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Effects of Mineral Nitrogen on Host Control in Legume-Rhizobium Symbiosis

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by

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DEDICATION

I dedicate this dissertation to my wife, Heather. Without her love and support this would not have been possible. Whatever good I am is because of you.

ABSTRACT OF THE DISSERTATION

Effects of Mineral Nitrogen on Host Control in
Legume-Rhizobium Symbiosis

by

John Ulrich Regus

Doctor of Philosophy, Graduate Program in Evolution, Ecology and Organismal Biology
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Dr. Joel L. Sachs, Chairperson

Legume-rhizobium symbiosis has become a model system for studying beneficial symbiosis between eukaryotic hosts and bacterial symbionts. Hosts are predicted to exert control over beneficial bacterial symbionts to prevent the evolution and spread of exploitative genotypes. Since nitrogen is exchanged in legume-rhizobium symbiosis and legumes can acquire nitrogen from soils, there can be context dependent effects from nitrogen-enriched soils that can alter outcomes of legume-rhizobium symbiosis. Anthropogenic nitrogen deposition has been enriching terrestrial ecosystems for more than a century and is accelerating in some regions. One prediction is that nitrogen enrichment can relax selection to maintain host control traits, leading to evolution and spread of exploitative rhizobia and/or an breakdown in symbiosis if legumes cease to gain benefit from rhizobial infection.

Lotus strigosus is an annual legume that experiences variable nitrogen deposition and mineral nitrogen contexts across California. To examine the potential for adaptation

to mineral nitrogen enrichment in *L. strigosus,* inbred lines of *L. strigosus* from populations that have experienced either little nitrogen deposition or more than seven decades of intense nitrogen deposition were exposed to a simulated nitrogen deposition gradient and *Bradyrhizobium* that vary in growth benefit provided to *L. strigosus*. To examine the effects of mineral nitrogen saturation on a host control traits in a legume that has historically experienced nitrogen-poor soils, *L. strigosus* from a pristine site were exposed to growth saturation mineral nitrogen and infected with combinations of *Bradyrhizobium* strains that vary in benefit to *L. strigosus* and also with individual strains.

Lotus exhibited little evidence of adaptation to use mineral nitrogen more efficiently in response to simulated nitrogen deposition. Symbiosis with *Bradyrhizobium* was reduced at very high fertilizer levels, but nodule formation was not reduced when *Lotus* gained no benefits from infection.

Host control traits in *L. strigosus,* from a pristine site, were resilient to growth saturating nitrogen, contrary to expectations if such traits are costly. When inoculated with single strains of *Bradyrhizobium* in different mineral nitrogen contexts, *L. strigosus* exhibited fine tuned investment in *Bradyrhizobium* to prevent exploitation.

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General Introduction

Eukaryotes ubiquitously gain diverse benefits from mutualistic symbioses with environmental bacteria (Douglas 2010). However, such cooperative interspecific interactions are not free of conflict because fitness interests of the partners can diverge (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1999; Herre et al. 1999). Moreover, bacteria have an evolutionary advantage over hosts in terms of generation time and population size (Sachs et al. 2004). Symbiont populations can potentially generate mutants that exploit host resources but provide little or no benefit to the host in return (Herre et al. 1999; Simms & Taylor 2002; Sachs et al. 2004). Theory predicts that hosts must exhibit host control traits to prevent the evolution and spread of exploitative symbionts (Bull & Rice 1991; Denison 2000).

A dominant paradigm in eukaryote-bacterial symbiosis is that symbiotic interactions span a continuum for hosts from mutualistic (positive fitness benefits for both partners) to parasitic (fitness costs for hosts; Bronstein 1994; Johnson et al. 1997; Neuhauser & Fargione 2004). Fitness benefits that hosts receive can often be context dependent; varying with extrinsic environment, combinations of host and symbiont genotype and interactions among these factors (Bronstein et al. 1994, 2001). Against the backdrop of climate change and massive anthropogenic alterations of biologically relevant global nutrient cycles (e.g. nitrogen and carbon), it is critical to understand how host control over bacterial symbionts operate in variable environmental contexts. Yet, there is a paucity of work examining context dependence of host control in symbiosis.

The interaction between legume plants and soil bacteria, called rhizobia, has become a model system for studying beneficial symbiosis and host control (Denison 2000). Legumes become infected by rhizobia soon after germination. The host then forms a root tumor, called a nodule, where rhizobia reside. Inside the nodule, rhizobia fix atmospheric dinitrogen into forms usable by the host and receive host-fixed carbon from the plant. Rhizobia vary naturally in the benefit provided to hosts (Burdon et al. 1999; Simms et al. 2006; Sachs et al. 2010a) and the benefits hosts receive from rhizobia can depend on the extrinsic environment (Lau et al. 2012; Regus et al. 2014; Simonsen & Stinchcombe 2014), host/symbiont genotype combination (Burdon et al. 1999) and genotype x environment interactions (Heath et al. 2007). Legume hosts have been shown to exhibit host control traits that select for beneficial rhizobia in favor of less beneficial, exploitative rhizobia (Kiers et al. 2003, 2006; Simms et al. 2006; Sachs et al. 2010a) and theory suggests such traits can prevent the evolution and spread of exploitative rhizobia (Simms & Taylor 2002; West et al. 2002a,b; Sachs et al. 2004). Yet, most studies that find evidence for host control in legumes have used zero mineral nitrogen contexts, which are biologically unrealistic.

Nitrogen is the most abundant element in the atmosphere (~78%) in the form of diatomic N_2 , but N_2 is inert. Nitrogen is fixed into biologically active forms by several processes, including lightning and microbial fixation. Fixed nitrogen is cycled through terrestrial and aquatic ecosystems in various reactive nitrogen forms (e.g. NH_3, NH_4^+, NO_x, urea, amines, proteins, etc.) Before human agriculture and industrial activity, legume-rhizobium nitrogen fixation accounted for essentially all terrestrial biological

nitrogen fixation (BNF; Cleveland et al. 1999; Galloway et al. 2004). Human activity has more than doubled production of reactive nitrogen compounds and more than half of anthropogenic reactive nitrogen is emitted into the atmosphere (Galloway et al. 2004, 2008). Atmospheric reactive nitrogen is most often subsequently deposited into aquatic and terrestrial ecosystems (Vitousek et al. 1997). Such nitrogen deposition has been intense and global for more than a century and most deposition falls on natural (non-agricultural) ecosystems (Dentener et al. 2006; Holtgrieve et al. 2011). Natural ecosystems are often nitrogen-limited (Vitousek et al. 1997) and nitrogen deposition enriches soils (Padgett et al. 1999; Egerton-Warburton et al. 2001). Since nitrogen is exchanged in legume-rhizobium symbiosis, mineral nitrogen availability can potentially alter the balance of trade between legumes and rhizobial partners (Kiers et al. 2007, 2010).

Mineral nitrogen is relatively less costly for hosts to acquire relative to bacterial-fixed nitrogen because of carbon costs to support rhizobia and associated plant tissue (Silsbury 1977; Voisin et al. 2002). If hosts can switch to mineral nitrogen when available, selection to maintain host control traits can be relaxed (Kiers et al. 2010) or hosts can potentially abandon symbiosis entirely if rhizobia cease to be beneficial (Sachs & Simms 2006; Kiers et al. 2010). Spread of exploitative rhizobia or abandonment of symbiosis by legumes can potentially have dramatic consequences for global nitrogen cycles decreasing or eliminating a major terrestrial source of BNF. Moreover, characterizing contexts in which symbiosis can lead to the evolution and spread of

exploitative symbionts or abandonment of symbiotic interactions is important for a broader understanding of context dependence in symbiosis.

I sought to examine how variable extrinsic nitrogen contexts can affect host control of *Bradyrhizobium* in an annual California legume, *Lotus strigosus*. California experiences variable nitrogen deposition with intense deposition in some regions that overlap with the native range of *L. strigosus*. In the first chapter, I exposed four inbred lines of *L. strigosus* from two different locations, one a pristine site and the other a high deposition site, to an experimental nitrogen deposition gradient designed to mimic and exceed mineral nitrogen concentrations at host sites. Hosts were inoculated with one of two strains of *Bradyrhizobium* that vary greatly in growth benefits to *L. strigosus*, to span natural variation of rhizobial infection (Burdon et al. 1999; Simms et al. 2006; Sachs et al. 2010a). Uninoculated *L. strigosus* were used to assess the response of uninfected *L. strigosus* to nitrogen deposition. The goal of the experiment was to assess if *L. strigosus* from a site that has experienced long-term nitrogen deposition exhibit evidence of adaptation to elevated mineral nitrogen in terms of growth, mortality or ability to control or halt nodulation of *Bradyrhizobium*.

In the second chapter, I exposed *L. strigosus*, from a site historically depauperate for mineral nitrogen, to a fertilizer concentration that maximize uninfected plant growth, and coinoculated plants with pairs of *Bradyrhizobium* that varied in the benefit provided to the host. The goal of the experiment was to assess host control traits in *L. strigosus*, from a low mineral nitrogen site, over *Bradyrhizobium* when mineral nitrogen is abundant for the host. I examined two key host control traits, i) partner choice and ii)

sanctions. Partner choice is the ability of a host to restrict infection of particular strains when hosts are coinoculated with multiple strains and is measured as the relative presence or absence of strains in nodules irrespective of bacterial population size. Sanctions is the ability of a host to control proliferation of exploitative strains relative to beneficial strains in coinoculations in terms of per plant bacterial population size (Bull & Rice 1999; Denison 2000; Simms & Taylor 2002). I predicted that *L. strigosus* would potentially relax host control traits in response to growth maximizing mineral nitrogen if rhizobial infection ceases to provide benefit, especially if these traits are costly to express.

In the third chapter, I exposed *L. strigosus* to growth maximizing mineral nitrogen and inoculated hosts with individual strains of sympatric *Bradyrhizobium* that vary in growth benefit provided to this host. The goal of the experiment was to assess the potential for a mutualism-parasitism continuum in this symbiosis and the ability of hosts to modulate investment in rhizobial infection in a fitness-optimizing manner. If hosts continue to allow infection in mineral nitrogen contexts that eliminate the benefit of infection and hosting rhizobia is costly, then infected hosts can potentially experience growth depression (parasitism) relative to uninoculated hosts. Alternatively, hosts can modulate investment in rhizobia in a context dependent manner to prevent parasitism and optimize plant fitness.

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Chapter 1

An experimental nitrogen deposition gradient increases mortality and eliminates symbiosis in *Lotus*

Abstract

Over the last 150 years anthropogenic nitrogen fixation has dramatically altered the global nitrogen cycle. Soils enriched for nitrogen provide legumes with an alternative cheaper source of nitrogen relative to biological nitrogen fixation. We exposed the native annual, *Lotus strigosus*, to a simulated nitrogen deposition gradient designed to mimic and exceed current anthropogenic deposition patterns observed in the plant's natural range. We tested four inbred lines of *L. strigosus*, two from a low nitrogen site and two from a high nitrogen site that has experienced > 70 years of intense nitrogen deposition. Plants were grown axenically, or were inoculated with one of two root-nodulating rhizobial strains that span natural variation in nitrogen fixation. Comparing inbred lines among sites, we found little evidence of recent adaptation to nitrogen deposition in terms of plant growth, but did find varied susceptibility to mortality in the low nitrogen population. Growth benefits from rhizobial infection were eliminated by modest levels of mineral nitrogen, and all *Lotus* lines uniformly failed to form root nodules at high nitrogen concentrations. Failure to nodulate appeared to be a response to nitrogen toxicity rather than loss of symbiotic benefit. Our experiments suggest that current levels of nitrogen deposition are negating the benefits of *Lotus*-rhizobial symbiosis throughout much of Southern California. If nitrogen deposition increases, total collapse of the symbiosis could result.

Introduction

Prior to industrialization, biological nitrogen fixation (BNF) from legume-rhizobium symbiosis dominated natural inputs of nitrogen into terrestrial ecosystems (Cleveland et al. 1999). In this symbiosis, rhizobia form nodules on the roots of legume hosts and fix diatomic nitrogen (N_2) into reactive nitrogen forms that are biologically and chemically active (N_r; e.g., NH_3, NH_4^+, N_2O, NO_x; Galloway et al. 2013). Human industrial activity in the past 150 years has more than doubled N_r production globally and the rate of anthropogenic N_r production is accelerating (Vitousek et al. 1997; Cleveland 1999; Galloway et al. 2004, 2008). Most anthropogenic N_r is emitted into the atmosphere as gaseous NO_x and NH_3 (Galloway et al. 2004) that are eventually deposited into aquatic and terrestrial ecosystems (Vitousek et al. 1997). Such 'nitrogen deposition' has begun to have global impacts with the spread of industrialization over the last century (Galloway et al. 2004; Dentener et al. 2006; Holtgrieve et al. 2011).

