Mutualist-Provided Resources Increase Susceptibility to Parasites

Eunnuri Yi[1*], Nova Meng[1], and Corlett W. Wood[1]

[1]Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

*Correspondence

Eunnuri Yi, eunnuri@sas.upenn.edu

Abstract

Mutualistic microbes often increase their host's susceptibility to parasite infection. The phenomena of mutualisms increasing susceptibility to antagonists is a significant and especially well-documented subset of indirect costs. We tested two competing hypotheses to identify the cause of this phenomenon. First, in resource-exchange mutualisms in particular, mutualist-provided resources could attract antagonists. If mutualists provide hosts with key resources that are difficult to obtain otherwise, antagonists might preferentially attack hosts with mutualists because those hosts are more resource-rich. Second, mutualism establishment in and of itself might increase host vulnerability to antagonists, as some antagonists mimic mutualists to evade host defenses, and many hosts also downregulate their immune systems when establishing mutualisms. We independently manipulated mutualism establishment and resources traditionally provided by mutualists to directly test which mechanism underlies increased susceptibility to antagonists, using parasitic root-knot nematodes in the legume-rhizobia mutualism. We found little effect of mutualism establishment on parasite infection, as nodulation without nitrogen fixation did not significantly increase parasite infection. By contrast, nitrogen did increase

parasite infection. However, the resource effect was non-linear and was not fully explained by nitrogen assimilation into plant tissues. Our results suggest that mutualist-provided resources are an important driver of indirect ecological costs, but that this effect may be complex.

Key words: mutualism, parasitism, indirect costs, symbiosis, nitrogen, resource use, legume, rhizobia, root-knot nematode

Introduction

Mutualistic species interactions are ubiquitous (Janzen 1985). Mutualists provide fundamentally important biological functions for their partners and their ecosystems (Bronstein 2001, Traveset and Richardson 2014). These functions are diverse, spanning transportation, protection, reproduction, and nutrition (Kleipzig et al. 2009). But despite these wide-ranging benefits, mutualisms are also costly (Bronstein 2015). Internal costs arise directly from investment in specialized goods and services provided to partners (Bronstein 2001). Mutualisms also incur ecological costs, where external species interactions that the organism engages in can have adverse effects. These external costs can be highly significant, as mutualisms can facilitate competition, compromise or disrupt other mutualisms, or increase susceptibility to various antagonists (Richardson et al. 2000, Schmitt and Holbrook 2003, Hoek et al. 2016, Johnson 2021, Johnson and Bronstein 2019, Ness 2006, Hartley and Gang 2009).

The phenomenon of mutualisms increasing susceptibility to antagonists is a significant and especially well-documented subset of indirect costs. Examples of crosstalk between mutualists and antagonists spans many different fields under various terms such as priority effects, priming, and niche modification (Mauch-Many et al. 2017, Mhlongo et al. 2018, Tiwari et al. 2022, Pickard et al. 2017). The plant-pollinator and legume-rhizobia mutualisms can open up opportunities for increased predation and herbivory (Nauer et al. 2018, Simonsen and Stinchcombe, 2014, Freedman et al. 1987). The ant-fungus nutritional mutualism, ant-plant protection mutualism, and cleaner fish-client interactions are occasionally exploited by pathogenic fungi, herbivores, parasites, and predators that imitate the host's mutualist partner (Herrera et al. 2002, Frederickson and Gordon 2007, Little and Currie 2009, Meehan et al. 2009, Bshary 2002).

There are two broad hypotheses to explain why mutualisms may increase susceptibility to antagonists. First, in resource-exchange mutualisms in particular, mutualist-provided resources may attract antagonists. If mutualists provide hosts with key resources that are difficult to obtain otherwise, antagonists may preferentially attack hosts with mutualists because those hosts are more resource-rich. For example, enriching seawater with nutrients, which increased the density of mutualistic zooxanthellae symbionts and subsequently the growth of their coral hosts, also increased the density of a corallivore snail by an order of magnitude (Rice et al. 2021). A meta-analysis found that in the mutualism between plants and mycorrhizal fungi, the density and consumption of chewing insect herbivores was higher on plants colonized by mycorrhizae (Koricheva et al. 2009). Insect herbivores have also been found to cause greater damage on hosts associating with rhizobia that provide more nitrogen (Simonsen and Stinchcombe 2014).

Second, mutualism establishment in and of itself may increase host vulnerability to antagonists. Some antagonists mimic mutualists to evade host defenses. The ant-plant mutualism is exploited by ant and beetle species that mimic ant mutualists and effectively function as parasites (Janzen 1975, Raine et al. 2004). The predatory fang blenny can mimic the cleaner wrasse to feed on tissues of fish visiting cleaning stations, exploiting the client-cleaner fish mutualism to increase its foraging success (Moland and Jones 2004). Many hosts also downregulate their immune systems when establishing mutualisms, increasing their susceptibility to infection (Salem et al. 2015). This may be an especially important factor in symbiotic mutualisms, which involve the coordinated establishment of prolonged contact between partners. Cnidarian immunity is generally downregulated during symbiosis establishment and often upregulated during the loss of symbionts (Mansfield and Gilmore, 2019).

As the above examples illustrate, both mutualism establishment and mutualist-provided resources likely contribute to the indirect costs of mutualism. A definite test of these competing hypotheses, however, requires de-confounding mutualism establishment from mutualist-provided resources in a single system. This is especially important because the two mechanisms are not mutually exclusive: both could (and probably do) operate at the same time. Nevertheless, the two mechanisms would have very different implications for mutualism evolution. If mutualist-provided resources increase susceptibility to antagonists, then selection imposed by antagonists may constrain the evolution of resource exchange in mutualisms. Alternatively, if mutualism establishment in and of itself increases susceptibility to antagonists, then selection imposed by antagonists may favor the evolution mechanisms to discriminate friend from foe, but would not affect the subsequent exchange of benefits.

Here, we independently manipulated mutualist-provided resources and mutualism establishment to directly test which mechanism underlies increased susceptibility to antagonists. The legume-rhizobia mutualism is ideal for testing these competing hypotheses because the legume-rhizobia system allows for mutualist-provided resources (nitrogen) to be decoupled from mutualism establishment (nodulation). The legume *Medicago truncatula*, with its rhizobia partner *Ensifer meliloti*, is a model system for the study of resource exchange and symbiotic mutualisms. Rhizobia bacteria fix atmospheric nitrogen in exchange for plant carbon. To fix nitrogen, the rhizobia and plant must first form specialized structures called nodules on roots. Nodulation is a distinct stage of mutualism establishment that necessarily precedes resource exchange, and is controlled by different plant and rhizobia genes. This legume-rhizobia mutualism is also known to increase susceptibility to the northern root-knot nematode *Meloidogyne hapla*, a parasite that forms galls on plant roots and siphons resources from the host

(Wood et al. 2018). Rhizobia and root-knot nematodes both form specialized root structures and indirectly interact through the legume host (Catella et al. 2021). Both rhizobia-provided resources (nitrogen) and establishment of the rhizobia mutualism (nodulation) could be responsible for increasing susceptibility to nematodes. Rhizobial Nod factors, which signal to begin legume nodule formation, suppress plant immunity, and it has been shown that a Nod factor receptor previously thought to function solely during rhizobial symbiosis is also active during plant pathogen infection (Gourion et al. 2015). Plant-parasitic nematodes also have genes likely to be the product of horizontal gene transfer from a rhizobial origin, and it is hypothesized that these lateral gene transfers and duplications have facilitated the ability to parasitize plants (Danchin et al. 2010).

