRAITS

We confirmed, under controlled common garden conditions, that *L.* japonicus ecotypes Gifu and MG-20, and the near-isogenic mutants of MG-20, *plenty* and *har1*, each exhibit significant variation in the number of nodules formed ($F_{3,111} = 44.94$; $P < 0.0001$). MG-20 hosts formed the least number of nodules, followed in increasing order by Gifu, *plenty*, and *har1*, irrespective of the rhizobial inoculum that the host received (Fig. 2A). No nodules were formed on uninoculated controls, which were excluded from this analysis.

We calculated the growth benefit from nodulation in each of the host genotypes, measured as the difference in shoot biomass between inoculated and control hosts ($Host_{inoculum}$(g) – $Host_{control}$(g)). There were no significant ecotype effects, but these data showed that the mutant genotypes, *plenty* and *har1*, that formed more nodules on average, also gained less benefit from nodulation ($F_{3,111} = 7.944$; $P < 0.001$; Fig. 2B). These results demonstrate that mutations that induce the formation of additional root nodules can cause a significant reduction in the net benefits gained from root nodule symbiosis, consistent with past work that uncovered growth deficits associated with these mutants (Krusell et al. 2002; Nishimura et al. 2002; Yoshida et al. 2010).

We assessed the capacity of *M. loti* to fix nitrogen in symbiosis with *plenty* and *har1* mutants as deficiency in nitrogen fixation could be a pleiotropic effect of mutations to PLENTY or HAR1 that could confound our interpretation of fitness alignment or conflict between the partner species. We did not detect any effect of the PLENTY or HAR1 mutations on nitrogen fixation (compared to the wildtype MG-20 genotype) thus differences in the net benefit of the association among these plant genotypes would be driven by changes in costs of investment rather than

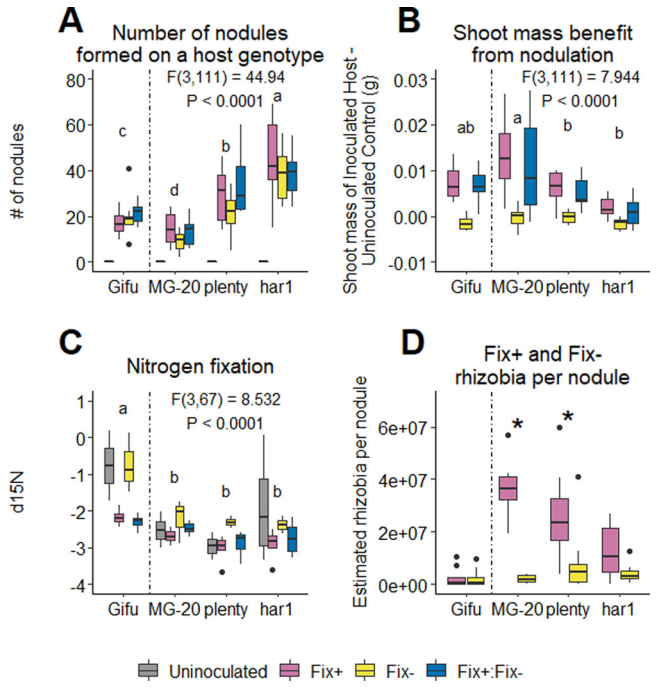

Figure 2. (A) Lotus japonicus nodulation varied significantly among host genotypes. (B) The benefits received from nodulation were most substantial with the MG-20 host. (C) The Fix+ symbiont fixed nitrogen with similar efficiency on all three MG-20 hosts, but nitrogen fixation was reduced in the Gifu ecotype (i.e., higher δ15N). (D) In planta symbiont fitness showed a trend towards favoring the Fix+ symbiont in all MG20 hosts with significant differences detected for the wildtype MG-20 ecotype and plenty mutant. Lowercase letters indicate ANOVA Tukey HSD post hoc significant differences among host genotypes. Asterisks represent significant differences between symbionts analyzed within a host genotype. Vertical dashed lines separate hosts with different ecotype genetic backgrounds.

alterations to nitrogen fixation capacity. Conversely, we did find a significant effect of host ecotype on nitrogen fixation ($F_{3,67}$ = 8.532; $P < 0.0001$), wherein Gifu fixed significantly lower levels of nitrogen than the three MG-20 background host genotypes (i.e., greater relative $\delta^{15}N$ content indicates less nitrogen fixation; Fig. 2C). The decrease in nitrogen fixation might be because the Gifu genotype develops at a slightly slower rate than MG-20 (Kawaguchi 2000), thus the hosts were at different stages of the symbiosis.