Most atmospheric N_r deposition on terrestrial ecosystems occurs on non-agricultural vegetation (50-80%; Dentener et al. 2006) leading to enrichment of historically nitrogen-limited soils (Vitousek et al. 1997; Padgett et al. 1999; Egerton-Warburton et al. 2001). N_r fertilization of soils can reduce plant species richness (Carroll et al. 2003; Roem et al. 2002; Clark & Tilman 2008; Maskell et al. 2006, 2010) by altering outcomes of competitive interactions among plants, and by making the environment unfavorable for a subset of sensitive species (Bobbink et al. 2010). N_r fertilization can also change the composition of soil fungal communities (Egerton-Warburton et al. 2001) and decrease bacterial populations that are critical for litter

decomposition (Janssens et al. 2010; Hobbie et al. 2012; Kamble et al. 2013). Finally, N_r deposition can negate beneficial plant-microbe species interactions in which root-associated bacteria and fungi provide N_r to plants in exchange for photosynthates. In N_r-enriched soils, mycorrhizal symbionts can become superfluous to host plants, providing little or no benefit to the infected hosts (Johnson et al. 1997; Hoeksema et al. 2010; Kivlin et al. 2013). Longitudinal analyses suggest that long term N_r deposition can promote shifts in mycorrhizal communities toward fungal species that are less beneficial for plant hosts (Egerton-Warburton et al. 2001). In contrast to mycorrhizal symbionts, relatively little work has examined consequences of N_r deposition for rhizobial symbiosis despite the central role of rhizobia in global terrestrial BNF.

Legumes initiate symbiosis with rhizobia soon after seed germination, whence the bacteria enter host roots, invade individual host cells, and begin to fix atmospheric diatomic nitrogen for the host, in exchange for host-fixed carbon (Denison 2000). Legumes can also acquire mineral nitrogen from soils, which can be less costly relative to BNF because of carbon costs for supporting rhizobia (Voisin et al. 2002). Increased soil N_r, as a result of anthropogenic N_r deposition, can alter the balance of trade between the symbiotic partners (Kiers et al. 2010).

Few predictions exist about how legumes will respond to long term N_r deposition. In terms of plant growth, we predict that legumes will evolve to use N_r more efficiently thus increasing growth rates under increased soil N_r concentrations. Similarly, we predict that legumes will evolve to tolerate increased N_r concentrations in terms of growth or mortality. Legume hosts must also exhibit host control traits to prevent infection and

spread of exploitative rhizobia (e.g. host sanctions; Denison 2000). Theory generates two alternate hypotheses about the evolution of legume control under N_r deposition. Firstly, if legumes gain little or no benefit from symbiosis in N_r enriched soils, selection to maintain sanctions traits can be relaxed, potentially leading to spread of less beneficial or even parasitic rhizobia (Kiers et al. 2007, 2010). Second, legumes can evolve to lose their ability to form nodules (Sachs & Simms 2006; Kiers et al. 2010). Relaxation of control mechanisms by legumes or wholesale loss of nodulation in response to anthropogenic N_r deposition could reduce or remove a major global contributor to N_r cycling (Galloway et al. 2008).

Here, we exposed *Lotus strigosus*, an annual legume, to an experimental gradient of mineral N_r concentrations. We tested inbred plant lines sourced from two populations; two lines from a site with negligible deposition, and two from a site that has experienced high N_r levels over the last century (Fenn et al. 2010). Experimental N_r fertilizer concentrations spanned and exceeded variation in soil nitrogen levels across the natural range of *L. strigosus* in California (Regus et al. 2014), ranging from N_r poor sites on the coasts and high deserts to regions with intense N_r deposition in the Los Angeles and Santa Ana River basins (Fenn et al. 2010). Plants were either grown axenically (without rhizobial infection) or were exposed to one of two single-strain rhizobial inoculation treatments. One effective *Bradyrhizobium* strain provides *L. strigosus* with significant benefit (~5x improved growth over controls; Sachs et al. 2010a) and the other strain is ineffective, providing zero benefit, thus bracketing the natural variation of rhizobial symbiotic quality (Burdon et al. 1999; Simms et al. 2006; Sachs et al. 2010a). Uninfected

plants were used to assess baseline response of *L. strigosus* to N_r deposition and also to characterize the relative benefits or costs of rhizobial infection for the host. We examined host responses including i) nodulation status, ii) host mortality and iii) differential investment in beneficial versus ineffective nodules. We tested how these traits varied in response to simulated N_r deposition and examined whether the response depended on the plant's past history of N_r deposition.

Materials and Methods

Plant population selection and preparation

Fruits of *L. strigosus* were collected from Bodega Marine Reserve (BMR) and University of California, Riverside (UCR). BMR has negligible N_r deposition (e.g., < 5 Kg N_r ha^{-1} yr^{-1}; Fenn et al. 2010), but the UCR site has experienced intense nitrogen deposition for more than 70 years (e.g., > 20 Kg N_r ha^{-1} yr^{-1}; Fenn et al. 2010) and exhibits 7x more total soil Nr and 5x more mineral N_r (NO_3 + NH_4) than BMR soils (Regus et al. 2014). Seeds were collected from parent plants at BMR in June 2005 (BMR05) and 2007 (BMR07) in a 25 m radius from sympatric sites as the two tested *Bradyrhizobium* strains (Sachs et al. 2009), and from UCR in April 2008 (UCR08) and April 2009 (UCR09) from parent plants at sites separated by 20 m. To generate inbred seed sets for the experiment, ~10 wild plants from each seed collection above (BMR05, BMR07, UCR08, UCR09) were grown in one gallon pots in sterile soil (UC Mix iii) from January to June, 2011. Plants were only allowed to self-pollinate (greenhouses were sprayed weekly with the insecticide Mavrik). Fruits were harvested as they matured over

several months from ~ 6 inbred plants per collection that had generated > 1500 seeds. We refer to these four seed sets as inbred lines (BMR05, BMR07, UCR08, UCR09).

Experimental Bradyrhizobium

Two genetically diverged *Bradyrhizobium* strains, referred to as #'s 2 and 49, were collected from *L. strigosus* at BMR and characterized in genotypic and phenotypic analyses (Sachs et al. 2009, 2010a,b; 2011). Strain #49 is highly effective on *L. strigosus* from BMR, providing ~500% increase in *L. strigosus* shoot biomass when hosts are grown in soil without soil nitrogen, and #2 is ineffective, not significantly affecting shoot biomass (Sachs et al. 2010a). Both strains readily infect this host in single strain inoculations, attain high population density within nodules in the absence of mineral nitrogen (Sachs et al. 2010a) and also when mineral nitrogen is present (Regus et al. 2014a). Inocula were generated from frozen stocks using published protocols (Sachs et al. 2009).

Inoculation experiment

Seedlings were prepared axenically per the methods of Sachs and colleagues (2009) and moved to the greenhouse one week prior to inoculation. After four days in the greenhouse, plants were fertilized with 10.0 mL nitrogen-free Jensen's solution with dissolved KNO_3 for nitrogen treatments 0.0 g L^{-1} to 5.00 g L^{-1}. Three days after initial fertilization, plants were inoculated with a 5.0 mL mixture of one strain or the other at a concentration of 1.0 x 10^8 cells mL^{-1} (Sachs et al. 2009) or uninoculated control plants

were given 5.0 mL of sterile ddH_2O. Four days after inoculation treatment, plants were fertilized per treatment as above and then once a week until harvest. At harvest, plants were carefully depotted, all 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.

The experiment ran for eight weeks, from inoculation treatment to harvest (03.12.12 - 05.07.12). For each inbred line, 126 size-matched sterile-grown seedlings were randomly assigned to treatments groups. Fertilizer treatments consisted of 0.00, 0.25, 0.50, 1.00, 3.00 and 5.00 g L^{-1} KNO_3. This range of concentrations exceeds the nitrogen deposition gradient that occurs between the two sites and soil N_r concentrations observed at the two sites. Previous work showed that BMR soils exhibit extremely low N concentrations (0.01% total N, ~ 4.00 ppm mineral N) and UCR exhibits total N concentrations comparable to tilled agricultural soils (0.1% total N, ~20.00 ppm mineral N; Bremner 1965; Regus et al. 2014). For comparison, the third fertilizer concentration (0.50 g L^{-1} KNO_3) in this experiment provides plants with approximately 15 ppm NO_3 per weekly fertilization or 75% of mineral nitrogen content at UCR. We used KNO_3 because plants most readily take up NO_3 in nature and soil processes convert most mineral nitrogen to NO_3 (Streeter 1988). The experiment included 504 plants in total (7 replicate plants per treatment, 4 inbred lines, 3 inoculation treatments, 6 N_r treatments).

Data analysis

Host growth response to infection was calculated as the percent difference in dry shoot biomass between inoculated plants and size-matched un-inoculated control plants (Sachs et al. 2010a). Net benefit or cost of infection was characterized by positive or negative growth response compared to size matched uninfected control plants. We tested whether growth response differed significantly from zero using a one-sample t-test (JMP 10.0; SAS Institute Inc. 2012). Differences in host growth response, nodule number and shoot weight of uninfected plants among inbred lines or fertilizer treatments were assessed with ANOVA in JMP 10.0 using pairwise analyses correcting for multiple comparisons using Tukey's Honestly Significant Distance test (HSD). Mortality was analyzed using multiple logistic regression in the Fit Model Platform (JMP 10.0; SAS Institute Inc. 2012).

Results

Response of uninoculated Lotus strigosus to N_r gradient

Shoot mass increased over the span of the four lower fertilizer concentrations (0.00- 1.00g L^{-1} KNO_3) and then decreased in the highest concentrations (3.00- 5.00 g L^{-1} KNO_3; Fig 1.1). Shoot mass was largest at 1.0 g L^{-1} for BMR07 and UCR08 ($p < 0.05$ for all pairwise comparisons). Shoot mass was largest for BMR05 at the same concentrations, but not significantly larger than 0.5 g L^{-1} ($p > 0.05$). For UCR09, 5.0 g L^{-1} yielded a slightly larger mean (166 mg) than 1.0 g L^{-1} (160 mg) but the difference was

not significant ($p > 0.05$). Shoot mass was never significantly different among the different inbred lines within any individual nitrogen treatment.

Multiple logistic regression of mortality with fertilizer treatment and inbred line as the main effects revealed significant effects of both (fertilizer $p < 0.0001$, inbred line $p < 0.001$). Mortality of uninoculated plants increased in the two highest fertilizer levels for all inbred lines, though BMR07 had only plant die in the highest fertilizer treatment (Table 1.1). Mortality was greater for BMR05 compared to other inbred lines (Table 1.1).

Lotus strigosus nodulation status and nodule growth

Lotus strigosus formed more nodules with the effective strain #49, but the nodulation patterns of each strain were roughly parallel across the fertilization gradient (Fig. 1.2). The number of nodules per plant increased with increasing fertilizer concentration for the lowest three fertilizer treatments (0.00- 0.50g L^{-1} KNO_3), for both *Bradyrhizobium* strains. Nodules per plant decreased in the fourth concentration (1.00g L^{-1} KNO_3) and nodulation was nearly or completely eliminated in the highest two concentrations (3.00, 5.00 g L^{-1} KNO_3; Fig. 1.2). When infected with the ineffective strain #2, *L. strigosus* nearly eliminated nodulation in 3.0 g L^{-1} KNO_3; 25 of 28 plants survived across all inbred lines but only 4 plants had any nodules, and none of those plants had more than 3 nodules (Table 1.1). In the highest fertilizer concentration, when inoculated with with strain #2, 22 of 28 plants survived across all inbred lines but none had any nodules. *L. strigosus* similarly prevented nodulation with strain #49 in the highest two fertilizer concentrations, though in the highest fertilizer concentration only 6

of 13 surviving plants formed nodules and never more than 4 nodules per plant (Fig. 1.2; Table 1.1).

It was not possible to make statistical comparisons for nodule number for the two highest fertilizer treatments because many plants did not form nodules or died (See mortality analysis below; Table 1.1). A GLM analysis of nodule number was performed including the four lowest fertilizer concentrations. Main effects were inoculation treatment, fertilizer concentration and inbred line. Inoculation treatment ($F_{1,193} = 25.79$, $p < 0.0001$) and fertilizer ($F_{3,191} = 16.68$, $p < 0.0001$) were significant effects on nodule number but inbred line was not significant.

In terms of nodule growth, *L. strigosus* formed larger nodules, by mean individual nodule mass, with the effective strain #49 (Fig. 1.3). Within strain, nodule size was not significatnly different for the first three fertilizer concentrations but *L. strigosus* formed smaller nodules with strain #49 in the fourth concentration. Including the four lowest nitrogen concentrations, a GLM of mean individual nodule mass, where inoculation treatment, fertilizer concentration and inbred line were main effects found significant effects of inoculation treatment ($F_{1,192} = 71.82$, $p < 0.0001$) and fertilizer ($F_{3,190} = 2.72$, $p < 0.05$) but not inbred line.

Growth benefits for Lotus strigosus of Bradyrhizobium infection

All inbred lines gained significant benefit from infection with the effective strain #49 in zero fertilizer ($p < 0.05$ for all inbred lines; Fig. 1.4). Growth benefit from infection with strain #49 was eliminated by nitrogen fertilization in most cases (except

for three treatment combinations; BMR05, 07 x 0.25 g L^{-1} and UCR09 x 1.0 g L^{-1}). No inbred line gained significant benefit from infection with the ineffective strain #2 in any fertilizer concentration (Fig. 1.4). Neither BMR inbred line exhibited significantly negative growth responses from infection by either *Bradyrhizobium* strain in any fertilizer treatment. Strain #49 caused a significantly negative growth response for both UCR inbred lines only in the highest fertilizer treatment. Strain #2 caused significantly negative growth response for both UCR inbred lines in zero fertilizer and for UCR08 in 0.25 and 1.0 g L^{-1} fertilizer (Fig. 1.4).

Mortality rates analysis for inoculated Lotus strigosus

Similar to axenic *L. strigosus*, mortality was negligible in the lowest four fertilizer concentrations (0.00- 1.00 L^{-1} KNO_3), but increased in the highest two fertilizer concentrations (3.00- 5.00 g L^{-1} KNO_3; Table 1.1). One inbred line had no mortality (UCR09 strain #2). Similar to axenic plants, BMR05 tended to have greater mortality than other lines regardless of inoculation treatment. Inoculation treatment had little effect on mortality except for BMR05 strain #49 and UCR09 #49. In the highest two concentrations (3.00- 5.00 g L^{-1} KNO_3), plants usually survived inoculation but many did not form nodules (Table 1.1).