In this study, we independently manipulated mutualist-provided resources (nitrogen) and mutualism establishment (nodulation) in the legume-rhizobia system to evaluate their relative impact on parasite infection. We found little effect of mutualism establishment on parasite infection; nodulation without nitrogen fixation did not significantly increase parasite infection. By contrast, mutualist-provided resources (nitrogen) did increase parasite infection. However, the resource effect was non-linear — the intensity of parasite infection peaked at intermediate nitrogen levels — and was not fully explained by nitrogen assimilation into plant tissues. Our results suggest that mutualist-provided resources are an important driver of indirect ecological costs, but that this effect may be complex.

Methods

Study System

Medicago truncatula is a Mediterranean legume that is a popular model system for the study of mutualisms. Rhizobia form specialized nodule organs on roots through a tightly

regulated process of signaling between the plant host and bacteria (Ferguson et al 2018). Inside the developed nodule, rhizobia convert atmospheric nitrogen into ammonia, which is a bioavailable form of nitrogen for the legume host. *M. truncatula* associates with rhizobia in the genus *Ensifer*, primarily *E. meliloti and E. medicae*.

The parasite we used in our experiment is the northern root-knot nematode *Meloidogyne hapla*. *M. truncatula* is susceptible to *M. hapla*, which is an extremely generalist obligate parasite that establishes galls on many plant and crop species' root systems (Castagnone-Sereno et al. 2013). Second-stage juvenile root-knot nematodes enter roots in the elongation region and migrate to their chosen spot, where they become sedentary and inject chemical effectors that cause plant cell nuclei to duplicate without cell division. Root-knot nematodes use their stylets to secrete saliva and feed on phloem rich in carbon and nitrogen from these giant cells. This resource extraction by root-knot nematode parasitism leads to a decrease in reproductive fitness in *M. truncatula* (Wood et al. 2018).

Greenhouse Experiment

We factorially manipulated nodulation and nitrogen availability to disentangle the effects of mutualism establishment and mutualist-provided resources on susceptibility to parasite infection. We manipulated mutualist-provided resources (nitrogen availability) via the application of nitrogen fertilizer. Our experiment consisted of three nitrogen fertilizer levels (0, 0.625, and 5 mM N, hereafter referred to as no-N, low-N, and high-N).

We manipulated mutualism establishment via two inoculation treatments: no rhizobia (R-; no nodulation) and rhizobia (R+; nodulation). We used four different rhizobia strains (A145, Rm41, Em1021, and Em1022) in our experiment. Our selection of rhizobia strains was intended

to maximize variation in nitrogen fixation (mutualist-provided resources), in order to deconfound nodulation and nitrogen fixation in inoculated plants. Rhizobia strains A145 and Rm41 can form small white nodules, but are incompatible for nitrogen fixation with our chosen genotype, *M. truncatula* Jemalong A17 (Wang et al 2018). This is possible because rhizobia have distinct genes for mutualism establishment and resource exchange: Nod genes induce the legume host to initiate nodule formation by lengthening root hairs and increasing cell division, while nif and fix genes are involved in nitrogen fixation after nodulation (Shamseldin 2013, Jimenez-Guerrero et al. 2017). Rhizobia strains Em1021 and Em1022 both nodule and fix nitrogen on *M. truncatula*, although Em1022 is known to be a generally better partner than Em1021 (Batstone et al. 2020).

Briefly, we germinated seeds of *M. truncatula* genotype A17 ("Jemalong") under sterile conditions in a sequence of scarification, sterilization, and stratification (Barker et al. 2006). To prepare for planting, we filled 66-mL Cone-tainer pots (Stuewe and Sons, Inc., Tangent, OR, USA) with a 1:4 (volume:volume) mixture of perlite and sand. These filled Cone-tainer pots were sterilized by autoclaving at 121 C twice for 90 minutes. We planted one germinant in each Cone-tainer and fertilized each plant with 5 mL of Fahraeus fertilizer (Batstone et al. 2017) corresponding to its respective nitrogen treatment. The experiment was set up in a temperature-controlled greenhouse at the University of Pennsylvania's Carolyn Lynch Laboratory. We fertilized plants with 5 mL of fertilizer twice a week using a peristaltic pump with tubing that was sterilized before and after each use. We grew the plants in an incomplete block design to minimize blocking effects and the risk of cross-contamination across rhizobia strains. To additionally reduce contamination, we used 12 in tall transparent plexiglass sheets to separate plants of different rhizobia strains.

Two weeks after planting, we inoculated all plants with rhizobia and nematodes. To collect M. *hapla* nematode eggs, galled roots were collected from a cv. Rutgers tomato greenhouse cultured with the LM line of *M. hapla* nematodes procured from North Carolina State University. This line was originally collected in France, the native range of *M. truncatula*. To extract eggs, we cut the roots, shook them in 10% bleach for two minutes, and strained them over stacked 75 and 25 µm pore sieves to filter out large particles and collect the eggs. After decanting the eggs off the bottom sieve with tap water, we counted samples of the collected eggs under a microscope to calculate egg density in solution. We then diluted the culture with water to inoculate each plant with approximately 300 nematode eggs suspended in 5 mL DI water.

To inoculate plants with rhizobia, we grew strains from glycerol stocks and stab cultures in sterile tryptone yeast liquid media in a shaking incubator at 180 rpm and 30 °C. After two days, we streaked the cultures onto solid tryptone yeast agar media in a biosafety cabinet. These plates were incubated at 30 °C until distinct colonies formed, at which point we selected individual colonies from each strain to grow in tryptone yeast liquid media. After these single-colony rhizobia cultures grew for two days in the shaking incubator, we diluted the liquid media to an OD600 of 0.1 using a spectrophotometer. Each plant was inoculated with 1 mL of diluted culture containing either A145, Rm41, Em1021, or Em1022. Plants in the R- treatment received 1 mL of sterile tryptone yeast liquid media as a control.

Data Collection

Every week, the number of fully opened leaves were counted on each plant as a proxy for growth rate. We harvested all plants 7.5 weeks after the start of the experiment (53 days; May 28-July 20, 2021). Shoots were placed into labeled brown paper bags and dried in a drying oven

at 60°C for at least a week before being weighed to the nearest 0.0001g. We counted seed pods and flowers on dried shoots. Root tissue was rinsed in tap water, patted dry with paper towels, and stored in plastic bags at 4°C to preserve nodule and gall root structures. We counted nematode galls and rhizobia nodules under a dissecting microscope to quantify nematode infection and rhizobia colonization (Wood 2018).

We scored nodule color as a proxy for active nitrogen fixation (Ott et al. 2005). White nodules indicate the absence of active leghemoglobin, which is pink in color and is necessary for N-fixation by binding oxygen (Wang et al. 2016, Wang et al. 2019). We classified pink and yellow nodules as N-fixing, and white nodules as non-N-fixing. We did not observe any senesced nodules, which are typically dark brown. After classification, nodules were plucked off with dissecting tweezers and stored in 1mL microcentrifuge tubes at 4°C. Nodules were kept in their microcentrifuge tubes with the lid ajar, and both roots and nodules were dried in a drying oven at 60 °C for at least a week and weighed to the nearest 0.001g.

Of the 22 plants in the uninoculated treatment, eight formed nodules, with five plants forming fewer than 13 nodules (Figure S1A). By contrast, rhizobia-inoculated plants had a mean of 34 nodules. Contamination was thus minimal, as most of the rhizobia contamination was limited to a few nodules, suggesting that they were likely contaminated fairly late into the experiment. Contrary to our expectation, some plants inoculated with A145 and Rm41, the "non-N-fixing" strains, formed pink (nitrogen-fixing) nodules. However, they formed significantly fewer N-fixing nodules than Em1021 and Em1022, and formed slightly more small nodules (Figure S1C and S1D). It is possible that a small number of these N-fixing nodules on plants inoculated with A145 and Rm41 may have been due to contamination by other strains.

