JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12579

Lotus hosts delimit the mutualism–parasitism continuum of *Bradyrhizobium*

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Keywords:

Bradyrhizobium;
host control;
legume;
Lotus;
mutualism;
parasitism.

Abstract

Symbioses are modelled as evolutionarily and ecologically variable with fitness outcomes for hosts shifting on a continuum from mutualism to parasitism. In a classic example, rhizobia fix atmospheric nitrogen for legume hosts in exchange for photosynthetic carbon. Rhizobial infection often enhances legume growth, but hosts also incur interaction costs because of root tissues and or metabolites needed to support symbionts *in planta*. Rhizobia exhibit genetic variation in symbiotic effectiveness, and ecological changes in light or mineral nitrogen availability can also alter the benefits of rhizobial infection for hosts. The net effects of symbiosis thus can range from mutualistic to parasitic in a context-dependent manner. We tested the extent of the mutualism–parasitism continuum in the legume–rhizobium symbiosis and the degree to which host investment can shape its limits. We infected *Lotus strigosus* with sympatric *Bradyrhizobium* genotypes that vary in symbiotic effectiveness. Inoculations occurred under different mineral nitrogen and light regimes spanning ecologically relevant ranges. Net growth benefits of *Bradyrhizobium* infection varied for *Lotus* and were reduced or eliminated dependent on *Bradyrhizobium* genotype, mineral nitrogen and light availability. But we did not detect parasitism. *Lotus* proportionally reduced investment in *Bradyrhizobium* as net benefit from infection decreased. *Lotus* control occurred primarily after infection, via fine-scale modulation of nodule growth, as opposed to control over initial nodulation. Our results show how divestment of symbiosis by *Lotus* can prevent shifts to parasitism.

Introduction

The stability of symbioses is shaped by interplay between evolutionary and ecological parameters, and only recently have these factors been studied in concert. Soil-acquired microbes enhance the health and growth of diverse plants (Johnson *et al.*, 1997; Soto *et al.*, 2009; Douglas, 2010; Medina & Sachs, 2010; Friesen *et al.*, 2011), but these hosts can also incur interaction costs, at minimum because of root tissues and or metabolites needed to support symbionts *in planta* (Kouchi & Yoneyama, 1984; Vance & Heichel, 1991; Bourion *et al.*, 2007). The fitness benefit that plants receive from these interactions is often conditional and can vary depending on genetic variation in microbial and/or plant populations (Bever, 1999; Sachs *et al.*, 2010a), local environmental variation (Lau *et al.*, 2012; Regus *et al.*, 2014; Simonsen & Stinchcombe, 2014) and interactions among these factors (Heath *et al.*, 2010). A dominant paradigm of plant–microbial symbiosis models these interactions as a 'mutualism–parasitism continuum', defined here as variation in the fitness outcomes of symbiosis that range from mutualistic (i.e. net fitness benefits of infection) to parasitic (i.e. net fitness cost; Thompson, 1988; Bronstein, 1994, 2001; Neuhauser & Fargione, 2004). Some host inoculation experiments have found evidence for a mutualism–parasitism continuum (Hoeksema *et al.*, 2010; Lau *et al.*, 2012). But the ecological and evolutionary relevance of these kinds of data has been debated (Karst *et al.*, 2008), as some tested conditions might never occur in nature (e.g. geographically distant microbe–plant

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genotype combinations, extreme soil nutrient parameters). Moreover, models of mutualism stability predict that hosts are favoured by natural selection to optimize the net benefit from symbiosis, by supporting symbionts when infection provides net fitness rewards and/or by divesting in symbiosis when it is costly (Bull & Rice, 1991; Denison, 2000; Simms & Taylor, 2002; Sachs *et al.*, 2004; Sachs & Simms, 2006). Hence, a key unanswered question is whether plant hosts can modulate investment to prevent mutualistic symbioses from shifting into parasitism. If plants can delimit the continuum to prevent parasitism, does it occur by preventing infection in contexts where benefit is reduced, or by modulating investment in symbionts post-infection?