We also measured differences among host genotypes in nodule histology when infected with the Fix+ symbiont. We examined whether *plenty* and *har1* undergo premature senescence as a pleiotropic effect of the mutations, as host legumes can induce nodule senescence when symbiosis does not provide sufficient benefits (Quides et al. 2017; Regus et al. 2017). We found no evidence of senescence but nonetheless found that the infected and uninfected plant cells of the wildtype MG-20 nodules were significantly larger than the two MG-20 mutants, *plenty* and *har1*, while Gifu was intermediate to the MG-20 wildtype and mutants (infected: $F_{3,10} = 9.562$, $P = 0.003$; uninfected: $F_{3,10} = 7.556$, $P = 0.006$; Fig. S1). These results suggest that significant changes to infected and uninfected cell size within nodules are the main driver of nodule size variation, consistent with past studies (Regus et al. 2017).

Finally, we examined whether *plenty* and *har1* exhibit effects on *in planta* control over uncooperative rhizobia, in clonal or mixed strain infections (Quides et al. 2017; Regus et al. 2017). Here, we found modest differences among the genotypes, but no clear relationship between number of nodules formed, and the capacity to punish the Fix- symbionts. When Fix+ and Fix- symbionts were clonally inoculated on hosts we found that the mean population sizes of the Fix- symbionts in nodules showed a trend toward reduction compared to the Fix+ symbionts for all MG-20 backgrounds, but the differences were only significant for MG-20 ($t = 7.812$, $df = 6.154$, $P < 0.001$) and *plenty* ($t = 2.352$, $df = 14.918$, $P < 0.05$; Fig. 2D). In contrast, we did not detect differences in the mean rhizobial population sizes of the Fix+ and Fix- nodules in the *har1* ($t = 2.070$, $df = 7.120$, $P = 0.077$) or Gifu genotypes ($t = 0.081956$, $df = 13.882$, $P = 0.936$). When hosts were co-inoculated, we estimated the Fix+ symbiont's proportional presence within nodules and per plant population size to be at a greater proportion than expected (i.e., 50%) on all four hosts, but this difference was only significant for the *har1* and *plenty* genotypes, respectively (Table S2). Gifu hosts exhibited little or no capacity to bias in planta fitness of uncooperative rhizobia, potentially because it had not developed to the point where sanctions traits are expressed (Regus et al. 2017). Conversely, we found the *plenty* and *har1* hosts maintained some ability to sanction as they were able to significantly bias rhizobial infection towards the beneficial, wild-type rhizobia (Quides et al. 2017).

HOST AND SYMBIONT FITNESS FUNCTIONS DEPENDENT ON THE NUMBER OF NODULES FORMED

We modeled the relationship between the number of nodules formed and proxies of host and symbiont fitness. We used the Fix+ symbiont to incorporate both benefits and costs of nodulation and excluded the Gifu host ecotype to focus only on variation among MG-20 and its near-isogenic nodulation mutants (*plenty*, *har1*).

Dry shoot biomass rather than seed production was used as a host fitness proxy since our analysis required us to simultaneously estimate host and symbiont fitness before fruit production, at which point nodules senesce and rhizobial fitness cannot be practically measured (Puppo et al. 2004). We tested the fit of four different functions for host shoot biomass including negative exponential (Sachs et al. 2018), square root, Log_{10}, and linear (Table 1). The first three are consistent with diminishing