However, the low nodule counts for the few uninoculated plants that were contaminated suggest that contamination would only account for a small proportion of these N-fixing nodules.

We counted nematode galls as a proxy for parasite load. A single reproductive female resides in a gall structure and will generally lay a single egg sac. However, multiple egg sacs can be present on what looks to the human eye like a single continuous gall. To confirm that gall number is an accurate proxy for parasite load, we counted both galls and the number of egg sacs (a direct proxy for the number of females) using a dyeing method with red food coloring (Kandouh et al. 2019). We found that egg sacs and galls were extremely highly correlated (r^2=0.969).

To quantify shoot nitrogen and carbon composition, we performed elemental combustion analysis. Dried shoot tissue was pulverized using a mortar and pestle. Samples of homogenized shoot tissue were weighed on an ultra-micro balance (CP2P Microbalance, Sartorius GmbH, Goettingen, Germany) in 4-5 mg increments and packaged into tin capsules. For shoots smaller than 4 mg in weight, we packaged the entirety of the sample available, excluding samples that were smaller than 2 mg. Elemental combustion analysis was run on a Costech ECS4010 Elemental Analyzer (Costech Analytical Technologies, Inc. Valencia, CA, USA) using atropine as a standard. Each run consisted of about 45 shoot samples, with five atropine standards per run which were used to generate a C:N curve. With this curve, the elemental analyzer calculated carbon and nitrogen composition (%) for each sample. Forty-two plant samples were replicated across runs, which were consistent (r^2=0.999). By multiplying carbon and nitrogen composition by shoot biomass, we calculated the estimated total nitrogen in the shoot.

Statistical Analysis

We fit generalized linear mixed models in R version 4.3.1 and RStudio 2023.09.0+463 using the glmmTMB package (Brooks et al. 2017) unless otherwise noted. In preliminary analyses, we used rhizobia as a fixed effect, but found no significant variation across rhizobia strains (Table 1, Figure S2). For models with nematode gall number (our proxy for parasite load) as the response variable, we fit a zero-inflated negative binomial error distribution.

We performed two complementary analyses to test whether mutualist-provided resources (nitrogen) or mutualism establishment (nodulation) increased parasite load. First, we tested for an effect of our nitrogen and nodulation treatments on total parasite load in a model with gall number as the response variable and nitrogen treatment, nodulation treatment (rhizobia presence or absence), the nitrogen-by-nodulation interaction, and block as fixed effects. When using nodulation as a fixed effect, we fit rhizobia strain as a nested random effect. We ran a second version of this analysis that corrected for plant size, by adding root biomass as an additional fixed effect. We did so because greater nitrogen availability increased total biomass (Figure S3). We view size-adjusted parasite load (hereafter referred to as the intensity of parasite infection) as a more appropriate estimate of the relative cost of infection. We did not directly measure fitness costs of infection in this experiment.

Second, we tested for an association between non-nitrogen-fixing nodules and parasite load. Because these nodules did not provide nitrogen, a significant relationship between the two would support the mutualism establishment hypothesis. We standardized the number of non-N-fixing and N-fixing nodules within each nitrogen treatment using a z-score, by subtracting the treatment mean from the raw counts and then dividing by the standard deviation to convert them to a mean of zero and a variance of one. Nodulation is known to vary depending on nitrogen availability (Van Schreven 1959, Weese et al. 2015), which is why we standardized

nodule counts within each nitrogen treatment. We included nitrogen treatment, block, the number of non-N-fixing nodules, the number of N-fixing nodules, and the interaction between nitrogen treatment and the number of non-N-fixing nodules as fixed effects.

Finally, we tested whether the effect of nitrogen on intensity of parasite infection was explained by nitrogen assimilation into plant tissues. We converted percent compositions of nitrogen and carbon to z-scores (mean of zero and a variance of one), after which they were incorporated as fixed effects for analysis. Nitrogen treatment, nitrogen composition, carbon composition, root biomass, rhizobia strain, and block were included as fixed effects.

We confirmed all residual errors as normally distributed, homoscedastic, and properly dispersed using the DHARMa package (Hartig 2020). Post-hoc Tukey tests and least-squares means and confidence intervals were calculated with emmeans (Leoth 2018). All figures were created in ggplot2 (Wickham 2016).

Results

Mutualist-provided resources, not mutualism establishment, increased parasite load

We found a significant effect of nitrogen on total parasite load (Figure 1A, Table 1A). Increasing nitrogen increased parasite load from no-N to low- and high-N. This effect was qualitatively similar across inoculated and uninoculated plants, and across rhizobia strains.

We found a significant effect of nitrogen on the intensity of parasite infection (size-adjusted parasite load) (Figure 1B, Table 1B). Larger plants had more parasites overall, such that size-adjusted parasite load peaked at low-N, with no-N and high-N plants having similar intensities of infection. We did not find a significant effect of rhizobia strain or symbiosis on intensity of infection (Figure 1B, Table 1B).

We did not find a significant effect of nodulation (mutualism establishment) on total parasite load (Figure 1A, Table 1A). Inoculated and uninoculated plants did not significantly differ in total parasite load (Figure 1A, Table 1A). Some variation within the rhizobia strains did exist in their proportion of N-fixing nodules, but strains did not differ significantly in nodule number, nodule weight, resulting plant biomass, or total shoot nitrogen (Figure S1, Table S1).

We found no significant effect of non-nitrogen-fixing nodule count on total parasite load. This suggests that in the absence of nitrogen fixation, nodulation does not increase parasite infection. This is consistent with the analysis above, in which we observed no difference in infection between nodulation treatments. We did observe a significant effect of nitrogen-fixing nodule count on total parasite load: more nitrogen-fixing nodules corresponded to a higher total parasite load.

Mechanism of the nitrogen effect

We observed a significant effect of tissue nitrogen composition on intensity of parasite infection ($p=0.005$). Higher tissue nitrogen composition led to a lower intensity of parasite infection. Yet, significant differences in the intensity of parasite infection between nitrogen treatments remained even after accounting for plant size and tissue nitrogen composition. There was no relationship between tissue carbon composition and the intensity of parasite infection.

Discussion

In this study, we found that the resources provided by mutualistic symbionts facilitated parasite infection. However, the resource effect was more complicated than we anticipated, in two ways. First, the effect on the intensity of parasite infection was non-linear, peaking at an intermediate nitrogen level. Second, the effect of nitrogen on parasite load was not fully

explained by nitrogen assimilation into plant tissues, suggesting that mutualist-provided resources may impact susceptibility to parasite infection via multiple physiological pathways. We also found that mutualism establishment had little effect on parasitism, despite the fact that legumes downregulate their local immune response when forming a symbiosis with rhizobia (Grundy et al. 2023, Cao et al. 2017). In sum, the results of this study reflect the importance of resources on the indirect costs of mutualism.

Resources increase parasite load, with the greatest intensity of parasite infection at intermediate resource levels

Under the mutualist-provided resource hypothesis, we predicted that parasites would preferentially infect resource-rich hosts. We expected this to manifest in our experiment as higher parasite loads at higher levels of nitrogen supplementation. We found, however, that nitrogen supplementation had a non-linear effect on parasite load. Although total parasite load increased from no-N to the low- and high-N fertilizer treatments, it leveled off between low- and high-N (Figure 1A).[1]

In contrast to total parasite load, size-adjusted parasite load (i.e., the intensity of infection) peaked in the low-N treatment, and declined in the high-N treatment. This pattern suggests that the relative cost of infection is highest at intermediate nitrogen levels, because although low- and high-N plants had similar numbers of parasites, low-N plants were more heavily infected given their overall size. Plants in the no-N treatment also had low parasite loads, but we suspect that this is simply because these plants were so small that they could not support any appreciable parasite load.