The legume–rhizobium interaction is a classic model of plant–microbial symbiosis with both evolutionary and ecological variation in the effects of symbionts upon the host (Burdon *et al.*, 1999; Heath & Tiffin, 2007; Sachs *et al.*, 2010a; Lau *et al.*, 2012; Regus *et al.*, 2014). Rhizobial populations exhibit extensive genotypic variation in symbiotic effectiveness, varying from highly effective (fixing nitrogen, greatly enhancing host growth) to ineffective (fixing little or no nitrogen, providing zero growth benefits; Quigley *et al.*, 1997; Moawad *et al.*, 1998; Burdon *et al.*, 1999; Denton *et al.*, 2000; Chen *et al.*, 2002; Collins *et al.*, 2002; Simms *et al.*, 2006; Heath & Tiffin, 2007; Sachs *et al.*, 2010a; Schumpp & Deakin, 2010). Moreover, the net fitness effect of a rhizobial infection varies dependent upon the host's local environment (GxE interactions; Lau *et al.*, 2012; Regus *et al.*, 2014) and interactions between the rhizobial and plant genotypes (GxG interactions; Burdon *et al.*, 1999; Heath & Tiffin, 2007). Enhanced light availability can increase the net benefit of rhizobial infection (Lau *et al.*, 2012), as the host has a larger pool of carbon to feed into rhizobial metabolism. Conversely, nitrogen enrichment of soil can decrease the net benefit of rhizobial infection because uptake of mineral nitrogen can offer energetic savings to the legume relative to biologically fixed nitrogen (Silsbury, 1977; Voisin *et al.*, 2002). Unlike these variable benefits, the physiological costs of rhizobial symbiosis (i.e. forming nodules and maintaining rhizobia *in planta*) are unlikely to substantially vary among contexts (Kouchi & Yoneyama, 1984; Vance & Heichel, 1991; Bourion *et al.*, 2007). The context dependency of plant–microbial symbioses is of critical importance as global change alters nutrient inputs and shifts seasonality (Kiers *et al.*, 2010). Recent anthropogenic inputs such as nitrogen deposition have caused mineral nitrogen to increase rapidly in many soils (Tilman, 1999; Dentener *et al.*, 2006; Fenn *et al.*, 2010), leading to scenarios where legumes gain no benefit from rhizobial infection, and enhancing risk of breakdown for the symbiosis (Regus *et al.*, 2014).

Natural selection is predicted to favour plant traits that optimize investment in microbial symbiosis depending on the net fitness benefit that the host receives from the infection (Denison, 2000; Simms & Taylor, 2002; West *et al.*, 2002; Sachs *et al.*, 2004; Akcay & Simms, 2011). Nodule formation and maintenance impose energetic costs for legumes (Kouchi & Yoneyama, 1984; Vance & Heichel, 1991; Bourion *et al.*, 2007); hence, those plants should only invest resources in rhizobia when the benefits of symbiosis outweigh these costs (Denison, 2000; West *et al.*, 2002; Kiers *et al.*, 2003; Simms *et al.*, 2006; Heath *et al.*, 2010; Sachs *et al.*, 2010b). Legumes can modulate investment in rhizobia at two key stages of the interaction: by regulating the formation of root nodules and then by controlling nodule growth and metabolism (Streeter, 1988; Parsons *et al.*, 1993). Legume control over nodule formation can be regulated depending on host specificity for rhizobial genotypes (Endre *et al.*, 2002; Radutoiu *et al.*, 2003), the host's nodulation status (Caetano-Anolles & Gresshoff, 1991), the soil nitrogen content (Streeter, 1988) and presence of ineffective strains (Devine *et al.*, 1990; Heath & Tiffin, 2009; Sachs *et al.*, 2010b). After nodule formation, hosts can modulate resource allocation to nodules, dependent on the amount of nitrogen fixed by the resident rhizobia (Kiers *et al.*, 2003; Lodwig & Poole, 2003; Lodwig *et al.*, 2003). As a whole, host investment in rhizobia is thought to vary with the plant's budget of fixed nitrogen and photosynthetic carbon (Singleton & van Kessel, 1987; Denison, 2000; Kiers *et al.*, 2003, 2006; Simms *et al.*, 2006; Sachs *et al.*, 2010a; Voisin *et al.*, 2010). But host control has most often been examined under conditions of near zero soil nitrogen, which are biologically unrealistic.

Here, we investigated the mutualism–parasitism continuum between *Lotus strigosus*, an annual legume native to California, and sympatric *Bradyrhizobium* symbionts by varying rhizobial genotype, mineral nitrogen and seasonal light input under ecologically realistic conditions. We infected *L. strigosus* with four *Bradyrhizobium* genotypes that vary in symbiotic capacity from highly effective to ineffective, spanning the full range of host fitness effects that were sampled from the host population (Sachs *et al.*, 2009, 2010a,b). We manipulated soil nitrogen to bracket concentrations that *L. strigosus* encounter in California, by growing hosts in either zero added mineral nitrogen or fertilized with a concentration of nitrogen determined to maximize *L. strigosus* growth in the absence of rhizobial infection (Regus *et al.*, 2014). We replicated the experiment temporally, covering a seasonal span of light regimes that *Lotus* hosts can experience. We examined host growth response and nitrogen uptake from infection by comparing infected plants to matched, uninfected controls. We investigated host investment in rhizobial infection (nodule number) and maintenance (nodule size) dependent on rhizobial genotype, mineral nitrogen treatment and season. Our goals were to (i) test whether *Bradyrhizobium* can act as context-dependent

parasites to *Lotus* hosts, (ii) examine the degree to which modulation in investment by *Lotus* can delimit the mutualism–parasitism continuum of *Bradyrhizobium* and (iii) discern whether host control occurs over initial nodule formation or via modulation of nodule metabolism and growth.