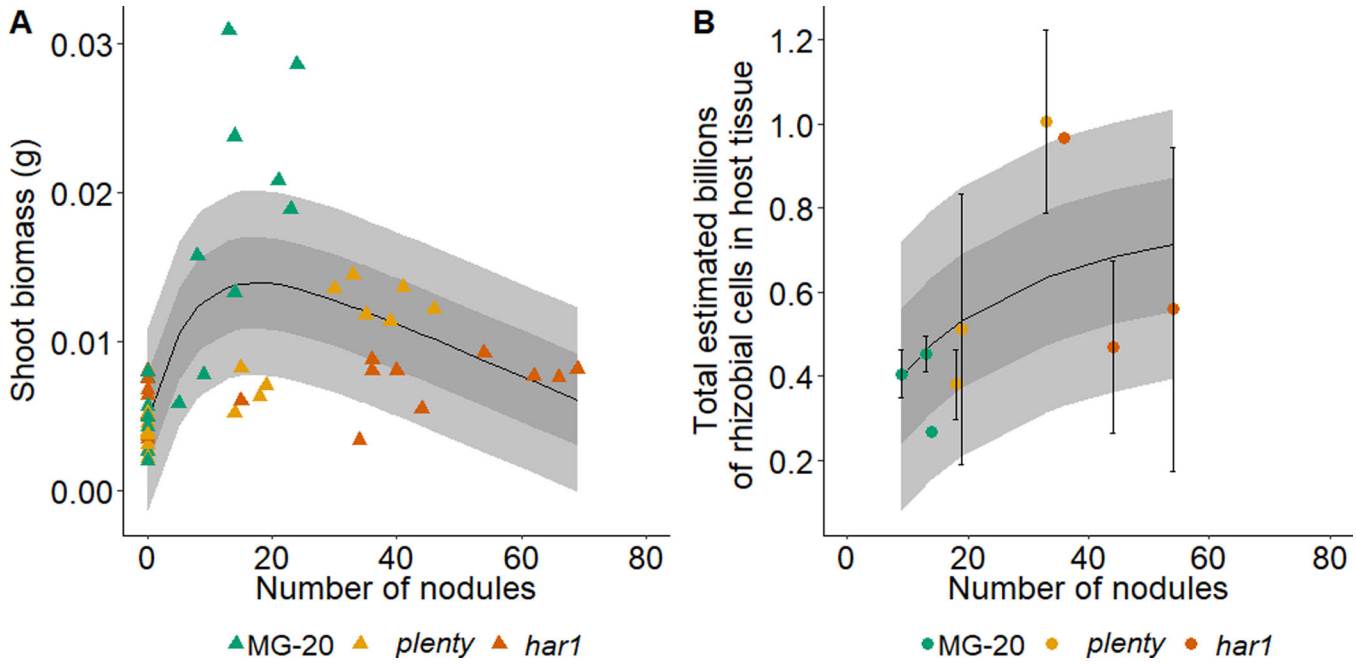

Figure 3. **(A) The negative exponential function for the host fitness proxy is graphed. (B) The square root function for the symbiont fitness proxy is depicted. Graphed functions are those with the lowest MSE (Fig. S2). Error bars represent one standard error calculated from a maximum of 3 nodules cultured for a given plant (data point). Green data points = MG-20 host, orange = plenty, and red = har1. The best fit functions are graphed in black with dark shading representing one standard error of the regression and lighter shading representing two standard errors of the regression.**

benefits and a fixed cost, while the linear function implies both benefits and costs are fixed. Based upon likelihood model selection using Akaike information criteria, we determined that the Log_{10} (AICc = −456.945; AICcw = 0.425), square root (AICc = −456.685; AICcw = 0.373), and negative exponential (AICc = −455.462; AICcw = 0.202) unimodal functions for host fitness fit our data set equally well, with host growth optimized at approximately 16–18 nodules formed (negative exponential visually depicted, lower MSE; Fig. 3A, Fig. S2A).

Rhizobial fitness was estimated as population size within nodules at the whole plant level. The same four functions modeled for the host were also modeled for the symbiont and compared using AICc values. Functions were set to have a y-intercept of zero because zero nodules formed would equate to no *in planta* rhizobia. We determined that the linear function (AICc = 8.332; AICcw = 0.252), Log_{10} function (AICc = 7.884; AICcw = 0.315) and square root function (AICc = 7.884; AICcw = 0.357) fit our data set equally well (square root visually depicted, lower MSE; Fig. 3B, Fig. S2B) with symbiont fitness conservatively optimized around 91 nodules (Fig. S2B), in any case well beyond the observed range of number of nodules formed in this study.