[1] This leveling off might have reflected our experimental design, where all plants were inoculated with a finite number of eggs such that the number of resulting galls could have been maximized at both low- and high-N, but the number of galls (around 10-30) was much smaller than the 300 egg dose of inoculation.

It is important to note that mutualist-provided resources increasing susceptibility to parasites does not necessarily mean that mutualists would be imposing a net cost, resulting in mutualists becoming antagonistic. Future research should explore at what resource level the net benefits of mutualism are maximized, integrating the effect of resources on parasite infection as well as the direct benefit of resources.

The resource effect is not fully explained by nitrogen assimilation into host tissues

Our original reasoning for the mutualist-provided resource hypothesis was that mutualist-provided resources enrich host tissues, making them more attractive to parasites. However, although nitrogen increased parasite load, our results differed from the prediction in two significant ways.

First, we found that increased tissue nitrogen composition (%N) actually decreased parasite load (Figure 3, Table 3). This was surprising, given the extensive literature on preferential feeding on higher nitrogen content due to its better nutritional quality (Van Alystyne et al. 2009, Chen et al. 2010 — but see Tomas et al 2011). Our experiment, however, was a no-choice experiment (parasites were not offered a choice between a nitrogen-poor and nitrogen-rich host), which may explain why our results differ from some of these past studies.

The negative relationship between nitrogen composition and parasite load in our experiment suggests that nitrogen is protective. Higher nitrogen composition in tissue may be indicative of nitrogen-rich host defense compounds. Many anti-herbivory defense compounds also utilize nitrogen, such as alkaloids, cyanogenic glycosides, glucosinolates, and benzoxazinoids (Harborne 1991; Zenk and Juenger 2007). This might also account for the observed peak in the intensity of parasite infection at intermediate nitrogen levels (Figure 3).

This intermediate peak could represent a balance between increased success of parasite infection on N-rich plants and resistance to parasites by some extremely N-rich plants. Plant hosts growing with high-N appear to face tradeoffs between either allocating their additional nitrogen to growing larger in size or increasing density of nitrogen composition. Larger size could reduce relative density and costs of parasites, while N-rich compounds or forms of dense storage may have deterred parasites outright (Figure S5).[2]

Second, assimilated nitrogen does not fully explain the effect of nitrogen resources. Even after accounting for the significant effect of nitrogen composition (N%) on total parasite load and intensity of infection, there remained a persistent effect of nitrogen treatment. This persistent effect of nitrogen treatment suggests that nitrogen is functioning in a way that affects parasite infection beyond assimilation into tissues. Nitrogen is known to affect plant hosts in many diverse, interlinked, and cascading ways which could impact parasite infection. As an essential element of amino acids, which are the building blocks for proteins, enzymes, and nucleic acids, nitrogen is necessary for cellular maintenance and developmental growth. In plants, it is also an essential component of chlorophyll which is necessary for photosynthesis. Various forms of nitrogen (nitrate and ammonium) function as signaling molecules within a complex network regulating uptake, metabolism, developmental phase change, and plant defense and immunity among other processes (Vidal et al. 2020, Hancock 2020, Xuan et al. 2017). Broadly, nitrogen can be utilized for size, photosynthesis, signaling, and defense. The pattern we identified of an intermediate peak may help to explain the wide range of effects of mutualisms on parasitism; while we focused on increasing susceptibility to parasites, many mutualisms do deter parasites (Braquart-Varnier et al. 2015, Gonzalez-Teuber et al. 2014, Jander 2015, Clay 1988, Wang et al.

[2] It has been noted, however, that if plant growth rate is greater than biomass removal by antagonism, increased plant defenses appear not to be necessary (Lou and Baldwin 2004).

2023). Nitrogen as a resource could both attract as well as defend against antagonists depending on the resource landscape, availability, and use.

This persistent effect of nitrogen on parasite infection could also be due to differing resource use and allocation across nitrogen treatments. Nitrogen can be used for signaling or defense, or allocated towards growth and reproduction. Biomass and growth rate can be interpreted as broad investment in size and growth (Figure S3). Plant hosts might allocate their resources differently depending on resource availability, such that defense may only become a priority over growth at a certain threshold of resource richness. Resources can also be differentially allocated to shoots, roots, leaves, or reproductive organs like seeds and fruit. Increased use for photosynthesis can be inferred from a high leaf count, shoot:root ratio, and possibly shoot nitrogen composition (Figure S3 and S4). Carbon:Nitrogen ratios are commonly used to reflect nitrogen use efficiency, such that a high C:N indicates high efficiency in use of nitrogen, as more carbon is photosynthesized per unit of nitrogen to allow for more efficient growth (Zhang et al. 2019, Dovrat et al. 2020). However, a low C:N could also potentially indicate higher tissue quality as high nitrogen composition indicates higher resource density (Figure S4). Variation in resource use — which we see here for plants of the same genetic background — could thus have effects on structuring species interactions.

Little effect of mutualism establishment on parasite load

In this study, mutualism establishment alone did not have a significant effect on parasite load. We found no statistical differences in parasite load between host plants inoculated and uninoculated with rhizobia, or between host plants inoculated with rhizobial strains of differing nitrogen fixation. One possibility for this was that our analysis was underpowered for the initial

test between rhizobia-inoculated plants and uninoculated controls. A relatively small number of plants formed zero rhizobia nodules, making our comparison between non-nodulated plants and nodulated plants unbalanced. However, we also showed that there was no relationship between non-nitrogen-fixing nodules and parasite load, consistent with our conclusion that mutualism establishment (nodulation) alone did not increase parasite load. By contrast, there was a significant increase in parasite load with nitrogen-fixing nodules, consistent with our conclusion that mutualist-provided resources increase susceptibility to parasite infection in our system. Thus, root-knot nematode parasite load responds much more strongly to nitrogen resources than to mutualist establishment.

This finding is surprising because it is well known that mutualist hosts undergo significant changes in their immune system while forming symbioses with microorganisms. Cnidarian immunity is generally downregulated during symbiosis establishment and often upregulated during the loss of symbionts (Mansfield and Gilmore, 2019). This is true in the legume-rhizobia mutualism as well. It is known to be tightly regulated, with the host systematically downregulating its innate immune system to prevent plant defense responses upon detecting rhizobial Nod factors (Grundy et al. 2023). Future research should interrogate why mutualism establishment alone seems to play a minor role in susceptibility to infection in the legume-rhizobia mutualism.

Implications for mutualism ecology

In our experiment, nitrogen had a significant and complex effect on susceptibility to antagonists. This finding has potentially wide-ranging implications because nitrogen is a common mutualist-provided resource. Organisms are driven to acquire nitrogen because it is a

necessary element for molecular, developmental, and ecological processes of life. However, because it is relatively limited in the environment, nitrogen is often provisioned biotically through species interactions (Knops et al. 2002, Fagan et al. 2002). Rhizobia fix nitrogen for legumes; mycorrhizal fungi also form symbiotic associations with plant roots, benefiting plants' ability to uptake nitrogen from the soil (Govindarajulu et al. 2005). Some cyanobacteria symbionts fix nitrogen for lichen, benefiting the fungus that provides the cyanobacteria a sheltered, protected environment (Zehr 2011). Similarly, algal turf communities fix nitrogen on coral reefs (Williams and Carpenter 1997). Nitrogen provisioning is also a common aspect of other mutualisms: in ant-plant protection mutualisms and bird-plant pollinator mutualisms, animals will deposit nitrogen-rich droppings to benefit the plant (Pinkalski et al. 2015; Defossez et al. 2010; Whelan et al. 2015). Nitrogen as a resource thus has effects on nutrient cycling, species interactions, and broader ecosystem functioning (Wiedenmann et al. 2023). Within nitrogen-limited environments, these mutualisms offset some environmental resource constraints, providing critical padding by increasing host and ecosystem nitrogen resource budgets. The legume-rhizobia symbiosis, for instance, fixes 21.5 Tg of nitrogen each year, and the cyanobacterial genus Trichodesmium is responsible for 240 Tg of nitrogen fixation per year (Guo et al. 2023, Berman-Frank et al. 2003).