In multiple logistic regression of mortality with inoculation treatment (including axenic plants), fertilizer concentration and inbred line were main effects, both fertilizer ($p < 0.0001$) and inbred line ($p < 0.0001$) were significant, while inoculation treatment was not significant ($p > 0.15$). We then performed multiple logistic regression of mortality

within each inoculation treatment separately, with fertilizer and inbred line as main effects. Both effects were significant for each *Bradyrhizobium* strain (strain #2 fertilizer $p < 0.01$, inbred line $p < 0.001$; strain #49 fertilizer $p < 0.0001$, inbred line $p < 0.001$).

Discussion

Over the past century anthropogenic activity has more than doubled global N_r output (Galloway et al. 2004) leading to intense N_r deposition in natural ecosystems (Dentener et al. 2006; Holtgrieve et al. 2011). N_r deposition has enriched soils that were historically nitrogen-limited, potentially saturating plants with mineral nitrogen (Dentener et al. 2006; Vitousek et al. 1997). In southern California, deposition has been intense for more than 70 years (Fenn et al. 2010) and some soils have been greatly enriched for N_r over that time (Egerton-Warburton et al. 2001). Nonetheless, *L. strigosus* plants from southern California (two UCR lines) did not exhibit evidence of adaptation in terms of more efficient use of mineral nitrogen for growth. Shoot growth universally increased for all inbred lines up through N_r concentrations currently experienced by UCR plant populations (i.e. 1.0 g L^{-1}; all inbred lines, axenic and inoculated) and then decreased in N_r concentrations much greater than observed at UCR (3.0 g L^{-1}, 5.0 g L^{-1}), consistent with toxicity. One inbred line from a region with low soil nitrogen (BMR05) had significantly greater mortality than other lines, and had more dead plants in the highest two fertilizer concentrations for both axenic plants and inoculated plants (Table 1.1). Since inbred lines were raised in greenhouse conditions, it is unlikely that seed quality or other maternal effects can explain the mortality response in BMR05. But it is

important to acknowledge the limited scope for interpreting differences in mortality response because we tested only two *L. strigosus* populations and results were not consistent among inbred lines at the low N_r site (BMR05 vs. BMR07). However, our results suggest the potential for within site genetic variation in the sensitivity of *L. strigosus* to high N_r concentrations.

Our experimental N_r deposition gradient significantly reduced or eliminated the growth benefit of rhizobial infection at the lowest fertilization concentration for all inbred lines but only caused significantly decreased growth in the UCR inbred lines. Infection with the beneficial strain #49 caused significant growth decreases in three instances for UCR08 in 1.0 g L^{-1} and for both UCR lines at 5.0 g L^{-1}, suggesting the possibility of costs associated with hosting rhizobia when environmental N_r is abundant. Infection with the ineffective strain #2 caused a growth decrease only for UCR at the two ends of the simulated deposition gradient (Fig. 1.4) suggesting that mineral nitrogen availability can reduce the impacts of exploitative rhizobia in low N_r contexts. Since all negative growth responses were observed in UCR inbred lines and both *Bradyrhizobium* used in this study were isolated from *L. strigosus* at BMR (Sachs et al. 2009), it is likely negative growth responses were caused by G x G interactions between plant host and allopatric rhizobia. Such G x G effects can cause up to 90% differences in growth effects of rhizobia, when tested on related legume species (Burdon et al. 1999). It is worth noting that in the highest fertilizer concentration, both UCR lines experienced negative growth effects from inoculation with strain #2 but did not form any nodules, suggesting that halting nodulation is not without systemic cost for legume hosts. Heil and colleagues (2000)

found induced systemic resistance to pathogens was costly in terms of growth and seed production for corn.

Populations of *L. strigosus* in southern California have experienced long term N_r deposition (Fenn et al. 2010). Several sites exhibit mineral N_r concentrations (Regus et al. 2014) comparable to the middle concentrations used in this study at which *L. strigosus* gained no benefit from *Bradyrhizobium* infection. Our results suggest that many *L. strigosus* populations across Southern California are gaining little or no benefit from *Bradyrhizobium* symbiosis. Nonetheless, mature *L. strigosus* are always nodulated in the field sites surveyed for this study. More work is needed to characterize the benefits that sympatric rhizobia provide to *L. strigosus* and other legumes at N_r polluted sites.

We experimentally assessed mineral N_r concentrations past those observed for *L. strigosus* test sites to model predicted increases in the intensity of N_r deposition (Galloway et al. 2008). Some regions, particularly in China, can experience nitrogen deposition rates more than 5x that of California (Fenn et al. 2010; Ti et al. 2012; Tu et al. 2014). For comparison, the middle two of our six N_r treatments bracketed concentrations observed at the high deposition *L. strigosus* site in this study and the highest fertilizer treatment represented approximately 6x times observed concentrations. The UCR site has experienced high N_r deposition for more than 70 years (Fenn et al. 2010). Yet, we found little evidence of differential adaptation to high soil N_r by *L. strigosus* from UCR compared to the BMR population, a site with historically little N_r deposition and significantly lower mineral N_r concentrations. If annual plants are slow to evolve in response to elevated mineral N_r driven by anthropogenic deposition then it is unlikely

that longer-lived perennials will fare better, especially in the face of rapid and intense N_r deposition. If global N_r deposition continues to increase, as predicted (Galloway et al. 2008), then it will become increasingly important to characterize the potential effects of extreme nitrogen enrichment on natural biological nitrogen fixation if we are to understand and properly assess global and regional N_r cycles and budgets.

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Table 1.1. Plant mortality and nodulation status. Seven total replicates per treatment combination. 'Control' plants are un-inoculated. 'Nodules' columns show the number of plants that formed nodules irrespective of the number of nodules. 'Survive' column shows the number of plants that were alive at the end of the experiment.

		0 g L^{-1} KNO_3		0.25 g L^{-1} KNO_3		0.5 g L^{-1} KNO_3		1.0 g L^{-1} KNO_3		3.0 g L^{-1} KNO_3		5.0 g L^{-1} KNO_3	
		Nodules	Survive	Nodules	Survive	Nodules	Survive	Nodules	Survive	Nodules	Survive	Nodules	Survive
Control	BMR05	n.a.	6	n.a.	6	n.a.	6	n.a.	5	n.a.	4	n.a.	3
	BMR07	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	6
	UCR08	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	6	n.a.	5
	UCR09	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	6	n.a.	4
Strain 2	BMR05	6	6	5	6	5	6	5	6	0	5	0	4
	BMR07	7	7	7	7	7	7	7	7	2	6	0	6
	UCR08	6	7	7	7	7	7	6	7	1	7	0	5
	UCR09	7	7	7	7	6	7	5	7	1	7	0	7
Strain 49	BMR05	6	6	6	6	6	6	6	6	2	4	0	1
	BMR07	7	7	7	7	7	7	7	7	3	6	2	5
	UCR08	7	7	7	7	6	7	2	7	5	7	3	5
	UCR09	7	7	7	7	7	7	7	7	4	7	1	2

Figure 1.1. Shoot mass response of un-inoculated *L. strigosus* to increasing mineral nitrogen. Error bars are ± standard error.

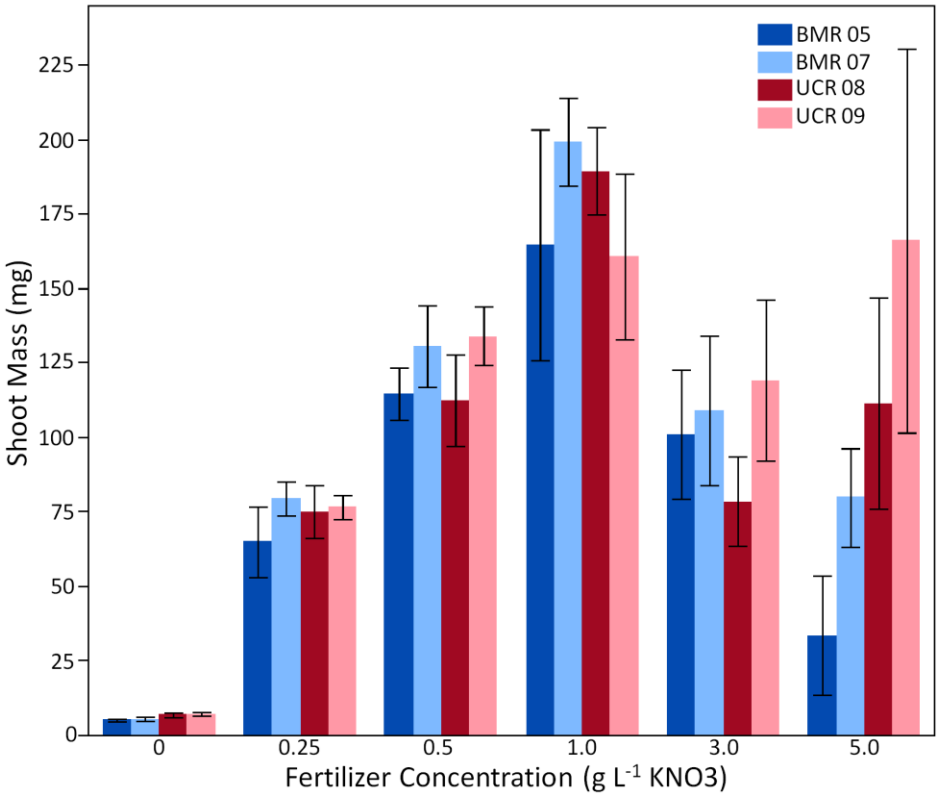


Figure 1.2. Mean nodules per plant. Error bars are ± standard error.

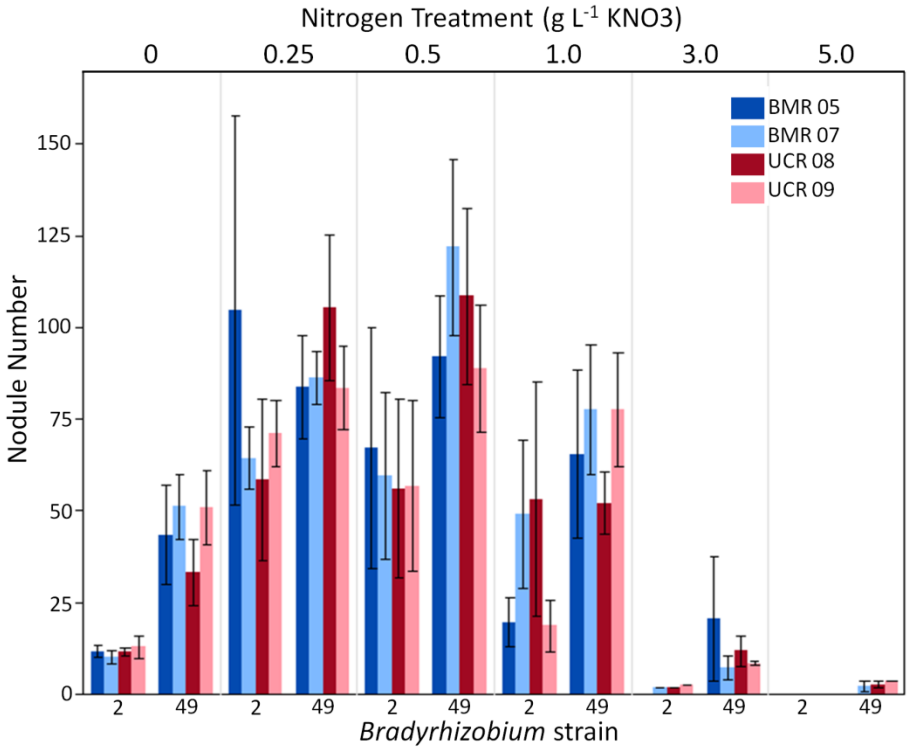


Figure 1.3. Mean individual nodule mass. Error bars are ± standard error.