When considering fitness functions from the host and symbiont datasets, we observed that both host and symbiont have increasing fitness up to the optimal number of nodules for the host (fitness alignment), but the fitness of each partner diverges as host fitness declines with additional nodules formed (fitness conflict; Fig. 3), consistent with a previous model that predicted zones of cooperation and conflict dependent on the number of nodules formed (Sachs et al. 2018). Uninoculated control plants may have skewed our models when zero nodules are formed, thus we also examined the host dataset without uninoculated controls and compared the fit of our generated functions with and without the added parameter of a forced y-intercept (Fig. S3). The negative linear function was the best fit among functions without a forced y-intercept, and it was among the three best-fitting models of the eight tested, thus we cannot rule out the potential for fitness conflict at all levels of nodule formation.

We also assessed fitness functions for each host genotype separately to remove sources of fitness variance driven by the genetic differences in host regulation of nodulation, and thus only observing variation within each host genotype (Fig. S4). We determined linear functions best fit the individual host genotype datasets and had a significantly positive association between the number of nodules formed and shoot mass (fitness alignment), showing that the partnership maintains a net benefit in all three host genotypes (i.e., mutualism). However, the shoot mass benefit received from forming additional nodules was significantly reduced in the mutant hosts (ANCOVA of the slopes: $F_{2,53} = 47.997$; $P < 0.0001$), thus our inference of fitness conflict between hosts and symbionts is driven by dysregulation of the

plenty and *har1* mutants that form more than the optimal number of nodules (Fig. 3). Importantly, natural populations of *Lotus japonicus* commonly form from 10 to over 100 nodules (Bamba et al. 2019, Bamba *personal communication*), so we were able to simulate some of the natural variation expressed in this species, albeit under highly controlled biotic and abiotic conditions.

Discussion

Herein, we provided empirical evidence of fitness conflict over nodule formation in the association between *Lotus japonicus* and *Mesorhizobium loti*. Our fitness data suggest that *L. japonicus* MG-20 hosts combined with near-isogenic nodulation mutants *plenty* and *har1* reveal a unimodal fitness function for nodule number, consistent with a previous model (Sachs et al. 2018). Our study found support for stabilizing selection on the host for the number of nodules formed, as host mutants that form more nodules gain less benefit from infection on a per nodule basis (Fig. S4). Specifically, shoot biomass (a proxy for plant fitness) increased as additional nodules were formed up to the optimal number of nodules (ca. 17; Fig. 3A, Fig. S2A), but subsequently decreased beyond that point. In contrast, our results support continuously increasing fitness of beneficial rhizobial symbionts (within our data range of 9–54 nodules) suggesting that the Fix+ symbiont experiences directional selection to increase nodule number well beyond the optimal number of nodules for *L. japonicus*. Combined, these fitness functions are consistent with an alignment of fitness between *L. japonicus* and the Fix+ *M. loti* up to the optimal number of nodules for the host (i.e., a zone of cooperation), but suggest conflict when more than that number of nodules is formed (i.e., zone of conflict; Fig. 1; Sachs et al. 2018), which in our case was induced by mutations that dysregulate nodulation control (Krusell et al. 2002; Nishimura et al. 2002; Yoshida et al. 2010). In natural populations in Japan, *Lotus japonicus* commonly form up to 100 nodules (Bamba et al. 2019; Bamba personal communication), so it is possible that the conflict we induced over nodule formation occurs in natural populations.