Human activity in the age of the Anthropocene can also affect the ecology and outcomes of species interactions, as agricultural inputs contribute about 100 Tg of Nitrogen per year through synthetic fertilizer and organic inputs of manure and crop waste. These human inputs have dramatic downstream effects on ecosystems and species interactions: for example, fertilization reduces the need for plant hosts to engage in mutualism with arbuscular mycorrhizal fungi, leading to selection of inferior mutualists and potentially contributing to mutualism

breakdown (Johnson, 1993). Yet we cannot yet predict the outcomes of these effects on other species interactions without a sense of the resource dynamics structuring them. It has been proposed that the ecophysiology of carbon resource allocation can explain the ecology and evolution of mutualisms (Pringle 2015). Nitrogen resource dynamics of resource production, exchange, and use can likewise contribute to our understanding of indirect species interactions and ecosystem structure in a time of rapid anthropogenic change.

More generally, mutualist-provided resources may contribute to greater costs by antagonists. These dynamics of mutualist-provided resource use or allocation might also affect other species interactions outcomes, whether in priming, priority effects, or niche modification. These hypotheses can and should be tested in the future; to do so will require drawing upon and synthesizing various subfields of biology.

Mutualist-provided benefits can increase indirect costs through antagonistic species interactions. This concept of ecological costs has been demonstrated up to this point primarily within herbivory and predator-prey interactions; our study broadens this idea across antagonisms in a comprehensive test of resource and establishment hypotheses in the soil microbiome's broad, diffuse network of multi-species interactions. Microbial mutualists appear to facilitate parasite infection by way of mutualist-provided resources as opposed to through mutualism establishment in and of itself. Nitrogen is an essential and commonly limited elemental resource commonly provided by mutualists, including in the legume-rhizobia symbiosis tested here, as well as by mycorrhizae fungi and cyanobacteria to their respective ecosystem partners (Stacey et al. 1992). However, the actual resource effect of nitrogen here was a complex effect not fully explained by traditional proxy effects of resource assimilation or total host size. Although it is clear that antagonistic costs from herbivores, parasites, and predators deplete host resource budgets, these

costs must be contextualized. Especially for nitrogen, with its massive mutualist and anthropogenic inputs, mutualist-provided resource use, its costs, and its relationship to changing resource budgets should be considered as a new aspect undergirding species interactions and their outcomes.

Acknowledgements

We would like to thank Chigozie Ibe and Yoon Chang for data collection and their help in maintaining the experiment. We also thank McCall Calvert, Anisa Robinson, Addison Buxton-Martin, and Wood Lab members for their comments and feedback on the manuscript. The Plant Seminar group at the University of Pennsylvania provided helpful feedback on the experimental analysis and interpretation of results. We are grateful to Adrienne Gorny at North Carolina State University for *M. hapla* nematode lines, Nevin Young and the *Medicago* HapMap project for seeds, Qiulin Qin and Hongyan Zhu at the University of Kentucky for Rm41 and A145 rhizobia strains, and the USDA for Em1021 and Em1022 rhizobia strains. David Vann and the Instrumentation Core at the University of Pennsylvania's Earth and Environmental Science Department were instrumental in their expert assistance with elemental analysis. We are also grateful to Samara Gray and Kathryn Weber for their management of the greenhouses at the University of Pennsylvania. This work was funded by University of Pennsylvania startup funds for Corlett Wood and a PURM fellowship award to Nova Meng.

References

Barker, D.G., Pfaff, T., Moreau, D., Groves, E., Ruffel, S., Lepetit, M., *et al.* (2006). Growing M. truncatula: choice of substrates and growth conditions.

Batstone, R.T., Dutton, E.M., Wang, D., Yang, M. & Frederickson, M.E. (2017). The evolution of symbiont preference traits in the model legume *Medicago truncatula*. *New Phytologist*, 213, 1850–1861.

Batstone, R.T., O'Brien, A.M., Harrison, T.L. & Frederickson, M.E. (2020). Experimental evolution makes microbes more cooperative with their local host genotype. *Science*, 370, 476–478.

Berman-Frank, I., Lundgren, P. & Falkowski, P. (2003). Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Research in Microbiology*, 154, 157–164.

Braquart-Varnier, C., Altinli, M., Pigeault, R., Chevalier, F.D., Grève, P., Bouchon, D., *et al.* (2015). The Mutualistic Side of Wolbachia–Isopod Interactions: Wolbachia Mediated Protection Against Pathogenic Intracellular Bacteria. *Front. Microbiol.*, 6.

Bronstein, J.L. (2001). The Costs of Mutualism. *American Zoologist*, 41, 825-839.

Bronstein, J.L. (2015). *Mutualism*. Oxford University Press, United States.

Brooks, M., E., Kristensen, K., Benthem, K., J. ,van, Magnusson, A., Berg, C., W., Nielsen, A., *et al.* (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, 9, 378.

Cao, Y., Halane, M.K., Gassmann, W. & Stacey, G. (2017). The Role of Plant Innate Immunity in the Legume-Rhizobium Symbiosis. *Annu. Rev. Plant Biol.*, 68, 535–561.

Castagnone-Sereno, P., Danchin, E.G.J., Perfus-Barbeoch, L. & Abad, P. (2013). Diversity and Evolution of Root-Knot Nematodes, Genus *Meloidogyne* : New Insights from the Genomic Era. *Annu. Rev. Phytopathol.*, 51, 203–220.

Catella, S.A., Olmsted, C.F., Markalanda, S.H., McFadden, C.J., Wood, C.W. & Kuebbing, S.E. (2022). A generalist nematode destabilises plant competition: no evidence for direct

effects, but strong evidence for indirect effects on rhizobium abundance. *New Phytologist*, 233, 2561–2572.

Chen, Y., Olson, D.M. & Ruberson, J.R. (2010). Effects of nitrogen fertilization on tritrophic interactions. *Arthropod-Plant Interactions*, 4, 81–94.

Clay, K. (1988). Fungal Endophytes of Grasses: A Defensive Mutualism between Plants and Fungi. *Ecology*, 69, 10–16.

Danchin, E.G.J., Rosso, M.-N., Vieira, P., De Almeida-Engler, J., Coutinho, P.M., Henrissat, B., *et al.* (2010). Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes. *Proc. Natl. Acad. Sci. U.S.A.*, 107, 17651–17656.

Defossez, E., Djiéto-Lordon, C., McKey, D., Selosse, M.-A. & Blatrix, R. (2011). Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. *Proc. R. Soc. B.*, 278, 1419–1426.

Dovrat, G., Bakhshian, H., Masci, T. & Sheffer, E. (2020). The nitrogen economic spectrum of legume stoichiometry and fixation strategy. *New Phytologist*, 227, 365–375.