Materials and methods

Bradyrhizobium inocula

Four *Bradyrhizobium* genotypes, referred to as #'s 2, 14, 38 and 49 (Sachs *et al.*, 2010a), were selected based on their natural variation in symbiotic effectiveness on *L. strigosus*, under conditions of high light intensity and zero soil nitrogen availability (Sachs *et al.*, 2009, 2010a, b, 2011). Under these conditions that optimize the fitness benefits of rhizobial infection, genotypes #49, #38 and #14 provide a net growth benefit to *L. strigosus* (increase in shoot biomass relative to uninfected controls) of ~500%, ~350% and ~200%, respectively, whereas genotype #2 forms nodules, but does not enhance host growth (i.e. ineffective; Sachs *et al.*, 2010a). Genotypes #2, #38 and #49 were isolated from *L. strigosus* nodules at Bodega Marine Reserve (BMR), CA, USA, and genotype #14 was isolated from *Lotus micranthus* collected at Sonoma Coast State Park, CA, USA, adjacent to BMR (Sachs *et al.*, 2009). Inocula of each genotype were generated per published protocols (Sachs *et al.*, 2009).

Host plant preparation

Lotus strigosus fruits were collected at BMR in June 2011 in a 25-m radius from sympatric sites as *Bradyrhizobium* isolates #2, #38 and #49 (Sachs *et al.*, 2009). Host seed sets were comprised of equal mixes from different parental plants to reflect local genetic diversity. This approach allows us to study mean host response to a rhizobial genotype in a specific environment, averaging G × G interactions between hosts and rhizobia. But as we have not surveyed host genetic diversity, it is possible that the sampled plants represent only a small subset of potential genotypes. Thus, experimental responses reported here represent a sample of sympatric hosts as opposed to a species-level response to the infection and nitrogen treatments. Seed preparation, planting and plant maintenance followed published protocols (Sachs *et al.*, 2009).

Inoculation experiments

Replicated experiments were performed in fall 2011 (17 October 2011–12 December 2011) and winter 2012 (23 January 2012–19 March 2012), hereafter referred to as the Fall and Winter experiments. Sterile-grown *L. strigosus* seedlings were arranged by size and divided into two blocks per experiment to minimize the effects of initial plant size. Within blocks, size-matched sterile-grown seedlings were randomly assigned to treatments: Bacterial treatments consisted of single infections of the four rhizobial genotypes (#2, #14, #38, #49) and uninfected control plants. Nitrogen fertilizer treatments included fertilization with 5.0 mL nitrogen-free Jensen's solution per plant per week with dissolved potassium nitrate (KNO_3; 0.5 g L^{-1}, fertilized plants) or no KNO_3 (unfertilized plants). The KNO_3 fertilization treatment parallels the highest soil nitrogen levels observed at *L. strigosus* sites and maximizes *Lotus* shoot growth in the absence of rhizobial infection (growth saturating nitrogen or GSN; Regus *et al.*, 2014). Other forms of nitrogen are rapidly converted into nitrate in the soil (Streeter, 1988), making KNO_3 fertilization ecologically relevant. Each inoculation experiment included 180 plants (nine replicate plants per treatment, 10 treatments, two replicate blocks). All plants were grown in prewashed, autoclave-sterilized quartzite sand that provides no mineral nitrogen to hosts.

Seedlings were hardened to greenhouse conditions for one week and were inoculated with *Bradyrhizobium* (1.0×10^8 cells mL^{-1} in 5 mL ddH_2O) three days after their initial fertilization. Plants were fertilized per nitrogen treatment weekly thereafter until harvest. Seasonal ambient light input varied naturally between the Fall and Winter experiments (~598 vs. 637 h of total daylight; 33.98°N). Day length changes were opposite between the two experiments, so Fall plants had longer days at the beginning of the experiment, whereas the Winter plants had longer days at the end. Each experiment lasted 8 weeks from inoculation to harvest, at which time plants were removed from pots, sand was washed from the roots, and nodules were dissected, counted and photographed. Roots, shoots and nodules were separated and dried in an oven (60 °C, > 4 days) before weighing dry biomass. Experiments were terminated prior to flowering because *L. strigosus* rapidly senesces nodules at flowering, making it impossible to collect data on nodules status. Dates for the two experiments overlap with *L. strigosus* growth periods over much of its habitat in the Pacific Southwest of the United States (www.calflora.org).

Statistical analysis

Net effects of *Bradyrhizobium* infection to the host were quantified in two ways. First, we assessed 'host growth response (%)', quantified as the mean percentage difference in total plant biomass between inoculated plants and matched uninoculated control plants [((Mass Inoculated Plant − Mass Control Plant)/Mass of Control Plant)*100; Sachs *et al.*, 2010a]. We tested whether host response differed significantly from zero using a one-sample *t*-test (JMP 10.0 SAS Institute Inc.; Regus *et al.*, 2014) to discern mutualism (greater than zero

growth response) from parasitism (less than zero growth response). We also compared plant dry shoot mass for inoculated plants with controls using ANOVA and pairwise *t*-tests. We measured leaf nitrogen content to examine the net effect of *Bradyrhizobium* nitrogen fixation on host nitrogen budget. The leaf economic spectrum theory predicts a linear relationship between leaf nitrogen and photosynthetic rate (Wright *et al.*, 2004), and variation in photosynthetic rate can affect plant fitness (Arntz *et al.*, 2000; Dodd *et al.*, 2005).