Examples of conflict in the legume-rhizobium symbiosis are often attributed to selfish genotypes of rhizobia (i.e., that enhance rhizobial fitness at the potential expense of the host; Sachs and Simms 2008; Sachs et al. 2018). For example, genotypes of the rhizobium *Ensifer fredii* (NGR234, USDA257) nodulate scores of legume species gaining access to host resources, while often providing negligible benefit in return (Pueppke and Broughton 1999). In a more host-specific association, *E. meliloti* strains bearing an *hrrP* plasmid display parasitic behavior on certain *Medicago truncatula* hosts, and are maintained via hypercompetitive nodulation (Crook et al. 2012; Price et al. 2015). Similarly, the soybean symbiont *Bradyrhizobium elkanii* USDA61 produces rhizobitoxine enabling the formation of many nodules that provide little fixed nitrogen for the host (Yuhashi et al. 2000). Some soybeans have evolved the *Rj4* resistance allele to protect against rhizobitoxine, but multiple USDA61 mutants have been identified that evade *Rj4* resistance (Faruque et al. 2015; Yasuda et al. 2016). The evolution of rhizobia that appear to selfishly enhance their fitness in association with legumes is in line with their predicted evolutionary advantage. Importantly, however, our data highlight a scenario where mutations to the host, as opposed to the symbiont, can uncover fitness conflict even with infection by beneficial nitrogen-fixing symbionts. Thus, rather than symbionts singlehandedly driving conflict by not reciprocating benefits, conflict can also occur when hosts disproportionately provide plant resources to beneficial symbionts. Therefore, hosts must regulate nodule numbers to optimize benefits from rhizobia as overinvestment can come with substantial costs.

Other studies have also uncovered evidence consistent with fitness conflict in the legume rhizobium mutualism. Using multiple populations of *Medicago lupulina* hosts and beneficial *Ensifer* rhizobia symbionts, Simonsen and Stinchcombe (2014) demonstrated a host tradeoff between nodule formation (a proxy for rhizobial fitness) and host fitness (measured as shoot mass and survival). The models they generated were consistent with host and symbiont fitness conflict when regressed against each other (Simonsen and Stinchcombe 2014). Studies on natural populations of the host *M. polymorpha* and *Acmispon strigosus* also suggested conflict with rhizobia symbionts, in the sense that rhizobial genotypes that occupied more nodule tissue biomass (i.e., superior fitness) provided less benefit to the host (Porter and Simms 2014; Gano-Cohen et al. 2019). In our study, by measuring nodule number and *in planta* rhizobial population size, we were able to directly compare host and symbiont fitness functions to examine fitness differences over the observed range of nodules formed. Furthermore, our use of near-isogenic host variants limits interactions of traits correlated with the number of nodules formed. Nonetheless, we cannot completely rule out the potential for pleiotropy, especially since the capacity to sanction nonfixing rhizobia varied among the host genotypes, as well as the shoot mass benefit received by hosts when comparable numbers of nodules were formed (Fig. S4). These patterns suggest that there were costs of the mutations that were not directly related to the number of nodules formed.

The exchange of net fitness benefits between interacting species is what defines a mutualism, but selection to maximize individual gains while minimizing costs creates the potential for conflicting fitness interests (Bronstein 2001; Sachs et al. 2004). Plant and animal hosts have evolved a diversity of symbiotic organs that house high-density populations of microbial partners, within which hosts mediate microbial colonization and control aspects of resource flow between hosts and microbes (Currie

et al. 2006; Markmann and Parniske 2009; Kaltenpoth et al. 2014; Belcaid et al. 2019). Our data support previous work that suggests that the formation of nodules, the legume symbiotic organ, can be affected by fitness conflict (Heath and Tiffin 2007; Porter and Simms 2014; Simonsen and Stinchcombe 2014). However, it is unclear whether fitness conflict over symbiotic organs is common in other systems. Hosts such as bobtail squid (McFall-Ngai 2014), beewolf wasps (Kaltenpoth et al. 2014), and ambrosia beetles (Skelton et al. 2019) allocate substantial amounts of host tissue to symbionts. One prediction might be that increased size or host physiological supply to light organs in the bobtail squid, antenna reservoirs of the beewolf wasp, or mycangia of ambrosia beetles could increase symbiont population sizes but would also have the potential to interfere with host performance. These three hosts also acquire their symbionts through horizontal transmission, exposing them to exploitative symbionts (Kaltenpoth et al. 2014; McFall-Ngai 2014; Skelton et al. 2019), thus increasing their chance of experiencing fitness conflict over symbiotic organs. In other mutualisms that may not have well defined symbiotic organs, direct measurements of ATP production and consumption (Dixon and Kahn 2004; Douglas 2016) or shared resources (Lodwig et al. 2003; Prell et al. 2009) could be used to quantify the gross benefits and costs of exchanges related to joint phenotypes.