Evans, C.S. (1991). Methods in plant biochemistry: 1. plant phenolics. Academic Press, New York and London, 1990. £65. Phytochem. Anal., 2: 48-48. https://doi.org/10.1002/pca.2800020110

Ferguson, B.J., Mens, C., Hastwell, A.H., Zhang, M., Su, H., Jones, C.H., *et al.* (2019). Legume nodulation: The host controls the party. *Plant Cell & Environment*, 42, 41–51.

Frederickson, M.E. & Gordon, D.M. (2007). The devil to pay: a cost of mutualism with *Myrmelachista schumanni* ants in 'devil's gardens' is increased herbivory on *Duroia hirsuta* trees. *Proc. R. Soc. B.*, 274, 1117–1123.

Freedman, H.I., Addicott, J.F. & Rai, B. (1987). Obligate mutualism with a predator: Stability and persistence of three-species models. *Theoretical Population Biology*, 32, 157–175.

González‐Teuber, M., Kaltenpoth, M. & Boland, W. (2014). Mutualistic ants as an indirect defence against leaf pathogens. *New Phytologist*, 202, 640–650.

Gourion, B., Berrabah, F., Ratet, P. & Stacey, G. (2015). Rhizobium–legume symbioses: the crucial role of plant immunity. *Trends in Plant Science*, 20, 186–194.

Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., *et al.* (2005). Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature*, 435, 819–823.

Grundy, E.B., Gresshoff, P.M., Su, H. & Ferguson, B.J. (2023). Legumes Regulate Symbiosis with Rhizobia via Their Innate Immune System. *IJMS*, 24, 2800.

Guo, K., Yang, J., Yu, N., Luo, L. & Wang, E. (2023). Biological nitrogen fixation in cereal crops: Progress, strategies, and perspectives. *Plant Communications*, 4, 100499.

Hancock, J.T. (2020). Nitric Oxide Signaling in Plants. *Plants*, 9, 1550.

Hartig, F. (2020). DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. *R package version 0.3*, *3*(5).

Hartley, S.E. & Gange, A.C. (2009). Impacts of Plant Symbiotic Fungi on Insect Herbivores: Mutualism in a Multitrophic Context. *Annu. Rev. Entomol.*, 54, 323–342.

Herrera, C.M., Medrano, M., Rey, P.J., Sánchez-Lafuente, A.M., García, M.B., Guitián, J., *et al.* (2002). Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. *Proc. Natl. Acad. Sci. U.S.A.*, 99, 16823–16828.

Hoek, T.A., Axelrod, K., Biancalani, T., Yurtsev, E.A., Liu, J. & Gore, J. (2016). Resource Availability Modulates the Cooperative and Competitive Nature of a Microbial Cross-Feeding Mutualism. *PLoS Biol*, 14, e1002540.

Jandér, K.C. (2015). Indirect mutualism: ants protect fig seeds and pollen dispersers from parasites. *Ecological Entomology*, 40, 500–510.

Janzen D. H. (1975). Pseudomyrmex nigropilosa: A Parasite of a Mutualism. *Science (New York, N.Y.)*, *188*(4191), 936–937. https://doi.org/10.1126/science.188.4191.936

Janzen, D.H. (1985). "The Natural History of Mutualisms." *The Biology of Mutualism: Ecology and Evolution*. Oxford University Press, United States.

Jiménez-Guerrero, I., Acosta-Jurado, S., Del Cerro, P., Navarro-Gómez, P., López-Baena, F., Ollero, F., *et al.* (2017). Transcriptomic Studies of the Effect of nod Gene-Inducing Molecules in Rhizobia: Different Weapons, One Purpose. *Genes*, 9, 1.

Johnson, C.A. (2021). How mutualisms influence the coexistence of competing species. *Ecology*, 102, e03346.

Johnson, C.A. & Bronstein, J.L. (2019). Coexistence and competitive exclusion in mutualism. *Ecology*, 100, e02708.

Johnson, N.C. (1993). Can Fertilization of Soil Select Less Mutualistic Mycorrhizae? *Ecological Applications*, 3, 749–757.

Kandouh, B.H., Hasan, A.E., Al-Hakeem, A.M.A. & Aridh, Z.H.J. (n.d.). A COMPLETE SAFE AND COST EFFECTIVE METHOD FOR STAINING ROOT-KNOT NEMATODES MELOIDOGYNE SPP.

Klepzig, K.D., Adams, A.S., Handelsman, J. & Raffa, K.F. (2009). Symbioses: A Key Driver of Insect Physiological Processes, Ecological Interactions, Evolutionary Diversification, and Impacts on Humans. *en*, 38, 67–77.

Knauer, A.C., Bakhtiari, M. & Schiestl, F.P. (2018). Crab spiders impact floral-signal evolution indirectly through removal of florivores. *Nat Commun*, 9, 1367.

Knops, J.M.H., Bradley, K.L. & Wedin, D.A. (2002). Mechanisms of plant species impacts on ecosystem nitrogen cycling. *Ecology Letters*, 5, 454–466.

Koricheva, J., Gange, A.C. & Jones, T. (2009). Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. *Ecology*, 90, 2088–2097.

Lenth, R., Singmann, H., Love, J., Buerkner, P., & Herve, M. (2018). Emmeans: Estimated marginal means. *AKA least-squares means*, 2-1.

Little, A.E. & Currie, C.R. (2009). Parasites may help stabilize cooperative relationships. *BMC Evol Biol*, 9, 124.

Mansfield, K.M. & Gilmore, T.D. (2019). Innate immunity and cnidarian-Symbiodiniaceae mutualism. *Developmental & Comparative Immunology*, 90, 199–209.

Mauch-Mani, B., Baccelli, I., Luna, E. & Flors, V. (2017). Defense Priming: An Adaptive Part of Induced Resistance. *Annu. Rev. Plant Biol.*, 68, 485–512.

Meehan, C.J., Olson, E.J., Reudink, M.W., Kyser, T.K. & Curry, R.L. (2009). Herbivory in a spider through exploitation of an ant–plant mutualism. *Current Biology*, 19, R892–R893.

Mhlongo, M.I., Piater, L.A., Madala, N.E., Labuschagne, N. & Dubery, I.A. (2018). The Chemistry of Plant–Microbe Interactions in the Rhizosphere and the Potential for Metabolomics to Reveal Signaling Related to Defense Priming and Induced Systemic Resistance. *Front. Plant Sci.*, 9, 112.

Moland, E. & Jones, G.P. (2004). Experimental confirmation of aggressive mimicry by a coral reef fish. *Oecologia*, 140, 676–683.

Ness, J.H. (2006). A mutualism's indirect costs: the most aggressive plant bodyguards also deter pollinators. *Oikos*, 113, 506–514.

Ott, T., Van Dongen, J.T., Gu¨nther, C., Krusell, L., Desbrosses, G., Vigeolas, H., *et al.* (2005). Symbiotic Leghemoglobins Are Crucial for Nitrogen Fixation in Legume Root Nodules but Not for General Plant Growth and Development. *Current Biology*, 15, 531–535.

Pickard, J.M., Zeng, M.Y., Caruso, R. & Núñez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunological Reviews*, 279, 70–89.

Pinkalski, C., Jensen, K.V., Damgaard, C. & Offenberg, J. (2018). Foliar uptake of nitrogen from ant faecal droplets: An overlooked service to ant‐plants. *Journal of Ecology*, 106, 289–295.

Pringle, E.G. (2016). Integrating plant carbon dynamics with mutualism ecology. *New Phytologist*, 210, 71–75.

Raine, N.E., Gammans, N., Macfadyen, I.J., Scrivner, G.K. & Stone, G.N. (2004). Guards and thieves: antagonistic interactions between two ant species coexisting on the same ant‐plant. *Ecological Entomology*, 29, 345–352.