To examine effects of *Bradyrhizobium* genotype and fertilizer on host investment in symbiosis, we used general linear models (GLM; Fit Model Platform in JMP 10.0 SAS Institute Inc., Cary, NC, USA) to test main effects (rhizobial genotype, fertilizer) and interactions among effects within each experiment. Seasonal variation is derived from separate experiments and so cannot be analysed as a manipulated treatment. We thus combined the data from both experiments keeping observations separated by experiment and assigned each experiment to a category (Fall or Winter). We added season as an additional main effect in the multivariate models above, but acknowledge the limited statistical power of comparing this effect from separate experiments.

Host investment in *Bradyrhizobium* infection was quantified in terms of nodule number and mean individual nodule size, both of which can correlate with fitness of the rhizobia (Heath & Tiffin, 2009; Sachs *et al.*, 2010a). A GLM was used to test effects of nitrogen fertilization, rhizobial genotype and interactive effects (Fit Model Platform in JMP 10.0). Both nodule number and total nodule dry mass can correlate with plant size, so plant biomass was added to GLM models as a covariate (Phillips *et al.*, 1976; Oono & Denison, 2010). We report least squares means of nodule number and mass from the model analysis to control for the effects of plant size on nodule number and size.

We tested the hypothesis that *Lotus* hosts modulate investment in *Bradyrhizobium* dependent on the host's net growth benefit from infection. To do this, we analysed the relationship between host growth response and both nodule number and mean nodule mass using GLM with host growth response from infection as a main effect. Plant biomass was also included in the GLM model not as an independent effect but as a covariate because previous analysis showed significant correlation between plant biomass and both nodule number and size.

Results

Net effects of *Bradyrhizobium* infection

Host growth response exhibited significant effects of *Bradyrhizobium* genotype (Fall $F3,140 = 8.58$, $P < 0.0001$; Winter $F3,139 = 9.26$, $P < 0.0001$), soil nitrogen treatment (Fall $F1,142 = 49.51$, $P < 0.0001$; Winter $F1, 141 = 52.34$, $P < 0.0001$) and interaction between these effects (Fall $F3,140 = 7.22$, $P < 0.001$; Winter $F3,139 = 7.07$, $P = 0.0002$). When season was included in the model, the effect was significant (Season $F1,285 = 10.39$, $P = 0.0014$).

We found no evidence of parasitism in terms of host growth response. Host growth response from infection was either significantly positive or not significant (i.e. no net fitness effect; Fig. 1; Table S1). Growth benefit from infection was completely eliminated by nitrogen fertilization for the effective *Bradyrhizobium* genotypes in Fall (Fig. 1; Table S1). In Winter, nitrogen fertilization significantly decreased, but did not eliminate, the growth benefit from the effective genotypes relative to unfertilized treatments (*t*-test among nitrogen treatments within *Bradyrhizobium* genotypes #'s 14, 38 and 49, $P < 0.05$). The ineffective genotype (#2) never affected host growth in any conditions. No uninoculated control plants were contaminated (nodulated) in either experiment.

Leaf nitrogen content exhibited significant effects of rhizobial genotype (Fall $F3,63 = 79.86$, $P < 0.0001$; Winter $F3,67 = 185.89$, $P < 0.0001$), nitrogen fertilization (Fall $F1,65 = 68.37$, $P < 0.0001$); Winter $F1,69 = 23.89$, $P < 0.0001$) and their interaction (Fall $F3,63 = 58.41$, $P = 0.0003$; Winter $F3,67 = 48.70$, $P = 0.0004$). When season was included in the model, the effect was significant (Season $F1,136 = 12.37$, $P = 0.0002$). Leaf nitrogen content was significantly decreased in only one treatment combination (Winter, #2; Fig. 2). Leaf nitrogen was not significantly correlated with host growth response from infection in individual

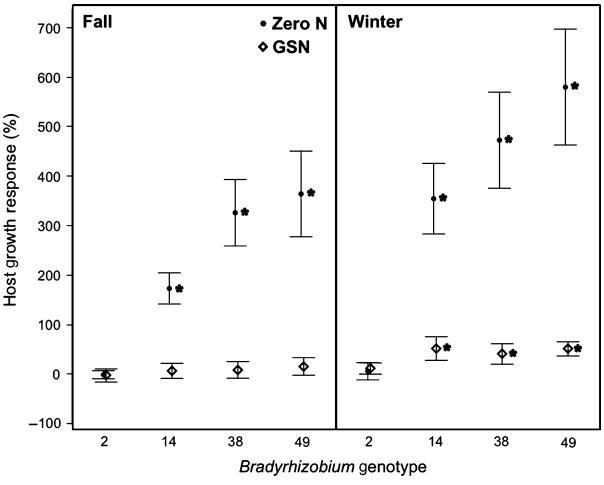

Fig. 1 Mean host growth response to infection (+standard error). Host growth response is the percent growth difference between inoculated plants and control plants [((Mass Inoculated Plant – Mass Control Plant)/Mass of Control Plant)*100]. *Significant difference from zero within each strain treatment in one sample *t*-test ($P < 0.001$). GSN is growth saturating nitrogen.