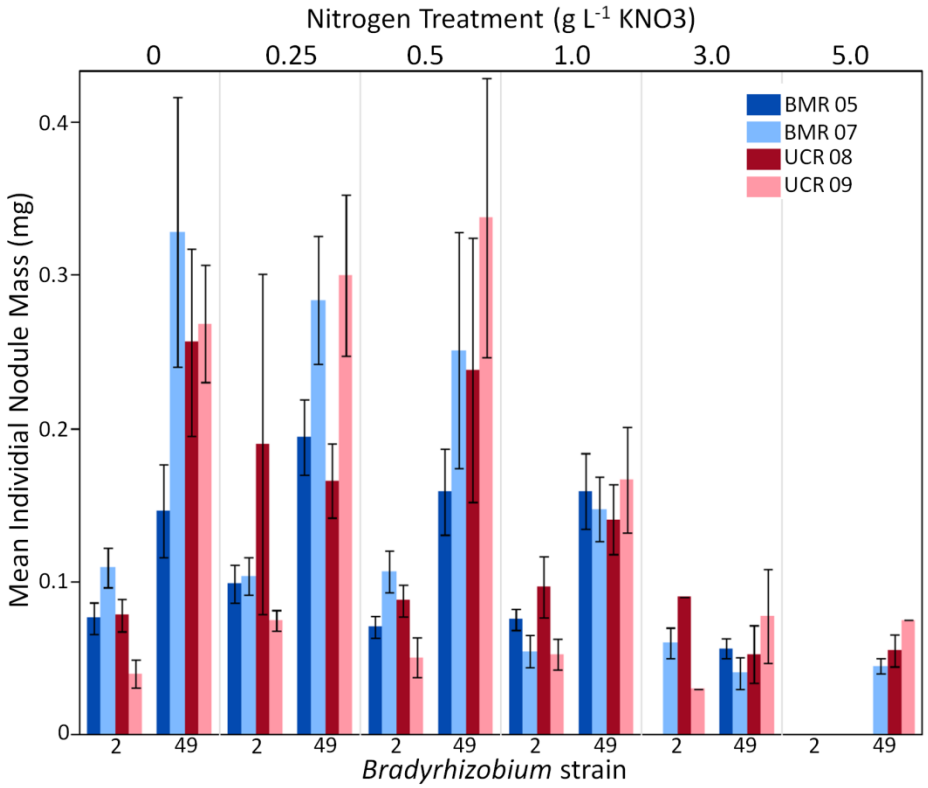
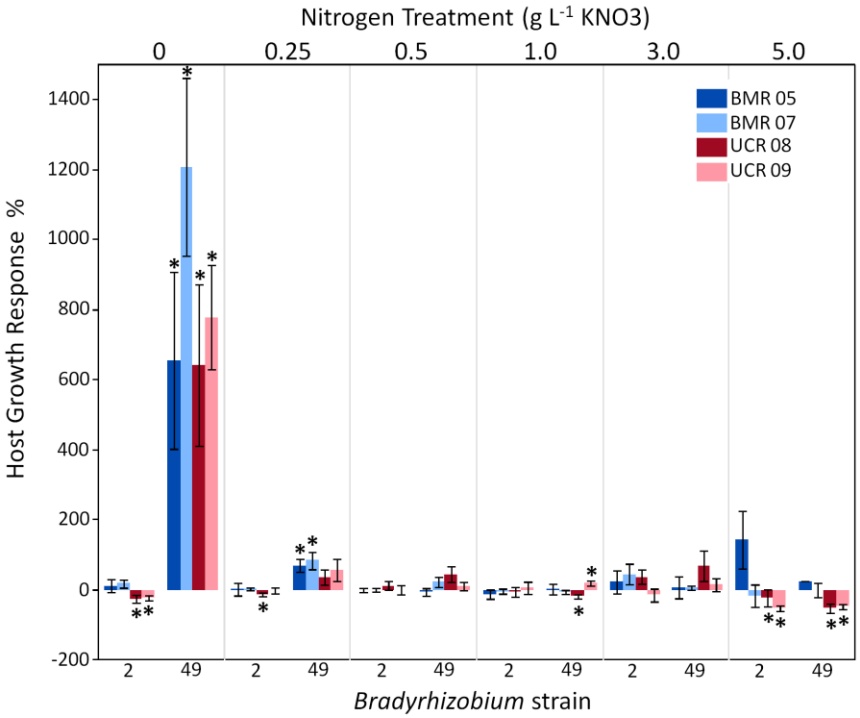


Figure 1.4. Host percent relative growth response from symbiosis. Percent response is ((shoot mass infected – shoot mass uninfected)/shoot mass uninfected) ± standard error. Asterisks show inbred lines that differ significantly from zero in one sample t-test ($p < 0.05$).



Chapter 2

Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization

Abstract

Eukaryotic hosts must exhibit control mechanisms to select against ineffective bacterial symbionts. Hosts can minimize infection by less effective symbionts (partner choice) and can divest of uncooperative bacteria after infection (sanctions). Yet, such host control traits are predicted to be context dependent, especially if they are costly for hosts to express or maintain. Legumes form symbiosis with rhizobia that vary in symbiotic effectiveness (nitrogen fixation) and can enforce partner choice as well as sanctions. In nature, legumes acquire fixed nitrogen both from rhizobia and soils and nitrogen deposition is rapidly enriching soils globally. If soil nitrogen is abundant, we predict host control to be downregulated, potentially allowing invasion of ineffective symbionts. We experimentally manipulated soil nitrogen to examine context dependence in host control. We coinoculated *Lotus strigosus* from nitrogen depauperate soils with pairs of *Bradyrhizobium* strains that vary in symbiotic effectiveness and fertilized plants with either zero nitrogen or growth maximizing nitrogen. We found efficient partner choice and sanctions regardless of nitrogen fertilization, symbiotic partner combination, or growth season. Strikingly, host control was efficient even when *L. strigosus* gained no significant benefit from rhizobial infection, suggesting that these traits are resilient to short-term changes in extrinsic nitrogen, whether natural or anthropogenic.

Introduction

Symbioses with environmentally acquired bacteria are critical to the health of many plant and animal species. Hosts must acquire these bacteria anew each generation, meaning that the fitness interests of the symbiont can be decoupled from those of the host (Herre et al. 1999; Sachs et al. 2004; Douglas 2010). Bacteria have a tremendous evolutionary advantage over eukaryotic hosts in terms of generation time and population size (Sachs et al. 2004), and thus mutations that allow exploitation of host resources without reciprocation can frequently arise in symbiont populations (West et al. 2002a). Moreover, the benefit that bacteria provide to hosts can be context dependent (Bronstein 2001). Bacterial genotypes that enhance fitness for one host genotype in one set of environmental conditions might provide little or no benefit in altered conditions or when infecting other hosts (Abd-Alla 1992; Burdon et al. 1999; Oliver et al. 2003; Stefanini & Duron 2012). To maximize benefits and minimize costs of symbiosis, hosts must exhibit 'host control' traits. Hosts can engage in partner choice by minimizing infection of ineffective symbionts and can enforce sanctions that selectively punish less effective symbionts after infection has occurred (Denison 2000; Simms & Taylor 2002; West et al. 2002a,b; Sachs et al. 2004). Empirical work has uncovered evidence of these host control mechanisms in diverse hosts including insects (Sachs et al. 2011a) and other invertebrates (Visick et al. 2000), mammals (Vaishnava et al. 2008; Petnick-Ocwieja et al. 2009; Salzman et al. 2009), and plants (Kiers et al. 2003, 2006, 2011; Simms et al. 2006; Sachs et al. 2010b; Oono et al. 2011). Yet, almost nothing is known about how host control functions in variable environmental contexts.

The legume-rhizobium interaction is a key model for eukaryotic control over bacterial symbionts. Rhizobia comprise several lineages of proteobacteria that have acquired the ability to infect legumes (Sawada et al. 2003). Symbiotic rhizobia most often infect roots and occupy host-derived tumors (nodules) where they fix atmospheric nitrogen in exchange for host-fixed carbon. Yet, many rhizobia can be ineffective. In these cases, nodule formation occurs but the rhizobia provide little or no fixed nitrogen for the host (Moawad et al. 1998; Burdon et al. 1999; Heath & Tiffin 2007; Sachs et al. 2010b; Schumpp & Deakin 2010). Ineffective rhizobia can potentially gain a metabolic advantage by redirecting plant carbon towards selfish ends (Hahn & Studer 1986; Lopez et al. 1995; Denison 2000) as opposed to engaging in energetically expensive nitrogen fixation (Trainer & Charles 2006).

Legume hosts can minimize the impact of ineffective rhizobia at two stages of the interaction. First, some legumes can discriminate against ineffective rhizobia during nodule formation (partner choice; Denison 2000; Simms & Taylor 2002). Subsequent to nodule formation, legumes can reduce within-nodule growth rates of ineffective rhizobia (sanctions; Denison 2000; Simms & Taylor 2002). Partner choice in legumes has received mixed empirical support; hosts that are coinoculated with effective and ineffective rhizobia that are closely related are often nodulated with equal frequency by both (Hahn & Studer 1986; Simms et al. 2006; Gubry-Rangin et al. 2010; Sachs et al. 2010b). Some legume hosts can engage in partner choice, especially when host discrimination is occurring among divergent rhizobial strains or populations (Heath & Tiffin 2009; Sachs et al. 2010b). By contrast, much empirical work has found evidence

for sanctions. Experiments on multiple host species have shown that when legumes are inoculated with mixed populations of rhizobia that vary in symbiotic quality, nodules with effective rhizobia typically grow large (and rhizobia within them proliferate) whereas nodules with ineffective rhizobia stay small (and rhizobia within them exhibit reduced growth; Kiers et al. 2003, 2006; Simms et al. 2006; Sachs et al. 2010b; Oono et al. 2011) but see (Gubry-Rangin et al. 2010; Marco et al. 2010).

Most work has investigated partner choice and sanctions in low or zero nitrogen contexts, which are biologically unrealistic. Soil nitrogen varies because of natural and anthropogenic inputs into soils and since the industrial revolution atmospheric deposition has dramatically polluted some soils with reactive forms of nitrogen (Tilman 1999; Egerton-Warburton 2001; Jumpponen et al. 2005; Dentener et al. 2006; Porras-Alfaro et al. 2007). Assuming that host control traits are costly to express (West et al. 2002b; Foster & Kokko 2006), such as other plant defenses against bacteria (Tian et al. 2003; van Hulten et al. 2006), we predict that host control will be downregulated in nitrogen rich soils, where hosts can gain nitrogen primarily from less costly mineral sources (Voisin et al. 2002) rather than symbionts. Yet, if partner choice and / or sanctions are downregulated, this could favor the spread of ineffective symbionts and a potential collapse of the symbiosis (Sachs et al. 2004; Sachs & Simms 2006). Few experiments have explored the efficiency of host control in varying environments. Most notably, research on soya beans found that sanctions were equally efficient in the presence of nitrate fertilizer, when a *Bradyrhizobium* strain was forced to fix less nitrogen by

replacing some or all of the air around nodules with a nitrogen-free atmosphere (Kiers et al. 2006).

Here, we tested the effects of nitrogen fertilization and other key variables on efficiency of both partner choice and sanctions in a wild legume. We studied *Lotus strigosus*, a native California annual legume, and four sympatric *Bradyrhizobium* symbionts that range in symbiotic quality from highly effective to ineffective. Hosts and rhizobia were gathered from a natural site with low soil nitrogen, which has likely experienced negligible effects of atmospheric nitrogen pollution (Fenn et al. 2010). We coinoculated *L. strigosus* with *Bradyrhizobium* populations comprised of an effective strain and one ineffective strain. Sachs and colleagues (2010b) previously established that, in zero soil nitrogen conditions, *L. strigosus* can exhibit both partner choice and sanctions when inoculated with mixed populations of these specific strains, favoring effective strains versus ineffective ones. In such experiments, it is critical to rule out the effects of competition among rhizobia causing differences in rhizobial fitness. Previous work showed that: (i) in single strain inoculations, the ineffective strain used here successfully nodulates *L. strigosus*, forming more nodules per plant and more rhizobia per nodule than tested effective strains and (ii) the ineffective strain exhibits similar population size to the tested effective strains in *in vitro* competition assays. These data suggest that inter-strain competition is unlikely to produce confounding evidence for partner choice or sanctions (Sachs et al. 2010b). We also report data from the current experiments from single strain inoculations that suggest it is unlikely that *L. strigosus*

exhibits strain-specific effects of nitrogen that could confound our interpretation of results presented here.

Plants were grown in zero nitrogen or were fertilized with a nitrogen concentration determined to maximize host growth in the absence of rhizobia. To test for partner choice, we examined whether hosts discriminated against ineffective rhizobia for nodule formation. To test sanctions, we compared within-nodule fitness of effective and ineffective *Bradyrhizobium* strains in coinoculated hosts. To estimate the relative contribution of symbiotic nitrogen fixation to plant growth, we measured $\delta^{15}N$ in leaf tissue of infected plants and compared it to uninfected plants in each treatment. The goals of the experiment were to (i) examine whether legume partner choice and sanctions are downregulated in growth-saturating nitrogen fertilization and (ii) to test whether partner choice and sanctions vary depending on rhizobial strain, exogenous nitrogen, season, or net fitness benefit of infection for the host.

Material and Methods

Selection and culturing of Bradyrhizobium strains

Four *Bradyrhizobium* strains, referred to as numbers 2, 14, 38 and 49 (Sachs et al. 2010a) were selected for this study based on previous genotypic and phenotypic analyses (Sachs et al. 2009; Sachs et al. 2010a,b; Sachs et al. 2011a). Strain 49 provides ~5x increase in host shoot biomass relative to uninfected control plants, whereas strains 38, 14, and 2 provided ~3.5x, ~2x and ~0.95x relative benefit, respectively for *L. strigosus*

(strain 2 is ineffective; Sachs et al. 2010a). Rhizobial inocula were generated using published protocols (Sachs et al. 2010b).

Lotus strigosus seed collection and preparation

In June 2011, *L. strigosus* fruits were collected at Bodega Marine Reserve (BMR), CA sympatric to where our *Bradyrhizobium* strains were originally collected, from coastal sand dunes that have little capacity to retain plant-available nitrogen (Cain et al. 1999). Host seed sets were comprised of equal mixes of seeds from different parental plants. Seed preparation and planting followed published methods (Sachs et al. 2009).

Soil nitrogen assays

We estimated total soil nitrogen concentration and soil mineral nitrogen (extractable NO_3 and NH_3) at BMR and ten other *L. strigosus* populations across California. Three soil cores (10 cm depth) per site were sampled from one square meter (where *L. strigosus* had been collected previously), then treated and analyzed per published methodology (Santiago et al. 2005). Nitrogen analysis was performed at the FIRM Isotope Facility at U.C. Riverside.