Rice, M.M., Baldwin, D.G., Fischer, J.N., Fuchs, C. & Burkepile, D.E. (2021). Complex interactions with nutrients and sediment alter the effects of predation on a reef‐building coral. *Marine Ecology*, 42, e12670.

Richardson, D.M., Allsopp, N., D'Antonio, C.M., Milton, S.J. & Rejmánek, M. (2000). Plant invasions – the role of mutualisms. *Biol. Rev.*, 75, 65–93.

Salem, H., Onchuru, T.O., Bauer, E. & Kaltenpoth, M. (2015). Symbiont transmission entails the risk of parasite infection. *Biol. Lett.*, 11, 20150840.

Schmitt, R.J. & Holbrook, S.J. (2003). Mutualism can mediate competition and promote coexistence. *Ecology Letters*, 6, 898–902.

Shamseldin, A. (2013). The Role of Different Genes Involoved in Symbiotic Nitrogen Fixation - Review.

Simonsen, A.K. & Stinchcombe, J.R. (2014). Herbivory eliminates fitness costs of mutualism exploiters. *New Phytologist*, 202, 651–661.

Tiwari, M., Pati, D., Mohapatra, R., Sahu, B.B. & Singh, P. (2022). The Impact of Microbes in Plant Immunity and Priming Induced Inheritance: A Sustainable Approach for Crop protection. *Plant Stress*, 4, 100072.

Tomas, F., Abbott, J.M., Steinberg, C., Balk, M., Williams, S.L. & Stachowicz, J.J. (2011). Plant genotype and nitrogen loading influence seagrass productivity, biochemistry, and plant–herbivore interactions. *Ecology*, 92, 1807–1817.

Traveset, A. & Richardson, D.M. (2014). Mutualistic Interactions and Biological Invasions. *Annu. Rev. Ecol. Evol. Syst.*, 45, 89–113.

Van Alstyne, K.L., Pelletreau, K.N. & Kirby, A. (2009). Nutritional preferences override chemical defenses in determining food choice by a generalist herbivore, Littorina sitkana. *Journal of Experimental Marine Biology and Ecology*, 379, 85–91.

Van Schreven, D.A. (1959). Effects of added sugars and nitrogen on nodulation of legumes. *Plant Soil*, 11, 93–112.

Vidal, E.A., Alvarez, J.M., Araus, V., Riveras, E., Brooks, M.D., Krouk, G., *et al.* (2020). Nitrate in 2020: Thirty Years from Transport to Signaling Networks. *Plant Cell*, 32, 2094–2119.

Wang, L., Rubio, M.C., Xin, X., Zhang, B., Fan, Q., Wang, Q., *et al.* (2019). CRISPR/Cas9 knockout of leghemoglobin genes in *Lotus japonicus* uncovers their synergistic roles in symbiotic nitrogen fixation. *New Phytologist*, 224, 818–832.

Wang, L., Wang, L., Tan, Q., Fan, Q., Zhu, H., Hong, Z., *et al.* (2016). Efficient Inactivation of Symbiotic Nitrogen Fixation Related Genes in Lotus japonicus Using CRISPR-Cas9. *Front. Plant Sci.*, 7.

Wang, M., Wang, Z., Guo, M., Qu, L. & Biere, A. (2023). Effects of arbuscular mycorrhizal fungi on plant growth and herbivore infestation depend on availability of soil water and nutrients. *Front. Plant Sci.*, 14, 1101932.

Wang, Q., Liu, J., Li, H., Yang, S., Körmöczi, P., Kereszt, A., *et al.* (2018). Nodule-Specific Cysteine-Rich Peptides Negatively Regulate Nitrogen-Fixing Symbiosis in a Strain-Specific Manner in *Medicago truncatula*. *MPMI*, 31, 240–248.

Weese, D.J., Heath, K.D., Dentinger, B.T.M. & Lau, J.A. (2015). Long-term nitrogen addition causes the evolution of less-cooperative mutualists: NITROGEN ADDITION DESTABILIZES MUTUALISM. *Evolution*, 69, 631–642.

Whelan, C.J., Şekercioğlu, Ç.H. & Wenny, D.G. (2015). Why birds matter: from economic ornithology to ecosystem services. *J Ornithol*, 156, 227–238.

Wickham, H. (2016). *ggplot2*. Use R! Springer International Publishing, Cham.

Wiedenmann, J., D'Angelo, C., Mardones, M.L., Moore, S., Benkwitt, C.E., Graham, N.A.J., *et al.* (2023). Reef-building corals farm and feed on their photosynthetic symbionts. *Nature*, 620, 1018–1024.

William F. Fagan, E.S., Charles Mitter, R.F.D., Andrea F. Huberty, H.A.W. & James J Elser. (n.d.). Nitrogen in Insects: Implications for Trophic Complexity and Species Diversification.

Williams, S.L. (n.d.). Grazing effects on nitrogen ®xation in coral reef algal turfs.

Wood, C.W., Pilkington, B.L., Vaidya, P., Biel, C. & Stinchcombe, J.R. (2018). Genetic conflict with a parasitic nematode disrupts the legume–rhizobia mutualism. *Evolution Letters*, 2, 233–245.

Xuan, W., Beeckman, T. & Xu, G. (2017). Plant nitrogen nutrition: sensing and signaling. *Current Opinion in Plant Biology*, 39, 57–65.

Zehr, J.P. (2011). Nitrogen fixation by marine cyanobacteria. *Trends in Microbiology*, 19, 162–173.

Zenk, M.H. & Juenger, M. (2007). Evolution and current status of the phytochemistry of nitrogenous compounds. *Phytochemistry*, 68, 2757–2772.

Zhang, J., He, N., Liu, C., Xu, L., Chen, Z., Li, Y., *et al.* (2020). Variation and evolution of C:N ratio among different organs enable plants to adapt to N‐limited environments. *Global Change Biology*, 26, 2534–2543.