Mutualistic interactions are defined by net positive fitness benefits, and these benefits are predicted to obscure costs associated with mutualisms (Trivers 1971; Axelrod and Hamilton 1981; Bull and Rice 1991). By altering the ecological context of a mutualism, we can shift the balance of a species interaction to expose these costs (Hoeksema et al. 2010; Chamberlain et al. 2014). Our study examined the effect of host genes that regulate nodulation and suggests that hidden conflicts can occur, as has been suggested as a general feature of many mutualistic interactions (Fonseca 1993; Bronstein 1998, 2001; Herre et al. 1999; Holland et al. 2002; Queller and Strassmann 2018). Studying joint phenotypes (e.g., symbiotic organs) offers a simple framework to characterize conflict in mutualisms and dissect the costs from the benefits (Currie et al. 2006; Markmann and Parniske 2009; Desbrosses and Stougaard 2011; Kaltenpoth et al. 2014; McFall-Ngai 2014; Ohbayashi et al. 2015; Belcaid et al. 2019; Skelton et al. 2019). Symbiotic organs allow for a close examination of costs (e.g., host tissue and symbiont metabolites) and benefits (e.g., growth and population size) that can be used to generate separate fitness models for each partner as we demonstrated here. The legume-rhizobium symbiosis is one model that can help us understand the potential for conflict that exists in mutualisms, but studies in other systems will help us understand common triggers of conflict in microbial mutualistic interactions. This will require a firm understanding of the symbiont diversity hosts are exposed to (i.e., transmission mode) and when to measure fitness (i.e., phenology of the interaction) so that we can develop novel approaches to study underlying conflict between coevolved species (Mueller 2002; McFall-Ngai 2014; Sachs et al. 2018). Just as evidence of fitness conflict between male and female *Drosophila melanogaster* heavily influenced how we now think about coevolution and genomic conflict (Rice 1996), our data highlights the often overlooked conflict in mutualistic interaction and provides a foundation for new research in mutualisms.

AUTHOR CONTRIBUTIONS

KWQ designed the experiment, collected and analyzed the data, and wrote the manuscript; FS conducted the experiment and collected data; RJ conducted the experiment and collected data; JLS designed the experiment and wrote the manuscript.

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CONFLICT OF INTERESTS

The authors have no conflict of interests regarding the publication of this paper.

DATA ARCHIVING

Raw data are available via Dryad (https://doi.org/10.5061/dryad.z08kprrbp)

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: (A-D) Light micrographs were taken to measure nodule histology traits when infected with the Fix+ symbiont.

Figure S2. Accompanying analyses for Figure 3 (A) The three unimodal functions were modeled as the best fit functions for host shoot biomass, a proxy for host fitness, as determined by AICc.
Figure S3. Generated functions without uninoculated control data.
Figure S4. Scatterplot with linear regressions for each host. Linear models fit our host datasets the best.
Table S1. Host phenotype data collected at the time of harvest
Table S2. Proportional in planta fitness of the Fix+ symbiont Fix+ at 5wpi in coinoculated hosts
Table S3. Estimate viable rhizobia per nodule
Table S4. Histological measurements of host nodules infected with MAFF at 5wpi
Table S5. Elemental and isotopic analysis of leaf tissue for plants harvested at 5wpi
Table S6. Fitness functions and corresponding AICc values for host shoot mass dependent on number of nodules formed
Table S7. Fitness functions and corresponding AICc values for estimated rhizobia population size per plant dependent on number of nodules formed
Table S8. Fitness functions and corresponding AICc values for host shoot mass dependent on number of nodules formed (Gifu ecotype not included)
Table S9. Fitness functions and corresponding AICc values for estimated rhizobia population size per plant dependent on number of nodules formed (Gifu ecotype not included)