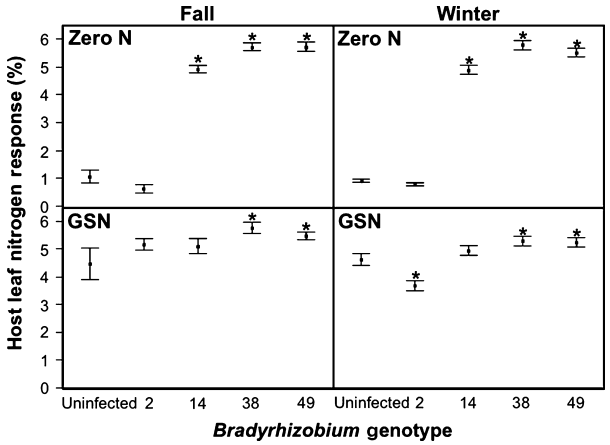

Fig. 2 Host leaf nitrogen response to *Bradyrhizobium* infection (+standard error). *Significant differences between inoculated and control plants (first column) per *t*-test controlling for multiple comparisons ($P < 0.001$).

treatment combinations, although sample sizes were small ($n \leq 9$ per treatment combination; $P > 0.05$). When combining leaf nitrogen data from all effective genotypes, leaf nitrogen was not significantly correlated with host growth response in either nitrogen treatment for both experiments ($P > 0.05$).

Lotus investment in Bradyrhizobium

Plant biomass exhibited a significant positive correlation with nodule number and nodule mass and thus was included as a covariate in GLMs (Table 1). *Bradyrhizobium* genotype had a significant effect on nodule number in both experiments (Table 1), with the ineffective genotype #2 tending to form the most nodules in both experiments (Table 2). Nitrogen fertilization had a significant negative effect on nodule number in Fall but not Winter (Table 1). Among genotypes, nitrogen fertilization tended to decrease nodule number in the Fall, although this was only significant for *Bradyrhizobium* genotype #49 (Table 2). In Winter, nitrogen fertilization increased nodule number significantly for genotype #2 only and had no significant effect for other genotypes (Table 2).

Table 1 Effects of *Bradyrhizobium* genotype and fertilization treatment on host investment in nodule formation and mass.

Experiment	Effect	d.f.	*F* – Nodule number	*F* – Mean individual nodule mass (mg)
Fall	Genotype	3	16.35***	32.39***
	Fertilizer	1	11.99**	70.20***
	Genotype × Fertilizer	3	0.96	2.38
	Plant biomass	1	104.97***	56.44***
Winter	Genotype	3	37.08***	17.78***
	Fertilizer	1	0.98	43.40***
	Genotype × Fertilizer	3	8.40***	3.24*
	Plant biomass	1	158.06***	26.32***

*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

Table 2 Variation in nodule formation and nodule mass. Predicted least squares mean estimates of nodule number and individual nodule mass.

Experiment	Bacteria	Nitrogen	Nodule number	Mean individual nodule mass (mg)
Fall	#2	Zero N	22.72 (1.48)	0.14 (0.02)*
		GSN	19.81 (1.33)	0.01 (0.02)
	#14	Zero N	16.31 (1.38)	0.37 (0.02)*
		GSN	11.55 (1.37)	0.14 (0.02)
	#38	Zero N	19.25 (1.32)	0.31 (0.01)*
		GSN	15.84 (1.37)	0.13 (0.02)
	#49	Zero N	16.52 (1.31)*	0.28 (0.01)*
		GSN	9.62 (1.40)	0.16 (0.02)
Winter	#2	Zero N	32.31 (2.34)*	0.19 (0.04)*
		GSN	45.05 (1.99)	0.05 (0.03)
	#14	Zero N	26.88 (2.06)	0.50 (0.04)*
		GSN	27.83 (2.13)	0.17 (0.04)
	#38	Zero N	23.17 (2.06)	0.51 (0.04)*
		GSN	24.11 (2.06)	0.20 (0.04)
	#49	Zero N	20.20 (1.95)	0.48 (0.03)*
		GSN	13.95 (2.22)	0.22 (0.04)

*Significant differences among nitrogen treatments within bacterial genotype in pairwise *t*-tests corrected for multiple comparisons ($P < 0.05$).