Inference of growth saturation of nitrogen-fertilized Lotus strigosus

Minimal growth saturating nitrogen (GSN), defined as the lowest concentration of KNO_3 in soil that maximizes *L. strigosus* growth in the absence of rhizobial infection,

was determined in eight-week greenhouse experiments (7 March 2011 – 2 May 2011; see below for fertilizer protocol, harvest and data collection).

Partner choice and sanctions experiments

Replicated experiments were performed in the same greenhouse in fall 2011 (17 October 2011 to 11 December 2011) and winter 2012 (23 January 2012 to 18 March 2012) to take seasonal variation into account, hereafter referred to as the fall and winter experiments. Each experiment comprised two blocks with a completely randomized factorial design. Axenic *L. strigosus* seedlings were arranged by size and divided into blocks accordingly. Within blocks, 144 size-matched seedlings were randomly assigned to treatments. Bacterial treatments consisted of single strain inoculations of the four strains (2, 14, 38, 49), coinoculations of each effective strain with the ineffective strain (14 x 2, 38 x 2, 49 x 2), and uninfected control plants. Each inoculation experiment consisted of 288 plants (nine replicate plants per treatment, sixteen treatments, two replicate blocks). Fertilization treatments were zero nitrogen fertilizer and fertilization with 0.5 g l^{-1} KNO_3 (GSN; Fig. 2.1). Each week, 10.0 ml of a nitrogen-free Jensen's solution was added to each plant (with KNO_3 for GSN treatments), beginning three days prior to inoculation. Seven days after placement in the greenhouse, plants were each inoculated with log-phase rhizobia (5.0 mL sterile ddH_2O, 1.0 x 10^8 cells ml^{-1}). Coinoculations comprised an equal mixture of each strain and uninfected plants received 5.0 ml sterile ddH_2O. Each experiment lasted 8 weeks from inoculation to harvest.

Harvest

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 before weighing (60 °C, more than 4 days). To identify rhizobial strains within the nodules of coinoculated plants, a random subset of nodules were cultured from two randomly selected plants, per fertilization treatment, per block, in both fall and winter. Five nodules (in the fall) and four nodules (in the winter) were randomly chosen per plant for culturing, resulting in 216 cultured nodules from 48 test plants. Nodules were surface sterilized, crushed, serially diluted in sterile ddH_20 (10^{-3}, 10^{-5}) and spread onto four to six MAG-agar plates. Colony counts from at least two plates were used to estimate nodule population size per nodule, at the whole plant level (rhizobial fitness). One hundred randomly selected colonies were then replica plated onto MAG-agar plates with streptomycin (100 μg ml^{-1}) to quantify relative population sizes of different strains within each nodule (Sachs et al. 2010b). Strains 14, 38, and 49 are sensitive to streptomycin whereas strain 2 is resistant (Sachs et al. 2010b). In winter, nodules were cultured from eight plants singly inoculated with strain 2. Four plants (two from each block) were randomly selected from each nitrogen treatment and three nodules from each plant were cultured and colonies were counted as above to estimate rhizobial population size within each nodule.

Leaf $\delta^{15}N$ assays

We compared leaf ^{15}N 'atom percent difference' ($\delta^{15}N$) between single infected and uninfected plants for each *Bradyrhizobium* strain, within each fertilization treatment. When plants incorporate symbiotically fixed nitrogen, leaves exhibit lowered $\delta^{15}N$ relative to uninfected plants because of isotopic fractionation by rhizobia (Yoneyama et al. 1986). Leaflets were removed from dried shoots, ground, and analyzed at the FIRM Isotope Facility at UC Riverside. We did not analyze coinoculated plants, because variation in $\delta^{15}N$ would be confounded by the plant's interaction with multiple rhizobial strains.

Data analysis

To test for partner choice, we quantified 'nodule occupancy' within coinoculated plants, defined as the proportion of nodules per plant occupied by the effective versus the ineffective strain (coinfected nodules were assigned to both categories) and tested for significance using a χ^2 test against a null of 0.50 (Sachs et al. 2010a). To test for sanctions, we compared per plant mean rhizobial population sizes of effective versus ineffective rhizobia in all sampled nodules, whether single or coinfected, of coinoculated plants (Sachs et al. 2010a), and tested for significance with analysis of variance (ANOVA) within each treatment combination (JMP v. 10.0 SAS Institute Inc. 2012). To test the effect of nitrogen on partner choice and sanctions, we tested for interactions between strain and nitrogen in terms of nodule occupancy (partner choice) or rhizobia nodule population sized (sanctions; Fit Model Platform, JMP 10.0). For single inoculated

plants, we compared per plant mean rhizobia population sizes among nitrogen treatments using ANOVA. Relative growth response of hosts to infection was calculated as the percent difference in total dry plant biomass between inoculated hosts (infected) and un-inoculated (uninfected) plants. Total soil nitrogen comparisons and $\delta^{15}N$ differences were analyzed using one-way ANOVA in JMP 10.0 and Student's t-test for pairwise comparisons with correction for multiple comparisons. Minimal GSN was analyzed using a one-way ANOVA and pairwise t-test comparisons of shoot mass between different soil fertilization treatments to determine the fertilizer concentration where increased fertilizer did not increase shoot mass.

Results

Soil nitrogen assays

Our *L. strigosus* collection site (BMR) exhibited total soil nitrogen levels comparable to the lowest concentrations observed in soils (i.e. approx. 0.01%; Bremner 1965; Sowden et al. 1977). Soil nitrogen at BMR was consistently lower than most southern California *L. strigosus* sites in terms of total nitrogen and mineral nitrogen (Table 2.1). Sites with the greatest total soil nitrogen, such as Bernard Field Station (Los Angeles County), exhibited total soil nitrogen levels comparable to tilled, agricultural soils (approx. 0.1%), consistent with the effects of nitrogen deposition (Bremner 1965; Sowden et al. 1977).

Growth saturating nitrogen

Nitrogen fertilization significantly increased *L. strigosus* growth (ANOVA; $F_{6,43}$ $p < 0.0001$). In pairwise comparisons among fertilization treatments, plant growth at 0.5 g L^{-1} KNO_3 was significantly greater than all lower concentrations. Concentrations more than 0.5 g L^{-1} did not significantly enhance host growth relative to 0.5 g L^{-1} (Fig. 2.1). None of the experimental plants exhibited evidence of rhizobial contamination (nodulation).

Single inoculation

When fertilized, *L. strigosus* formed more nodules then unfertilized plants in all cases and significantly so in six of eight comparisons across both experiments (see asterisks in Fig. 2.2). In zero nitrogen, the number of nodules formed did not differ among strains within each experiment, except for one treatment (fall strain 14 formed fewer). In GSN, strain 2 formed the most nodules in each experiment, but differences among strains were not always significant (Fig. 2.2). Per plant mean nodule population sizes for single infected strain 2 nodules were not significantly different in winter comparing nodules among fertilizer treatments (Table 2.2). Mean individual nodule mass decreased in response to nitrogen for all effective strains in each experiment and significantly so for strains 14 and 38 (t-test, $p < 0.05$) in each experiment (Fig. 2.3) and previous work showed population size (within strain) is positively correlated with nodule mass (Simms et al. 2006).

Partner choice efficiency

Only 13% (22 of 163; Table 2.2) of analyzed nodules were infected by the ineffective strain (including coinfected nodules), significantly less than expected by chance (0.50; Pearson χ^2 (1, n=183) = 108.64, $p < 0.0001$). Among the 216 test nodules, we could not recover data from 54, because they were damaged during dissection or culturing, or the cultures could not be analyzed because of contamination or sparse growth. No significant block effects or block x treatment interactions were detected, and blocks were combined for these analyses. No un-inoculated control plant showed evidence of contamination (nodulation). In all treatments but one (38x2 winter), the effective strains were significantly favored over the ineffective strains for nodule occupancy (Fig. 2.4; see Table 2.3 for proportional data and statistical significance). There was no significant strain x fertilizer treatment interaction within experiments (fall $F_{Strain*Nitrogen}(2,98) = 1.93$, $p > 0.15$; Winter $F_{Strain*Nitrogen}(2,71) = 0.88$, $p > 0.42$) suggesting the pattern of nodule occupancy (partner choice) was not significantly altered by fertilization.

Sanctions efficiency

The effective strains exhibited a fitness advantage (per plant) over the ineffective strain in all coinoculation treatment combinations but one, and these differences were significant in eight of the twelve treatment combinations (Table 2.2). There was no significant strain x fertilizer treatment interaction within experiments, suggesting that

sanctions was not altered by fertilizer treatment (fall $F_{Strain*Nitrogen}(2,92) = 0.33$, $p > 0.70$; winter $F_{Strain*Nitrogen}(2,65) = 1.14$, $p > 0.30$).

Relative host growth response

Plants grown in GSN gained significantly less growth benefit from rhizobial infection than plants grown in zero nitrogen in both experiments (Fig. 2.5). In the fall experiment, fertilized plants gained no net growth benefit from infection. Unfertilized plants gained significant growth benefit from infection in both experiments.

Leaf $\delta^{15}N$ analyses

Patterns of $\delta^{15}N$ leaf content were less pronounced in the fall experiment. In zero nitrogen, plants infected with effective strains most often exhibited significantly lower $\delta^{15}N$ relative to uninfected plants, consistent with substantial plant assimilation of symbiotically fixed nitrogen (Table 2.4). Conversely, nitrogen-fertilized plants assimilated relatively little or no symbiotically fixed nitrogen in both the fall and winter experiments, as indicated by small or no significant differences between the $\delta^{15}N$ values of infected plants versus uninfected controls. The $\delta^{15}N$ value for strain 2 was not significantly different from uninfected plants in all but one of the treatments, consistent with minimal or no symbiotically fixed nitrogen in these infections (Table 2.4).

Discussion

Plants and animals invariably encounter bacteria in their environment that can offer hosts a suite of fitness benefits, in particular nutrition and biological protection (Douglas 2010). To optimize the benefits from these infections and to minimize exploitation, the hosts often exhibit partner choice and or sanctions against ineffective symbionts. One main presumption is that these traits are costly for hosts to express and maintain (West et al. 2002b), and hence that they are downregulated or evolutionarily lost when not needed (Sachs & Simms 2006). Plant defense traits against bacterial pathogens often entail significant fitness costs to express, including R-gene mediated immunity (Tian et al. 2003) and induced direct defenses (van Hulten et al. 2006). In our experiments, *L. strigosus* hosts discriminated against ineffective rhizobia during nodulation (partner choice) and after nodule formation (sanctions), even when the hosts gained no symbiotically fixed nitrogen and no net fitness benefit from rhizobial infection. This result is consistent with previous work with soybeans that examined sanctions (Kiers et al. 2006) and together these data suggest that the expression of both partner choice and sanctions are canalized plant traits.

Our dataset supports the hypothesis that *L. strigosus* discriminates against ineffective rhizobia during nodule formation (partner choice), consistent with previous work in zero soil nitrogen (Sachs et al. 2010b). Among the 162 nodules examined from coinoculated plants, ~85% were infected by a single effective strain. Partner choice by *L. strigosus* was not affected by nitrogen fertilization of the soil. The ineffective strain that we used is genetically diverged from most effective *Bradyrhizobium* that infect *L.*

strigosus (Sachs et al. 2010a, 2011b), hence partner choice might be occurring through genotypic recognition by the host. Interestingly, the discrimination by the host is only evident in coinoculated plants, since the ineffective strain forms as many and often more nodules than effective strains in singly inoculated plants (Fig. 2.2; Sachs et al. 2010b). These data suggest that the host is detecting differences among rhizobia as they compete for infection sites on the root surface, but the signal that the host might be using to do this is unknown. An alternative explanation for biased nodulation rates is competition among the rhizobial strains for nodulation. Inter-strain competition cannot be ruled out, but previous *in vitro* competition assays on these same strains suggested that the ineffective strains can proliferate in direct competition with the effective rhizobia (Sachs et al. 2010a,b).

Phenotypic evidence of sanctions is now well supported by empirical data. But our knowledge is lagging in terms of the cellular or genetic mechanisms of legume sanctions. The dominant model of sanctions posits that legumes detect symbiotic nitrogen fixation at the nodule level (Denison 2000; West et al. 2002a,b), and sanction non-fixing rhizobia by reducing oxygen supply to those individual nodules (Kiers et al. 2003). But our data showed evidence of sanctions even when nodules contained both effective and ineffective rhizobia, inconsistent with the whole nodule model of sanctions. Focusing only on the subset of nodules in our experiments that were coinfected and comparing population size of the ineffective strain 2 to the effective strains, effective strains had a significant fitness advantage in terms of population size (Strain 2 mean population = 2.16×10^6 s.e. = 2.74×10^6, effective strains mean population = 2.42×10^7 s.e. = 2.29×10^7;

Matched Pairs t test; $T_{13} = 3.53$, $p = 0.004$; n = 14). Moreover, nodule number, nodule mass and population size data reported here suggests the absence of strain-specific effects of nitrogen that could confound interpretation of sanctions (Fig. 2.2, 2.3; Table 2.2). These results suggest that sanctions might be controlled at a cellular level within nodules. To date, research has not generated an explicit mechanistic model of legume sanctions that can account for nodules that contain more than one strain of rhizobia.

