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

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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 from both 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 co-inoculated 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.

1. 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 [1–3]. Bacteria have a tremendous evolutionary advantage over eukaryotic hosts in terms of generation time and population size [3], and thus mutations that allow exploitation of host resources without reciprocation can frequently arise in symbiont populations [4]. Moreover, the benefit that bacteria provide to hosts can be context dependent [5]. 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 [6–9]. 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 [3,4,10–12]. Empirical work has uncovered evidence of these host-control mechanisms in diverse hosts including insects [13] and other invertebrates [14], mammals [15–17] and plants [18–23]. 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 [24]. Symbiotic rhizobia most often infect roots and occupy host-derived tumours (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 [7,25–28]. Ineffective rhizobia can potentially gain a metabolic advantage by redirecting plant carbon towards
selfish ends [10,29,30] as opposed to engaging in energetically expensive nitrogen fixation [31].

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) [10,11]. Subsequent to nodule formation, legumes can reduce within-nodule growth rates of ineffective rhizobia (sanctions) [10,11]. Partner choice in legumes has received mixed empirical support; hosts that are co-inoculated with effective and ineffective rhizobia that are closely related are often nodulated with equal frequency by both [22,23,29,32]. Some legume hosts can engage in partner choice, especially when host discrimination is occurring among divergent rhizobial strains or populations [22,23]. 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) [19–23] but see [32,34].

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 [35–39]. Assuming that host-control traits are costly to express [12,40], similar to other plant defences against bacteria [41,42], we predict that host control will be downregulated in nitrogen-rich soils, where hosts can gain nitrogen primarily from less costly mineral sources [43] rather than symbionts. Yet, if partner choice and/or sanctions are downregulated, this could favour the spread of ineffective symbionts and a potential collapse of the symbiosis [3,44]. 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 [19].

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 [45]. We co-inoculated *L. strigosus* with *Bradyrhizobium* populations composed of an effective strain and an ineffective strain. Sachs et al. [22] 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, favouring 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 [22]. We also report data from the current experiments from single-strain inoculations which suggest that 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 co-inoculated hosts. To estimate the relative contribution of symbiotic nitrogen fixation to plant growth, we measured $^{15}\text{N}$ in leaf tissue of infected plants and compared it to uninfected plants in each treatment. The goals of the experiment were (i) to examine whether legume partner choice and sanctions are downregulated in growth-saturating nitrogen (GSN) 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.

2. Material and methods

(a) Selection and culturing of *Bradyrhizobium* strains

Four *Bradyrhizobium* strains, referred to as numbers 2, 14, 38 and 49 [27], were selected for this study based on previous genotypic and phenotypic analyses [13,22,27,46]. Strain 49 provides approximately 5-fold increase in host shoot biomass relative to uninfected control plants, whereas strains 38, 14 and 2 provide approximately 3.5-fold, approximately 2-fold and approximately 0.95-fold relative benefit, respectively, for *L. strigosus* (strain 2 is ineffective) [27]. Rhizobial inocula were generated using published protocols [22].

(b) *Lotus strigosus* seed collection and preparation

In June 2011, *L. strigosus* fruits were collected at Bodega Marine Reserve (BMR), CA, USA, sympatric to where our *Bradyrhizobium* strains were originally collected, from coastal sand dunes that have little capacity to retain plant-available nitrogen [47]. Host seed sets comprised equal mixes of seeds from different parental plants. Seed preparation and planting followed published methods [46].

(c) Soil nitrogen assays

We estimated total soil nitrogen concentration and soil mineral nitrogen (extractable NO$_3$ and NH$_3$) at BMR and 10 other *L. strigosus* populations across California. Three soil cores (10 cm depth) per site were sampled from 1 m$^2$ (where *L. strigosus* had been collected previously), then treated and analysed as per published methodology [48]. Nitrogen analysis was performed at the FIRM Isotope Facility at UC Riverside.

(d) Inference of growth saturation of nitrogen-fertilized *Lotus strigosus*

Minimal 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 an eight-week greenhouse experiment (7 March 2011 to 2 May 2011; see below for fertilizer protocol, harvest and data collection).

(e) Partner choice and sanctions experiments

Replicated experiments were performed in the same greenhouse in autumn 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 autumn 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 and 49), co-inoculations of each effective strain with the ineffective strain \((14 \times 2, 38 \times 2, 49 \times 2)\) and uninfected control plants. Each inoculation experiment consisted of 288 plants (nine replicate plants per treatment, 16 treatments, two replicate blocks). Fertilization treatments were zero nitrogen fertilizer and fertilization with 0.5 g l\(^{-1}\) KNO\(_3\) (GSN; electronic supplementary material, figure S1). Each week, 10.0 ml of a nitrogen-free Jensen’s solution was added to each plant (with KNO\(_3\) for GSN treatments), beginning 3 days prior to inoculation. Seven days after placement in the greenhouse, plants were each inoculated with log-phase rhizobia (5.0 ml sterile ddH\(_2\)O, \(1.0 \times 10^8\) cells ml\(^{-1}\)). Co-inoculations comprised an equal mixture of each strain and uninfected plants received 5.0 ml sterile ddH\(_2\)O. Each experiment lasted eight weeks from inoculation to harvest.

(f) 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 co-inoculated plants, a random subset of nodules were cultured from two randomly selected plants, per fertilization treatment, per block, in both autumn and winter. Five nodules (in the autumn) 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\(_2\)O \((10^{-3}, 10^{-5})\) and spread onto four to six MAG-agar plates. Colony counts from at least two plates were used to estimate 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 \((100 \mu g \text{ ml}^{-1})\) to quantify relative population sizes of different strains within each nodule [22]. Strains 14, 38 and 49 are sensitive to streptomycin, whereas strain 2 is resistant [22]. 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.