Bradyrhizobium genotype had a significant impact on mean individual nodule mass (nodule mass) with the ineffective genotype, #2, forming smaller nodules than the effective genotypes within nitrogen fertilizer treatment (Table 2). Nitrogen fertilization significantly decreased the least squares means of nodule mass for all genotypes in both experiments (Table 2). When season was included in the model, season had a significant effect on both nodule number ($F1,285 = 56.76$, $P < 0.0001$) and nodule mass ($F1,285 = 29.03$, $P < 0.0001$).

Lotus modulation of investment in *Bradyrhizobium*

Host growth response exhibited a significant positive correlation with nodule mass in both experiments. Host growth response was not correlated with nodule number. Rather, plant size was significantly and positively correlated with nodule number in both experiments (Fig. 3, Table 3).

Discussion

The net fitness effects of microbial symbioses can vary for plant hosts, dependent upon both evolutionary and

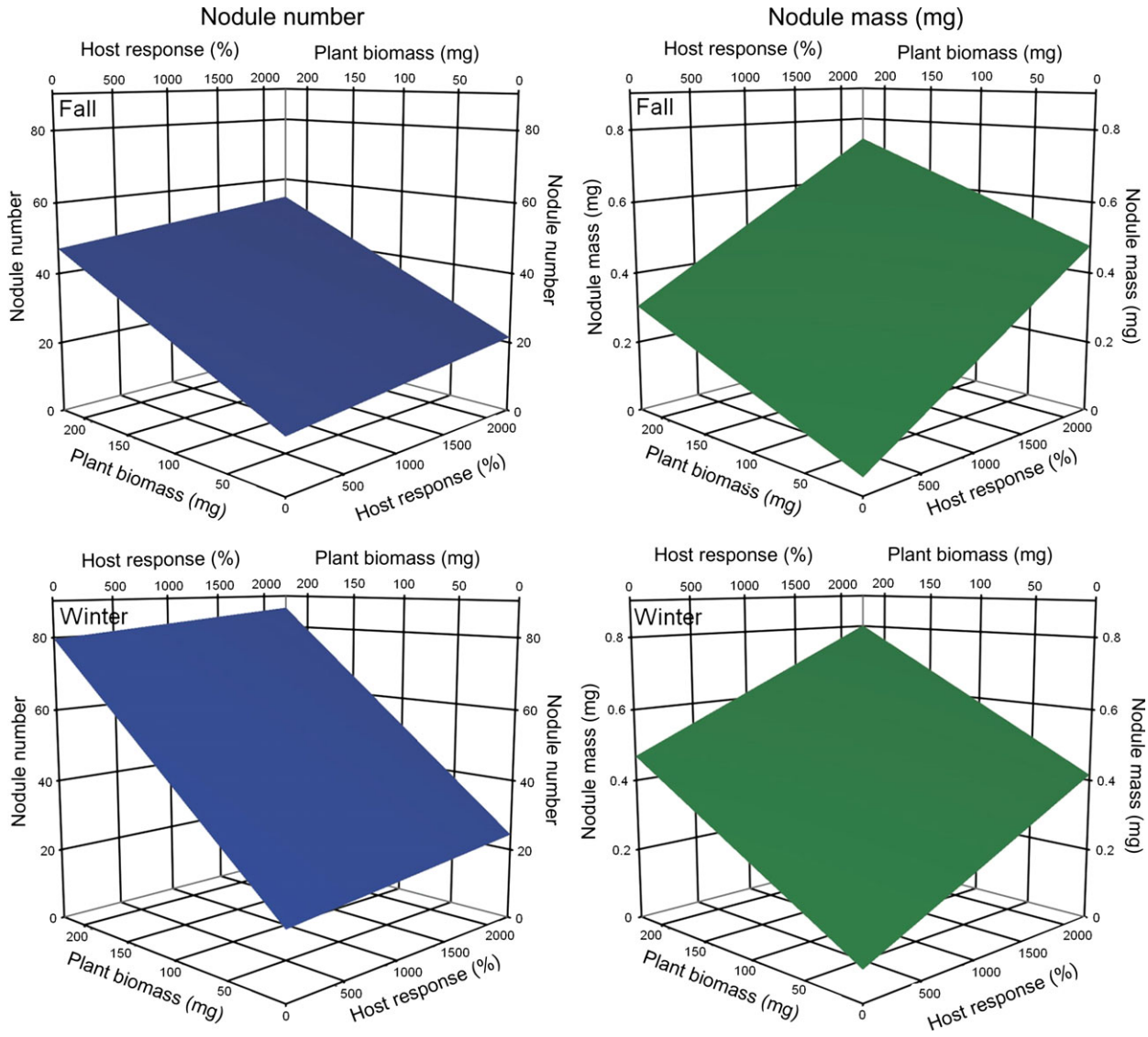

Fig. 3 Response surface plots of nodule number and nodule mass. Nodule number is positively correlated with plant biomass but not host growth response. Nodule mass is positively correlated with host growth response but not plant biomass. See Table 3 for statisitcs.