We examined wild *L. strigosus* hosts from soils with low nitrogen concentrations (Table 2.1) and with little ability to retain mineral nitrogen (Cain et al. 1999); hence the hosts are unlikely to have acclimatized to GSN conditions. Our data showed no significant downregulation of sanctions by *L. strigosus* in elevated nitrogen soils. This represents the first test of how a native legume from a nitrogen depauperate site can respond to elevated nitrogen and whether legume control mechanisms are robust to rapid, biologically relevant, shifts in soil nitrogen concentration. But, importantly, we did not test hypotheses about the evolution of partner choice or sanctions in nitrogen rich soils. Since the industrial revolution, chemical fertilization and atmospheric deposition have greatly increased reactive nitrogen concentrations in soils (Dentener et al. 2006). Global increases in soil nitrogen content are now driven by atmospheric deposition (Fenn et al. 2010), and our data suggest that these sources of pollution can easily lead to scenarios where infection with otherwise effective rhizobia would provide no net benefit to legume hosts. It remains an open question how legume hosts will respond evolutionarily to increased soil nitrogen. At least three possible evolutionary scenarios exist. One possibility is that host control traits would degrade under long-term conditions of GSN,

which could relax selection on the host to maintain sanctions. For instance, Kiers and colleagues (2007) found soya bean cultivars exhibited evidence consistent with evolutionarily relaxed sanctions over decades of selection in agricultural contexts. Conversely, host control might evolve to be even more efficient, thus selecting for rhizobia that still provide net benefit in the nitrogen enriched soils (Kiers et al. 2007). Finally, it is possible that legumes could lose the ability to nodulate rhizobia, as the net benefit that is provided by these symbionts decreases with increased soil nitrogen over time (Sachs & Simms 2006). Future work in this system must examine how host control evolves when hosts are exposed to increased nitrogen over many generations.

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Table 2.1. Soil nitrogen content from eleven sites in California with native *L. strigosus* populations. Total soil nitrogen samples collected winter 2011 and 2013. Mineral nitrogen (ppm = mg NO_3 + NH_3 per kg soil) samples collected summer 2013. Letter superscripts indicate significant differences in pairwise comparisons correcting for multiple comparisons ($p < 0.05$).

Site	Lat./Long. (DD)	CA. County	Mean Total Soil Nitrogen (%)	Mean Mineral Nitrogen (ppm)
Bodega Marine Reserve	38.319143 -123.063657	Sonoma	0.01 (0.01)^{c}	4.08 (2.38)^{c,d}
Anza-Borrego State Park	33.271264 -116.419368	San Diego	0.01 (0.01)^{c}	2.02 (0.20)^{d}
Burns-Pinyon Ridge Reserve	34.149309 -116.45523	San Bernardino	0.03 (0.01)^{c}	7.04 (0.30)^{b,c,d}
Mojave Desert Sweeney Granite Mountains Reserve	34.736199 -115.666199	San Bernardino	0.01 (0.01)^{c}	1.65 (0.07)^{d}
Guadalupe-Nipomo Dunes National Wildlife Refuge	35.010525 -120.604525	San Luis Obispo	0.03 (0.01)^{c}	1.69 (0.09)^{d}
Bernard Field Station of the Claremont Colleges	34.110525 -117.708916	Los Angeles	0.11 (0.01)^{a}	10.81 (1.78)^{b,c}
Griffith Park	34.122003 -118.308986	Los Angeles	0.04 (0.02)^{b,c}	6.70 (2.58)^{b,c,d}
Madrona Marsh Preserve	33.824819 -118.341558	Los Angeles	0.03 (0.01)^{c}	3.94 (0.88)^{c,d}
Motte-Rimrock Reserve	33.804816 -117.25802	Riverside	0.05 (0.01)^{b,c}	12.65 (1.88)^{b}
University of California Riverside	33.965938 -117.322903	Riverside	0.07 (0.02)^{b}	20.47 (1.54)^{a}
Whitewater Preserve	33.979842 -116.655478	Riverside	0.01 (0.01)^{c}	5.47 (0.64)^{b,c,d}

Table 2.2. Rhizobial population size estimates with nodule data. [1] Column shows simple ratio of per plant mean rhizobial population of effective strain (two columns previous) to per plant rhizobial population of ineffective strain (one column previous). Parentheses show one standard error. Asterisks show significant differences among per plant mean rhizobial population sizes within nodules (including all analyzed nodules) among effective and ineffective (* $p < 0.05$). Letter superscripts show significant difference of per plant mean rhizobial population among nitrogen treatments for plants singly infected with strain 2 (matching letters indicate no difference).

Experiment	Strains	Nitrogen	Plants Sampled	Nodules Analyzed	Co-infected	Effective Only	Ineffective Only	Nodules Plant^{-1} Sampled	Mean Rhizobia Nodule^{-1} Plant^{-1} Effective Strain	Mean Rhizobia Nodule^{-1} Plant^{-1} Ineffective Strain	Effective / Ineffective[1]
Fall	14x2	Zero N	4	16	2	14	0	14	2.44×10^7 (4.71×10^6)*	1.93×10^5 (1.47×10^5)	126.73
		GSN	4	19	1	18	0	32	3.13×10^7 (1.30×10^7)*	2.15×10^3 (2.15×10^3)	14558.14
	38x2	Zero N	4	10	1	8	1	17	7.98×10^5 (7.39×10^5)	4.17×10^4 (4.17×10^4)	19.14
		GSN	4	21	1	19	1	23	1.32×10^7 (6.27×10^6)*	8.19×10^4 (7.51×10^4)	1611.72
	49x2	Zero N	4	12	0	12	0	16	5.53×10^6 (1.70×10^6)*	0	NA
		GSN	4	17	3	13	1	21	2.04×10^7 (6.91×10^6)*	3.13×10^5 (2.55×10^5)	65.18
Winter	14x2	Zero N	4	15	2	13	0	18	3.58×10^7 (1.35×10^7)*	7.04×10^5 (7.04×10^5)	50.85
		GSN	4	13	3	10	0	37	3.82×10^7 (1.63×10^7)*	1.41×10^6 (8.11×10^5)	27.09
	38x2	Zero N	3	9	1	7	1	17	3.30×10^6 (3.23×10^6)	8.35×10^4 (8.33×10^4)	39.52
		GSN	3	11	0	8	3	34	9.42×10^5 (5.35×10^5)	4.99×10^6 (4.59×10^6)	0.19
	49x2	Zero N	4	9	0	8	1	18	1.03×10^6 (5.85×10^5)	2.75×10^5 (2.75×10^5)	3.75
		GSN	3	11	0	11	0	27	1.83×10^7 (8.44×10^6)*	0	NA
Totals			45	163	14	141	8	274	NA	NA	NA
Winter	2 Only	Zero N	4	11	NA	NA	11	12	NA	1.47×10^7 (2.98×10^6)[a]	NA
		GSN	4	12	NA	NA	12	12	NA	1.82×10^7 (2.40×10^6)[a]	NA

Table 2.3. Proportion of nodules infected with effective strain (partner choice). Asterisks show significant Pearson χ^2 deviation from expectation of 0.50 (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) and n.s. = non significant.

Strains	Fall Zero N	Fall GSN	Winter Zero N	Winter GSN
14x2	0.995 (0.045)***	0.948 (0.056)***	0.957 (0.069)***	0.954 (0.080)**
38x2	0.894 (0.056)*	0.904 (0.053)***	0.909 (0.080)**	0.726 (0.089)n.s.
49x2	1.000 (0.051)	0.935 (0.060)**	0.888 (0.088)*	1.000 (0.086)

Table 2.4. Mean $\delta^{15}N$ for leaf tissue of singly infected hosts. Letters are significant differences in t-test comparisons within fertilization treatment correcting for multiple comparisons ($p < 0.001$). Shaded areas highlight significant differences ($p < 0.05$) from uninfected control plants. Parentheses show one standard error.

	Fall		Winter	
Strain	Zero Nitrogen	GSN	Zero Nitrogen	GSN
Un-inoculated	-1.46 (0.44)[a]	-1.91 (0.35)[a]	2.89 (0.71)[a]	-1.20 (0.55)[a]
2	-1.86 (0.78)[a,b]	-2.34 (0.36)[a,b]	1.17 (0.58)[b]	-1.16 (0.31)[a]
14	-3.74 (0.54)[c]	-2.85 (0.30)[b]	-3.29 (0.23)[c]	-2.04 (0.31)[a]
38	-2.03 (0.06)[a,b]	-2.36 (0.21)[a,b]	-2.01 (0.21)[c]	-1.61 (0.22)[a]
49	-2.54 (0.14)[b]	-2.22 (0.14)[a,b]	-2.21 (0.19)[c]	-1.53 (0.34)[a]

Figure 2.1. Growth of *L. strigosus* in response to increasing soil nitrogen fertilizer in the absence of rhizobial infection. Letters represent significant differences in pairwise t-tests correcting for multiple comparisons ($p < 0.0001$ for all significant pairwise comparisons). GSN = Growth Saturating Nitrogen.

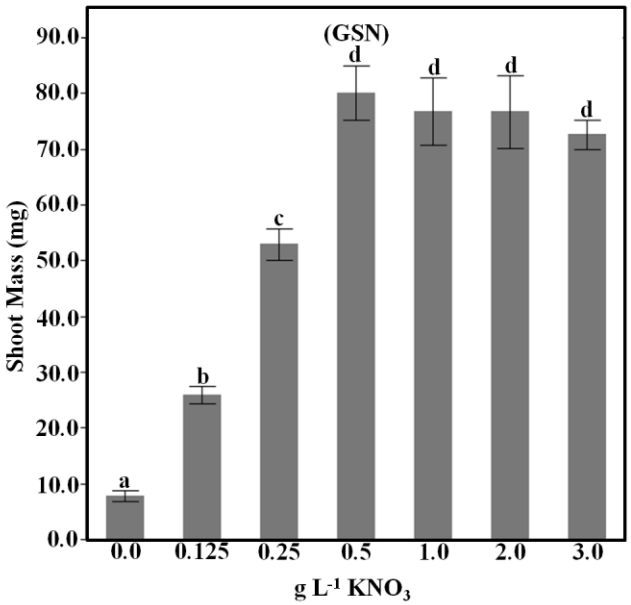


Figure 2.2. Mean number of nodules per plant for single inoculated plants. Light bars show zero nitrogen. Dark bars show GSN. Error bars show one standard error. Letters are significant differences among bacterial treatments within nitrogen treatment (pairwise t-test with Tukey's correction for multiple comparisons, $p < 0.05$). Asterisks show significant difference among nitrogen treatments within bacterial treatment (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

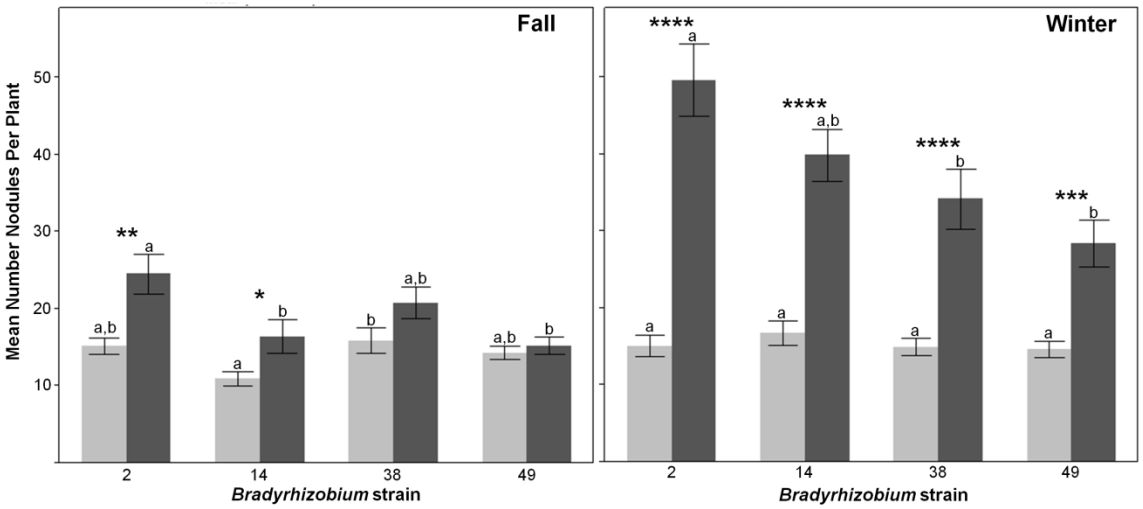


Figure 2.3. Mean individual nodule mass. Asterisks are significant differences among nitrogen treatments within bacterial treatment (t-test * = $p < 0.05$, ** = $p < 0.01$). Letters are significant differences among bacterial treatments within nitrogen treatment (pairwise t-test with Tukey's correction for multiple comparisons; $p < 0.05$).

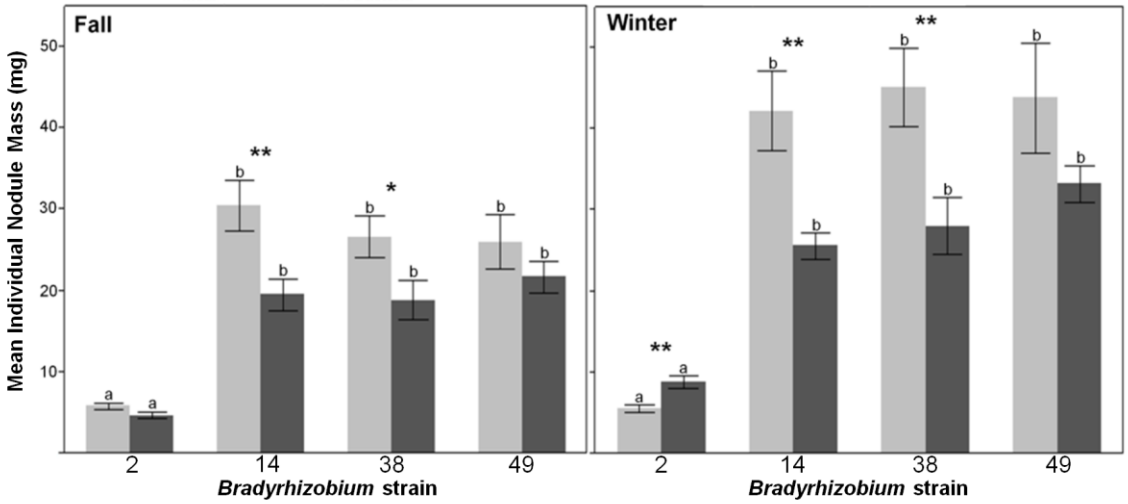


Figure 2.4. Nodule occupancy of effective versus ineffective strain in coinoculations (partner choice). Nodule occupancy measures presence-absence of the effective or ineffective strain in each nodule, and coinfected nodules count as both. Ratios of effective versus ineffective nodules per plant were tested using a χ^2 against a predicted ratio of 0.50. All tests are significant except where n.s.= non-significant ($p < 0.05$ or lower; see Table 2.3 for p values).