(g) Leaf \(^{15}\text{N}\) assays
We compared leaf \(^{15}\text{N}\) ‘atom per cent difference’ (\(\delta^{15}\text{N}\)) between single infected and uninfected plants for each *Bradyrhizobium* strain, within each fertilization treatment. When plants incorporate synthetically fixed nitrogen, leaves exhibit lowered \(\delta^{15}\text{N}\) relative to uninfected plants because of isotopic fractionation by rhizobia [49]. Leaflets were removed from dried shoots, ground, and analysed at the FIRM Isotope Facility at UC Riverside. We did not analyse co-inoculated plants, because variation in \(\delta^{15}\text{N}\) would be confounded by the plant’s interaction with multiple rhizobial strains.

(h) Data analysis
To test for partner choice, we quantified ‘nodule occupancy’ within co-inoculated plants, defined as the proportion of nodules per plant occupied by the effective versus the ineffective strain (co-infected nodules were assigned to both categories) and tested for significance using a \(\chi^2\)-test against a null of 0.50 [27]. To test for sanctions, we compared per plant mean rhizobial population sizes of effective versus ineffective rhizobia in all sampled nodules, whether single or co-infected, of co-inoculated plants [27], and tested for significance with analysis of variance (ANOVA) within each treatment combination (JMP v. 10.0, SAS Institute [50]). 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 nodules population size (sanctions; Fit Model Platform, JMP v. 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 per cent difference in total dry plant biomass between inoculated hosts (infected) and un-inoculated (uninfected) plants. Total soil nitrogen comparisons and \(\delta^{15}\text{N}\) differences were analysed using one-way ANOVA in JMP 10.0 and Student’s \(t\)-test for pairwise comparisons with correction for multiple comparisons. Minimal GSN was analysed 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.

3. Results

(a) 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%) [51,52]. Soil nitrogen at BMR was consistently lower than most southern California *L. strigosus* sites in terms of total nitrogen and mineral nitrogen (electronic supplementary material, table S1). Sites with the greatest total soil nitrogen, for example 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 [51,52].

(b) Growth-saturating nitrogen
Nitrogen fertilization significantly increased *L. strigosus* growth (ANOVA; \(F_{6,435}, p < 0.0001\)). In pairwise comparisons among fertilization treatments, host growth at 0.5 g l\(^{-1}\) KNO\(_3\) was significantly greater than at all lower concentrations. Concentrations more than 0.5 g l\(^{-1}\) did not significantly enhance host growth relative to 0.5 g l\(^{-1}\) (electronic supplementary material, figure S1). None of the experimental plants exhibited evidence of rhizobial contamination (nodulation).

(c) 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 figure 1). In zero nitrogen, the number of nodules formed did not differ among strains within each experiment, except for one treatment (autumn strain 14 formed fewer). In GSN, strain 2 formed the most nodules in each experiment, but differences among strains were not always significant (figure 1). Per plant mean nodule population sizes for single-infected strain 2 nodules were not significantly different in winter, comparing nodules among fertilizer treatments (table 1). 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 (electronic supplementary
material, figure S2) and previous work showed population size (within strain) is positively correlated with nodule mass [23].

(d) Partner choice
Only 13% (22 of 163; table 1) of analysed nodules were infected by the ineffective strain (including co-infected nodules), significantly less than expected by chance (0.50; Pearson $\chi^2 (1, n = 183) = 108.64, p < 0.0001$). 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 analysed because of contamination or sparse growth. No significant block effects or block $\times$ 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 (38 $\times$ 2 winter), the effective strains were significantly favoured over the ineffective strain (including co-infected nodules), especially in the autumn experiment. In zero nitrogen, plants infected with the effective strains most often exhibited significantly lower nodule occupancy (partner choice) and after nodule formation (sanctions), even when the hosts gained no net growth benefit from infection in both experiments (figure 3). In the autumn experiment, fertilized plants gained no net growth benefit from infection. Unfertilized plants gained significant growth benefit from infection in both experiments. 

(g) Leaf $\delta^{15}$N analyses
Patterns of $\delta^{15}$N leaf content were less pronounced in the autumn 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 S3). Conversely, nitrogen-fertilized plants assimilated relatively little or no symbiotically fixed nitrogen in both the autumn 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 S3).

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 [1]. 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 evolutionarily lost when not needed [44]. Plant defence traits against bacterial pathogens often entail significant fitness costs to express, including R-gene mediated immunity [41] and induced direct defences [42]. 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
Table 1. Rhizobial population size estimates with nodule data. Effective/ineffective 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 1 s.e. Asterisks show significant differences among per plant mean rhizobial population sizes within nodules (including all analysed 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). n.a., not applicable.

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symbiotically fixed nitrogen and no net fitness benefit from rhizobial infection. This result is consistent with previous work with soya beans that examined sanctions [19], 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 [22]. Among the 162 nodules examined from co-inoculated plants, approximately 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 ineffective strains in singly inoculated plants (figure 1) [27]. 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 [22, 27].

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 [4,10,12] and sanction non-fixing rhizobia by reducing oxygen supply to those individual nodules [20]. 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 co-infected 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 × 10^5; 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; see the electronic supplementary material, table S4, for population sizes of co-infected nodules). Moreover, nodule number, nodule mass and population size data reported here suggest the absence of strain-specific effects of nitrogen that could confound interpretation of sanctions (figure 1 and table 1; electronic supplementary material, figure S2). 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 rhizobium.

We examined wild *L. strigosus* hosts from soils with low nitrogen concentrations (electronic supplementary material, table S1) and with little ability to retain mineral nitrogen [47]; 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. 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 [35]. Global increases in soil nitrogen content are now driven by atmospheric deposition [45], 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 et al. [34] 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 [54]. 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 [44]. 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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