ecological factors, and these symbioses have been predicted to span a continuum from mutualism to parasitism (Bronstein, 1994; Johnson *et al.*, 1997; Neuhauser & Fargione, 2004). Our work supports the hypothesis of context-dependent fitness benefits for *Bradyrhizobium* infection on *Lotus*. In particular, nitrogen fertilization at a level that optimizes growth of uninfected *Lotus* plants (GSN) eliminated or drastically reduced growth benefits from effective *Bradyrhizobium* genotypes. Yet, no *Bradyrhizobium* infections were parasitic in terms of plant growth under any of the tested environments. This was true even for the ineffective *Bradyrhizobium* genotype that has been previously shown to exploit sympatric *Lotus* hosts, by forming more nodules than effective genotypes and attaining similar or higher per plant population sizes in nodules (Sachs *et al.*, 2010b; Regus *et al.*, 2014). Past work had also failed to find a net cost of infection, even with the ineffective strain (#2), but these previous experiments were conducted in zero nitrogen (Sachs *et al.*, 2010a,b). Under conditions with no soil nitrogen, the benefits of rhizobial infection are amplified, and costs are difficult or impossible to uncover as the uninfected controls are chlorotic and grow very little (and detecting costs means showing that inoculated plants are growing significantly less than the malnourished controls). When *L. strigosus* is fertilized, uninfected plants are robust and large. When ineffective rhizobia, such as strain #2, infect and proliferate within

Table 3 *Lotus* hosts modulate symbiotic investment with symbiotic benefit.

Experiment	Effect	d.f.	F – Nodule number	F – Mean individual nodule mass (mg)
Fall	Host growth response	1	1.31	24.58***
	Genotype	3	16.92***	28.97***
	Fertilizer	1	4.22*	14.81**
	Plant biomass	1	77.28***	23.71***
Winter	Host growth response	1	1.06	9.64**
	Genotype	3	31.52***	13.45***
	Fertilizer	1	1.40	15.93***
	Plant biomass	1	120.71***	16.39***

*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

a host, the rhizobia can sequester resources from the host (Denison, 2000; Denison & Kiers 2004; Lodwig *et al.*, 2003). Thus, it is possible that infection by ineffective rhizobia can be parasitic (reduced growth relative to uninfected plants) because of resource sequestration by rhizobia. But our host fitness data suggest that the symbiosis between *L. strigosus* and sympatric *Bradyrhizobium* can shift ecologically from mutualism to commensalism (i.e. no growth effect of infection), but we did not find evidence of parasitism, even under conditions in which it should have been easily detected.

We uncovered a strong, positive correlation between host growth response (to *Bradyrhizobium* infection) and nodule size, and this pattern occurred irrespective of the host's fertilization status. These data suggest that hosts can finely tune investment in nodules depending on the net growth benefit the host is receiving from those bacteria, and thus can delimit the fitness effects of rhizobial infection. Our data are consistent with host sanctions models (Denison, 2000; West *et al.*, 2002) but are inconsistent with models of automatic feedbacks between mutualist partners (Weyl *et al.*, 2010; Frederickson, 2013), as our fertilized hosts, whose roots are picking up significant mineral nitrogen, were nonetheless able to down-regulate metabolic support of some rhizobia (Fig. 1). Moreover, in both experiments, fertilized hosts formed smaller nodules (Table 2) even though mean plant biomass of fertilized hosts was always more than double of the unfertilized hosts (Table S1). If rhizobia in nodules were benefitting from positive feedbacks due to plant robustness, then nodules should be consistently larger when plants are larger.

Legumes can conceivably control rhizobial symbioses at two key stages of the interaction, by regulating nodule formation and then by mediating nodule metabolism and growth (Streeter, 1988; Parsons *et al.*, 1993). Classically, nodule formation has been shown to be down-regulated in response to soil nitrogen (Streeter, 1988; Bollman & Vessey, 2006). Mineral nitrogen is often cheaper for legumes to acquire relative to symbiotic nitrogen (Voisin *et al.*, 2002), so decreasing nodule formation when mineral nitrogen is abundant might offer hosts a metabolic cost savings. But the relationship between nodule number and host benefit was not significant in our experiments (Table 3), inconsistent with the hypothesis that *Lotus* adaptively modulates nodule number (Fig. 3, Table 3). Other researchers have also failed to find reduced nodulation under nitrogen fertilization (Davidson & Robson, 1986; Heath *et al.*, 2010), suggesting that inhibition of nodule formation is not a universal control mechanism among legumes. Unlike nodule number, we found strong evidence that *Lotus* hosts modulate nodule growth in a context-dependent manner, with plants that received the least net benefit forming minimally sized nodules (Table 3; Fig. 3). Several physiological mechanisms have been proposed for legume control over nodule metabolism and growth. Some legumes exhibit amino acid cycling, wherein host and symbiont depend on each other for certain amino acids, thus potentially enforcing mutualism between the two partners (Lodwig *et al.*, 2003). Another model proposes that legumes can decrease oxygen flux to nodules that contain ineffective rhizobia, thus constraining *in planta* growth of these rhizobia (Sheehy *et al.*, 1983; Kiers *et al.*, 2003). But there is controversy over both these mechanisms, especially when multiple rhizobia co-infect individual nodules (Regus *et al.*, 2014). Our data support the idea that hosts invest in nodule maintenance dependent on the carbon-to-nitrogen ratio within nodules (Puppo *et al.*, 2005), but the cellular and genetic bases of this control remain unknown.