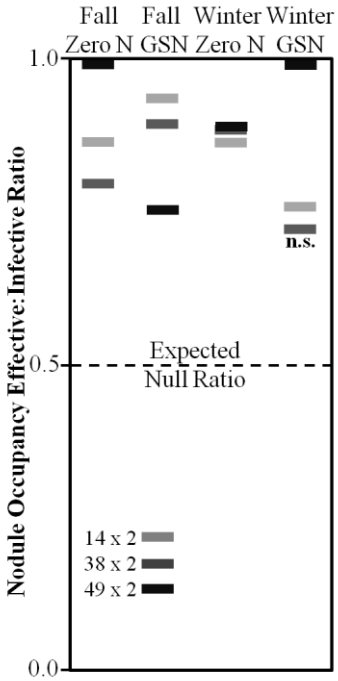
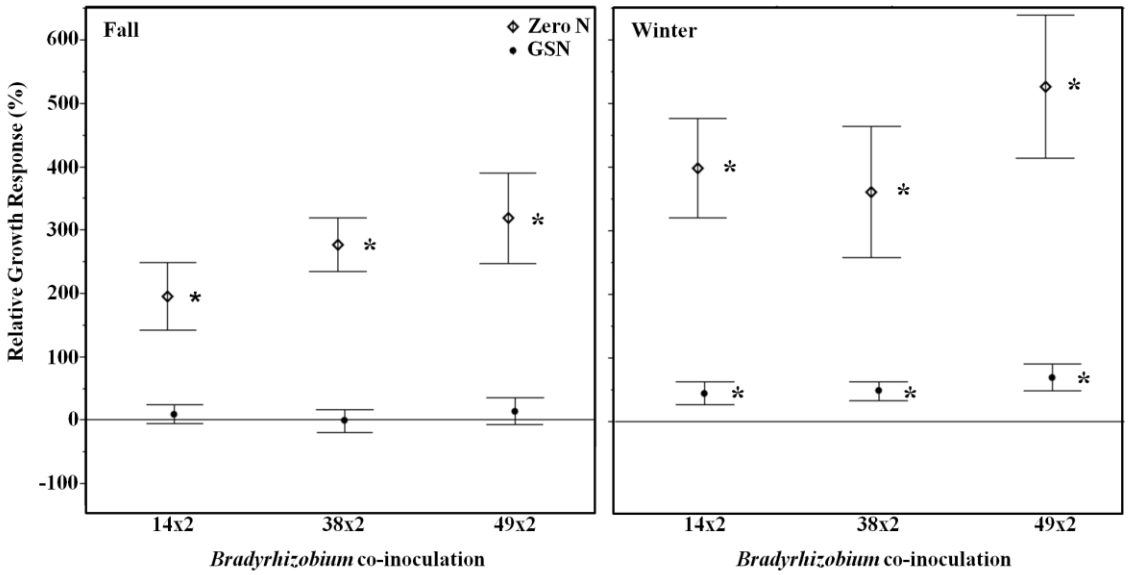


Figure 2.5. Relative host growth response to infection. Y-axis is percent increase in biomass relative to uninoculated plants. Error bars show one standard error. Asterisks show significant differences from zero in one sample t- test ($p < 0.001$).



Chapter 3

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

Abstract

Symbioses are modeled as ecologically variable such that fitness outcomes for hosts can shift on a continuum from mutualism to parasitism. In a classic example, rhizobia fix atmospheric nitrogen for legume hosts in exchange for photosynthetic carbon. Rhizobia vary genetically in symbiotic effectiveness and changes in light or mineral nitrogen availability can alter the benefits of rhizobial infection for hosts. Legumes must incur costs of infection and metabolic support of rhizobia within the plant host and thus the net effects of symbiosis 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 under different mineral nitrogen and seasons 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

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 benefits that plants receive from these interactions is often conditional, and can vary depending on the extrinsic environment (Lau et al. 2012; Simonsen & Stinchcombe 2014; Regus et al. 2014), the microbe and plant genotype combination (Bever 1999; Sachs et al. 2010a), and interactions among these factors *(*Heath et al. 2007*).* A dominant paradigm of plant-microbial symbiosis models these interactions as a 'mutualism-parasitism continuum', defined here as variation for the host 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 this continuum (e.g., Hoeksema et al. 2010; Lau et al. 2012). But the ecological relevance of these data has been debated (Karst et al. 2008), since some tested conditions might never occur in nature (e.g., geographically distant microbe-plant genotype combinations, extreme soil nutrient parameters). Models of mutualism stability are often in conflict with the mutualism-parasitism paradigm. Mutualism models predict that hosts are selected 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). Hence, a key unanswered question is whether plant hosts can modulate investment to prevent mutualistic symbioses from shifting into parasitism. Further, if plants can delimit parasitism, does it occur by preventing infection in contexts where benefit is reduced, or by modulating investment into symbionts post-infection?

The legume-rhizobium interaction is a classic model of plant-microbe symbiosis with context dependent effects upon the host (Burdon et al. 1999; Heath & Tiffin 2007; Sachs et al. 2010a; Lau et al. 2012; Regus et al. 2014). Rhizobial genotypes vary 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 the variable benefits of rhizobial symbiosis, which are based on fixed nitrogen that the host can also get from the soil, the physiological costs of of 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 the net effects of interaction (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).

Mutualism theory predicts that plants are selected to optimize investment into microbial symbiosis depending on the net fitness benefit that the host receives from the infection (Denison 2000; West et al. 2002; Simms & Taylor 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 that plants should only invest resources into 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 conceivably 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), any of which might optimize host fitness. 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 into 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. 2010b; 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). We manipulated soil nitrogen to bracket concentrations that *L. strigosus* can encounter across its range 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 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 if 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

Fruits were collected from *L. strigosus* 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 x G interactions between hosts and rhizobia. Seed preparation, planting, and plant maintenance followed published protocols (Sachs et al. 2009).

Inoculation experiments

Replicated experiments were performed in fall 2011 (17/10/11-12/12/11) and winter 2012 (23/01/12-19/03/12), 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 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 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 (9 replicate plants per treatment, 10 treatments, 2 replicate blocks). All plants were grown in pre-washed, 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 5ml ddH_20) 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 versus 637 hours 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. 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 percent difference in total plant biomass between inoculated plants and matched un-inoculated control plants

(Sachs et al. 2010a). We tested if 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 from parasitism. We also 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). We used general linear models (GLM; Fit Model Platform in JMP 10.0 SAS Institute Inc.) to test main effects (rhizobial genotype, soil nitrogen) within the experiments and interactions among effects for host growth response and percent leaf nitrogen content. Seasonal variation is derived from separate experiments, and so cannot be analyzed as a manipulated treatment. We thus combined data from both experiments keeping observations separate 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 mass, 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 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. To test the hypothesis that *Lotus* hosts modulate investment in *Bradyrhizobium* dependent on the host's net benefit from infection we analyzed the relationship between host growth response and both nodule number and mean nodule mass using GLM.

Results

Net effects of Bradyrhizobium infection

Host growth response exhibited significant effects of *Bradyrhizobium* genotype (Fall $F_{3,140} = 8.58$, $p < 0.0001$; Winter $F_{3,139} = 9.26$, $p < 0.0001$), soil nitrogen treatment (Fall $F_{1,142} = 49.51$, $p < 0.0001$; Winter $F_{1, 141} = 52.34$, $p < 0.0001$), and interaction between these effects (Fall $F_{3,140} = 7.22$, $p < 0.001$; Winter $F_{3,139} = 7.07$, $p < 0.001$). When season was included in the model, the effect was significant (Season $F_{1,285} = 10.39$, $p = 0.0014$).

Host growth response was either significantly positive or was not significantly different from zero (i.e., no net fitness effect of infection; Fig. 3.1). The symbiotically effective *Bradyrhizobium* genotypes (#14, #38, #49) failed to provide benefit to *Lotus* in some conditions, while the ineffective genotype (#2) never affected host growth in any conditions. Growth benefit from infection was completely eliminated by nitrogen fertilization for the effective *Bradyrhizobium* genotypes in Fall (Fig. 3.1). In Winter, nitrogen fertilization significantly decreased, but did not eliminate, the growth benefit from the effective genotypes (t-test among nitrogen treatments within *Bradyrhizobium*

genotypes #'s 14, 38 and 49, $p < 0.05$). No un-inoculated control plants were contaminated (nodulated) in either experiment.

Host leaf nitrogen content exhibited significant effects of rhizobial genotype (Fall $F_{3,63} = 79.86$, $p < 0.0001$; Winter $F_{3,67} = 185.89$, $p < 0.0001$), nitrogen fertilization (Fall $F_{1,65} = 68.37$, $p < 0.0001$); Winter $F_{1,69} = 23.89$, $p < 0.0001$) and their interaction (Fall $F_{3,63} = 58.41$, $p < 0.001$; Winter $F_{3,67} = 48.70$, $p < 0.001$). When season was included in the model, the effect was significant (Season $F_{1,136} = 12.37$, $p < 0.001$).

Host leaf nitrogen content was either significantly increased or not significantly different from uninoculated control plants for all genotype-nitrogen treatment combinations but one. In Winter, leaf nitrogen response significantly *decreased* when hosts were infected with the ineffective rhizobial genotype (#2; Fig. 3.2).

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 3.1). *Bradyrhizobium* genotype had a significant effect on nodule number in both experiments (Table 3.1), with the ineffective genotype #2 tending to form the most nodules in both experiments (Table 3.2). Nitrogen fertilization had a significant negative effect on nodule number in Fall but not Winter (Table 3.1). Among genotypes, nitrogen fertilization tended to decrease nodule number in the Fall, though this was only significant for *Bradyrhizobium* genotype #49. In Winter, nitrogen fertilization increased nodule number significantly for genotype #2 only and had no significant effect for other genotypes.

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 3.2). Nitrogen fertilization had a significant negative effect on nodule mass in both experiments (Table 3.1). Nitrogen fertilization significantly decreased the least squares means of nodule mass for all genotypes in both experiments (Table 3.2).

When included in the model, season had a significant effect on both nodule number ($F_{1,285} = 56.76$, $p < 0.0001$) and nodule mass ($F_{1,285} = 29.03$, $p < 0.0001$).

Host growth response exhibited a significant positive effect on nodule mass in both experiments but no effect on nodule number (Fig. 3.3, Table 3.3).

Discussion

The net fitness effects of microbial symbioses can vary for plant hosts, dependent on interactions among partner genotypes and the environment, 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 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. 2010; 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. 2010). Under conditions with no soil nitrogen, the benefits of rhizobial infection are amplified, and costs are difficult or impossible to uncover since the uninfected controls are chlorotic and barely grow (and detecting costs means showing that inoculated plants are growing significantly less than the malnourished controls). Our host fitness data here show that the symbiosis between *L. strigosus* and sympatric *Bradyrhizobium* can readily shift 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 show that that hosts can finely tune investment into 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), since our fertilized hosts, whose roots are picking up significant nitrogen, were nonetheless able to downregulate metabolic support of some rhizobia.

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 downregulated 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 3.3), inconsistent with the hypothesis that *Lotus* adaptively modulates nodule number (Fig. 3.3, Table 3.3) 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 3.3; Fig. 3.3). 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 coinfect 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 remains 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 extrinsic conditions in a specific attempt to detect parasitism. Lau and colleagues (2012) found mixed evidence of *Bradyrhizobium* parasitism on soybean, 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 x environment interactions (GxE). Simonsen and Stinchcombe (2014) found evidence of *Ensifer* parasitism (*Sinorhizobium*) 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 x genotype (GxG) interactions can mediate rhizobial parasitism. Parallel research of mycorrhizal symbioses has uncovered similar patterns. Hetrick (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 & Smith, 2003), soil phosphorous levels (GxE; Smith et al.