Testing whether plant–microbial symbioses exhibit a mutualism–parasitism continuum requires assessment of the net fitness effects of infection, for instance via comparisons between infected plants and uninfected control plants. Without such controls, it is difficult to distinguish parasitic infections from those that merely provide marginal or zero benefit to hosts. Several studies of legumes have calculated net effects and have also found neutral or beneficial effects of infection, and no evidence of rhizobial parasitism (e.g. Labandera & Vincent, 1975; Bromfield, 1984; Bromfield *et al.*, 1987; Sachs *et al.*, 2010a,b; Regus *et al.*, 2014). But unlike our research, these studies did not alter ecological conditions in a specific attempt to detect parasitism. Lau *et al.* (2012) found mixed evidence of *Bradyrhizobium* parasitism on soya bean, but they only detected net costs in terms of decreased root (but not shoot) mass in response to a shading treatment; hence, that parasitism was dependent on genotype × environment interactions (GxE). Simonsen & Stinchcombe (2014) found evidence of *Ensifer* (*Sinorhizobium*) parasitism on *Medicago lupulina*, but the same strain was found to be slightly beneficial or parasitic depending on the legume species (Bromfield *et al.*, 2010), suggesting genotype × genotype (GxG)

interactions can mediate rhizobial parasitism. Parallel research of mycorrhizal symbioses has uncovered similar patterns. Hetrick *et al.* (1992) found no evidence of mycorrhizal parasitism upon wheat, but other studies on agricultural hosts have found a continuum of response from mutualism to parasitism depending on host and symbiont combinations (GxG; Smith *et al.*, 2003), soil phosphorous levels (GxE; Smith *et al.*, 2004) and plant development (Li *et al.*, 2005). In nonagricultural host plants, Klironomos (2003) found that mycorrhizal taxa were often mutualistic for one host and parasitic for another (GxG). Two recent studies have employed meta-analyses to examine mean and variation in fitness effects of mycorrhizal symbiosis for host plants (Karst *et al.*, 2008; Hoeksema *et al.*, 2010) and both found a great majority of positive and or neutral effects of host inoculation, and net negative effects were rare and much less pronounced in effect size. A similar meta-analysis correlated measures of rhizobial effects on host fitness with data on the fitness of those rhizobia (i.e. nodule number and size; Friesen, 2012). This study examined a variety of ineffective rhizobia but found that rhizobial strains that caused lower host fitness also suffered reduced fitness; hence, that selection favours rhizobia that enhance host fitness in a broad variety of settings (Friesen, 2012). In summary, experiments that found evidence for the mutualism–parasitism continuum depended mostly on interactions between allopatric host–symbiont combinations. The evidence of parasitism in these various studies indicated that parameters exist in which symbionts can cause negative effects. But the ecological relevance of some of these treatments is difficult to assess, especially cross-inoculations of microbes from distant sites and inoculation at agricultural concentrations of fertilizer that rarely exist in natural soils (Karst *et al.*, 2008). We conclude that the mutualism–parasitism hypothesis must be considered with caution and is highly dependent on the ecological context.

Rapid ecological and evolutionary changes continue to amplify the relevance of context dependence in symbiotic interactions (Six, 2009). Here, we tested outcomes of symbiosis among sympatric host and symbiont genotypes in nutrient and light regimes that bracket wild populations. Anthropogenic inputs of nitrogen into ecosystems have dramatically increased in the past 150 years driven by combustion of fossil fuels (Vitousek *et al.*, 1997; Tilman, 1999; Dentener *et al.*, 2006). Our work suggests that nitrogen deposition could alter legume–rhizobium symbiosis by reducing or eliminating the benefit of rhizobial infections for host legumes. But our work does not address potential evolutionary consequences of nitrogen enrichment for legume–rhizobium symbiosis. Future work must address how environmental changes such as nitrogen deposition can potentially reshape the coevolutionary trajectories of legume–rhizobium symbiosis.

Acknowledgments

We acknowledge the help of numerous undergraduates for help with harvest. We thank the U.C. Natural Reserve System and Bodega Marine Reserve in particular. This research was supported by NSF DEB-1150278 to JLS and Mildred E. Mathias Graduate Student Research Award to JUR. The authors declare no conflict of interest in this work.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Shoot mass growth response from infection (+standard error).

Received 11 May 2014; revised 15 October 2014; accepted 22 December 2014