2004) and plant development (Li et al. 2005). In non-agricultural host plants, Klironomos (2003) found that mycorrhizal taxa were often mutualistic for one host and parasitic for another (GxG). Two recent studies have employed massive 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 favors 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 indicate that parameters exist in which symbionts can cause negative effects. But the ecological relevance of some of these treatments is questionable, 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 environmental 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 seasonality that bracket

wild populations. But rapid changes in climate and mineral nitrogen availability are shifting the environmental limits that plant species experience. Many plants exhibit phenological shifts in response to climate change (Walther et al. 2002). Earlier spring emergence exposes such plants to altered seasonal patterns relative to historic patterns. Our experiments showed that shifts to earlier emergence could reduce or eliminate the benefits of symbiosis for *Lotus* hosts (if seedlings emerge under shorter daylength conditions). Moreover, anthropogenic inputs of nitrogen into ecosystems have also massively increased in the past 150 years driven by combustion of fossil fuels (Vitousek et al. 1997; Tillman 1999; Dentener et al. 2006). Nitrogen deposition could impact legume-rhizobium symbiosis by reducing or eliminating the benefit of rhizobial symbiosis for host legumes. Since our data show that *Lotus* does not prevent *Bradyrhizobium* infection when saturated for mineral nitrogen, increased soil nitrogen could have deleterious long term effects on the evolution of *Lotus* and *Bradyrhizobium* populations. For instance, Kiers and colleagues (2007) found that soybeans evolved relaxed mechanisms of host control over decades of selection in agricultural contexts. But controversy exists in these predictions, host control has also been posited to evolve to be even more efficient under long-term fertilization, thus selecting for rhizobia that still provide net benefit in the nitrogen enriched soils (Kiers et al. 2007). Our data suggests that this latter possibility is unlikely since the growth benefit of *Bradyrhizobium* infection was minimal or zero in the fertilized treatment, and because *Bradyrhizobium* can downregulate or shut down nitrogen fixation when infecting a fertilized plant (Regus et

al. 2014). Future work must address how environmental changes such as nitrogen deposition reshape the coevolutionary trajectories of plant microbial symbioses.

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Table 3.1. Effects of *Bradyrhizobium* genotype and fertilization treatment on host investment into *Bradyrhizobium*. F ratio and significance from least square fit linear model for fixed effects of *Bradyrhizobium* genotype, nitrogen fertilization treatment, the interaction among these factors, and plant biomass. Single asterisks indicate $p < 0.05$, double asterisks indicate $p < 0.001$, and triple asterisks indicate $p < 0.0001$.

Experiment	Fixed Effect	Degrees of freedom	Nodule Number	Mean Individual Nodule Mass (mg)
Fall	Genotype	3	16.35 ***	32.39***
	Fertilizer	1	11.99 **	70.20***
	Genotype x 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 x Fertilizer	3	8.40***	3.24*
	Plant Biomass	1	158.06***	26.32***

Table 3.2. Variation in nodule formation and nodule mass among treatments. Predicted least square mean estimates of nodule number and individual nodule mass (see methods for details). Asterisks indicate significant differences among nitrogen treatments within bacterial genotype in pairwise t-tests corrected for multiple comparisons ($p < 0.05$).

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)

Table 3.3. Effects of host growth response on *Lotus* investment in nodule formation and nodule growth. Host growth response from infection predicts nodule mass but not nodule number. F-ratio and significance from general linear model. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. Genotype shows effect of rhizobial genotype. Fertilizer shows the effect of nitrogen fertilization.

Experiment	Fixed Effect	Degrees of Freedom	Nodule Number	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***

Figure 3.1. Mean host growth response to infection (± standard error). Host growth response is the percent growth difference between infected plants and uninfected control plants. Asterisks indicate significant difference from zero within each strain treatment in ones sample t-test ($p < 0.001$).

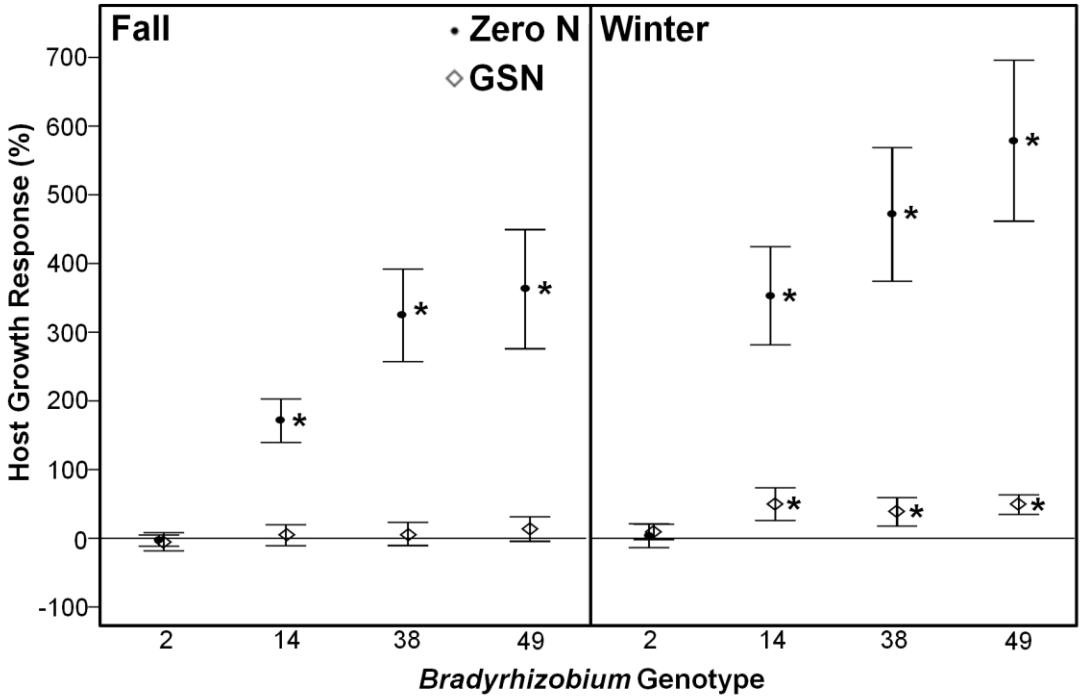


Figure 3.2. Host leaf nitrogen content (± standard error). Leaf nitrogen response is the mean percent leaf nitrogen by mass. Dashed lines show mean percent leaf nitrogen of uninfected control plants. Asterisks indicate significant differences between infected and uninfected plants (first column) per t-test controlling for multiple comparisons ($p < 0.001$) and n.s. is non-significant.

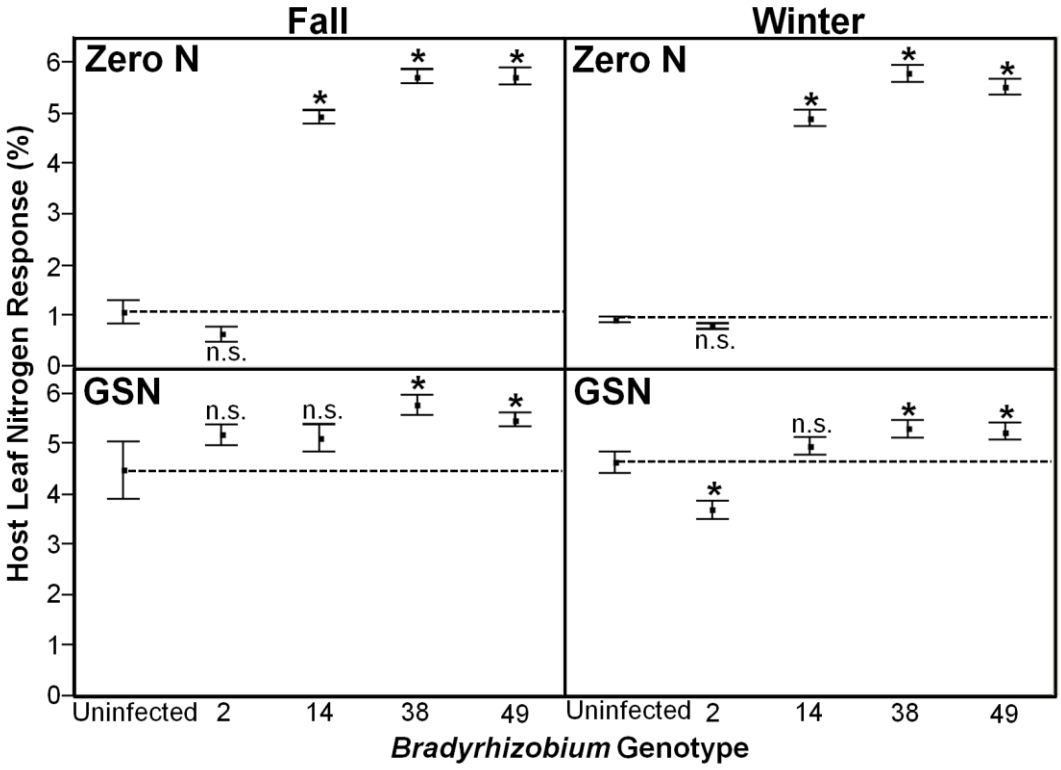
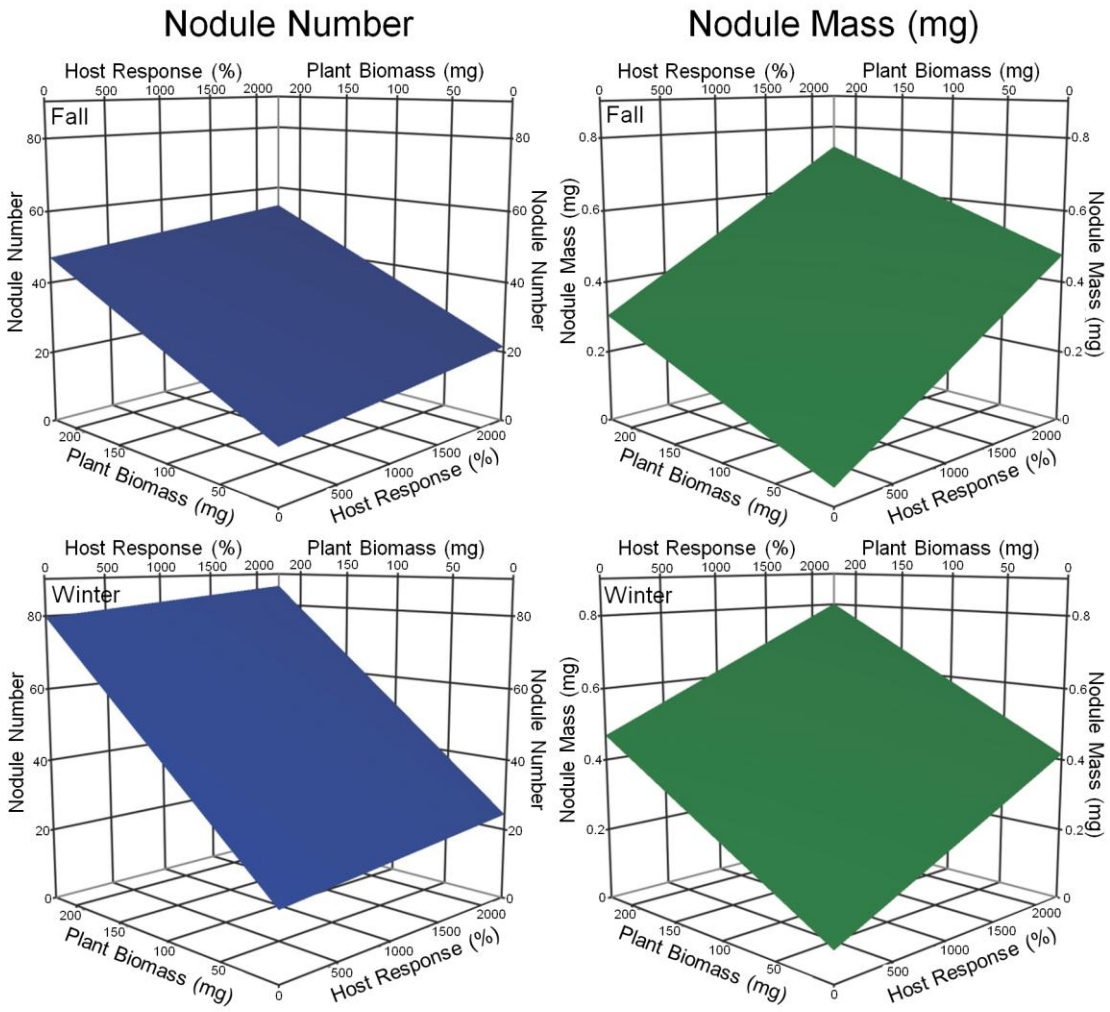


Figure 3.3. Response surface plots of nodule number and nodule mass. Graphs depict the relative contribution of Plant Biomass and Percent Host Response to both nodule number and nodule mass in both experiments. Graphs are derived from GLM analysis. Blue shows nodule number and green shows nodule mass.



General Conclusions

The benefits host receive from symbiotic bactrial infections can be context dependent (Bronstein 1994). Theory suggests hosts should control symbionts to prevent the evolution and spread of exploitative symbionts (Bull& Rice 1991; Sachs et al. 2004) We found that mineral nitrogen saturation can readily eliminate growth benefits of *Bradyrhizobium* infection for *L. strigosus* and the plant continues to allow infection even when growth benefits from infection are eliminated. *Lotus strigosus* from a historically nitrogen-pristine site exhibited host control traits that were resilient to nitrogen fertilization and hosts plants modulated investment in *Bradyrhizobium* nodules to delimit parasitism, even for *Bradyrhizobium* that provide no benefit to *L. strigosus*.

Taken together, these results suggest that if soils become nitrogen-enriched by anthropogenic nitrogen deposition, as is has been ongoing for more than a century (Vitousek et al. 1997; Dentener et al. 2006), *L. strigosus* at polluted sites may currently be gaining little or no benefit from rhizobial infection. It is possible that selection to maintain host control traits can be relaxed in nitrogen-enriched contexts, leading to the evolution and spread of exploitative rhizobia and the breakdown of symbiosis. Though our results suggest legumes can potentially prevent negative growth effects in high nitrogen contexts and continue to control less effective rhizobia relative to effective rhizoia, at least in the short term. More experimental work is needed to assess the evolutionary consequences for host control traits in legumes over long term selection in nitrogen-enriched contexts.

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