Figures & Tables

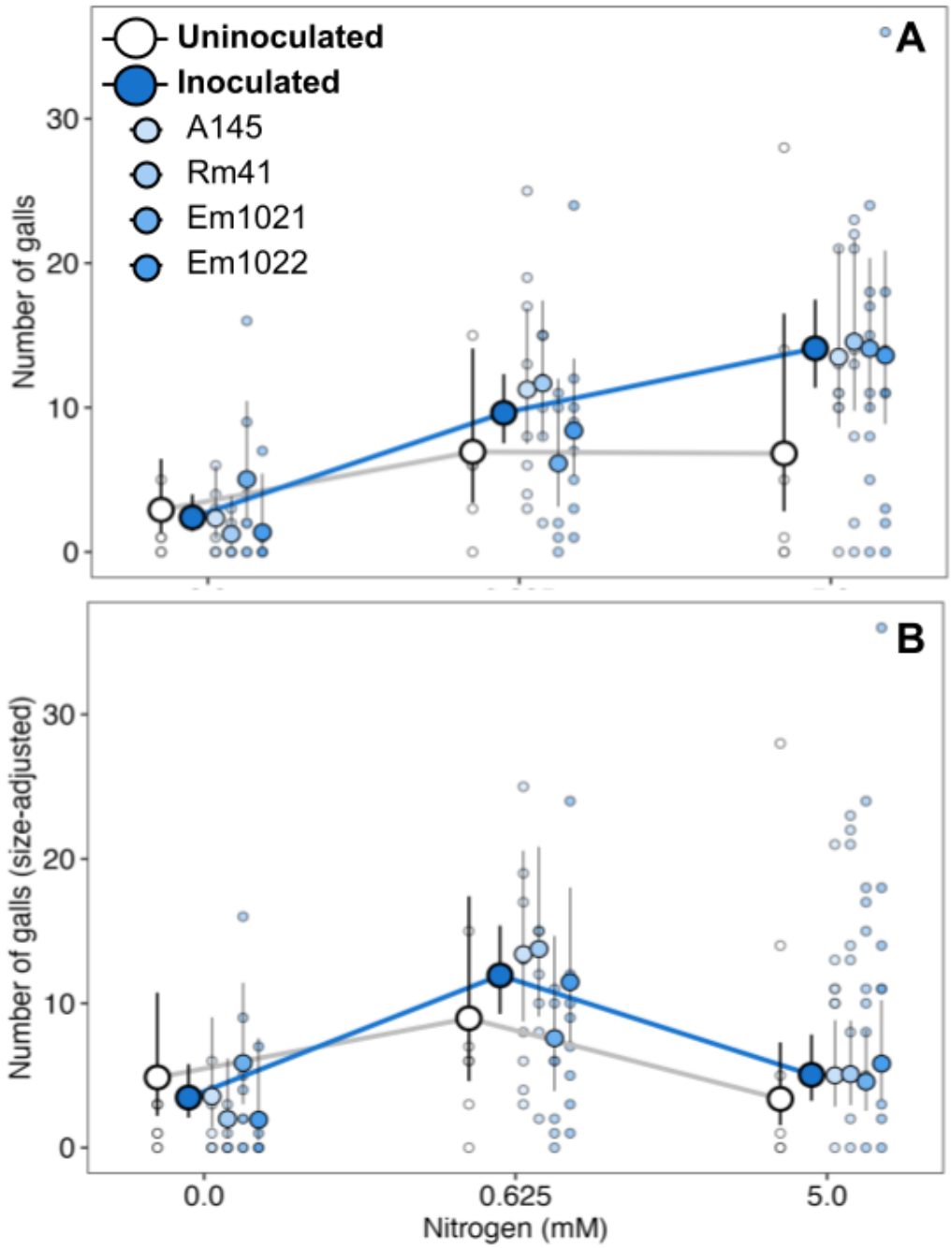

Figure 1: The effect of nitrogen and symbiosis treatments on A) total parasite load and B) size-adjusted parasite load (infection intensity). For all panels, smaller transparent points are raw data, while the larger opaque points are the estimated marginal means. Error bars on the estimated marginal means show the 95% confidence interval.

Table 1: The effect of nitrogen and symbiosis treatments on parasite load.

A1: The effect of rhizobia strain and nitrogen on total parasite load			
	χ^2	**Df**	**P**
Rhizobia	2.57	4	0.631
Nitrogen	40.0	2	<0.001
Block	4.55	3	0.208
Rhizobia:Nitrogen	9.86	8	0.275
A2: The effect of symbiosis (rhizobia inoculation) and nitrogen on total parasite load			
Symbiosis	1.27	1	0.259
Nitrogen	18.9	2	<0.001
Block	5.51	3	0.138
Symbiosis:Nitrogen	1.95	2	0.377
A3: The effect of symbiosis for each nitrogen treatment			
	Uninoculated - No rhizobia or nodulation		
	Ratio	SE	p.value
No-N and Low-N (0 - 0.625 mM)	0.421	0.226	0.240
No-N and High-N (0 - 5 mM)	0.428	0.259	0.339
Low-N and High-N (0.625 - 5 mM)	1.018	0.588	1.00
	Inoculated - No rhizobia or nodulation		
	Ratio	SE	p.value
No-N and Low-N (0 - 0.625 mM)	0.249	0.0701	<0.001
No-N and High-N (0 - 5 mM)	0.170	0.0474	<0.001
Low-N and High-N	0.683	0.113	0.0565

(0.625 - 5 mM)			
B1: The effect of rhizobia strain and nitrogen on size-adjusted parasite load			
	χ^2	**Df**	**P**
Rhizobia	0.809	4	0.937
Nitrogen	28.2	2	<0.001
Block	2.30	3	0.513
Root biomass (z-score)	26.2	1	<0.001
Rhizobia:Nitrogen	7.98	8	0.435
B2: The effect of symbiosis and nitrogen on size-adjusted parasite load			
Symbiosis	0.248	1	0.619
Nitrogen	14.3	2	<0.001
Block	1.76	3	0.623
Root biomass (z-score)	26.2	1	<0.001
Symbiosis:Nitrogen	1.71	2	0.425

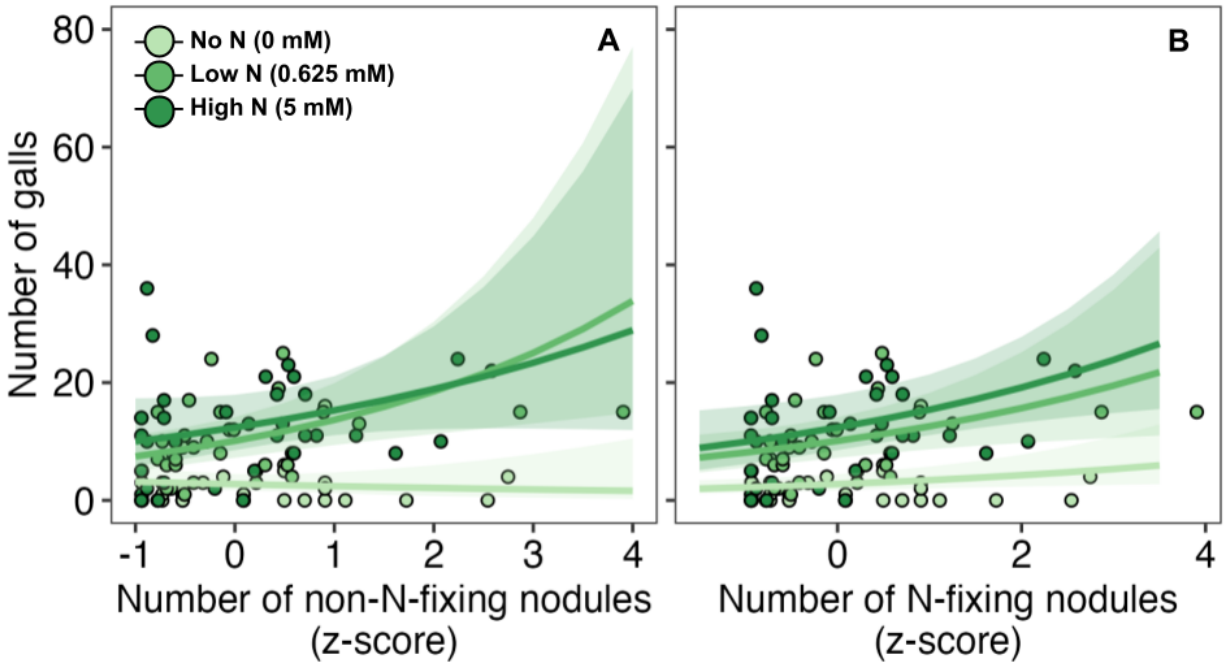

Figure 2: The relationship between nodulation (for N-fixing and non-N-fixing nodules) and gall number. Each point consists of a single plant sample, while lines of best fit are for plotted variables, not adjusted for the contribution of model covariates. Additional model covariates included block.

Table 2: The relationship between nodulation and gall number.

	χ^2	**Df**	**P**
Nitrogen	37.2	2	<0.001
Number of non-N-fixing nodules	2.05	1	0.153
Number of N-Fixing Nodules	6.27	1	0.0123
Block	3.57	3	0.312
Nitrogen:White Nodule	3.12	3	0.210

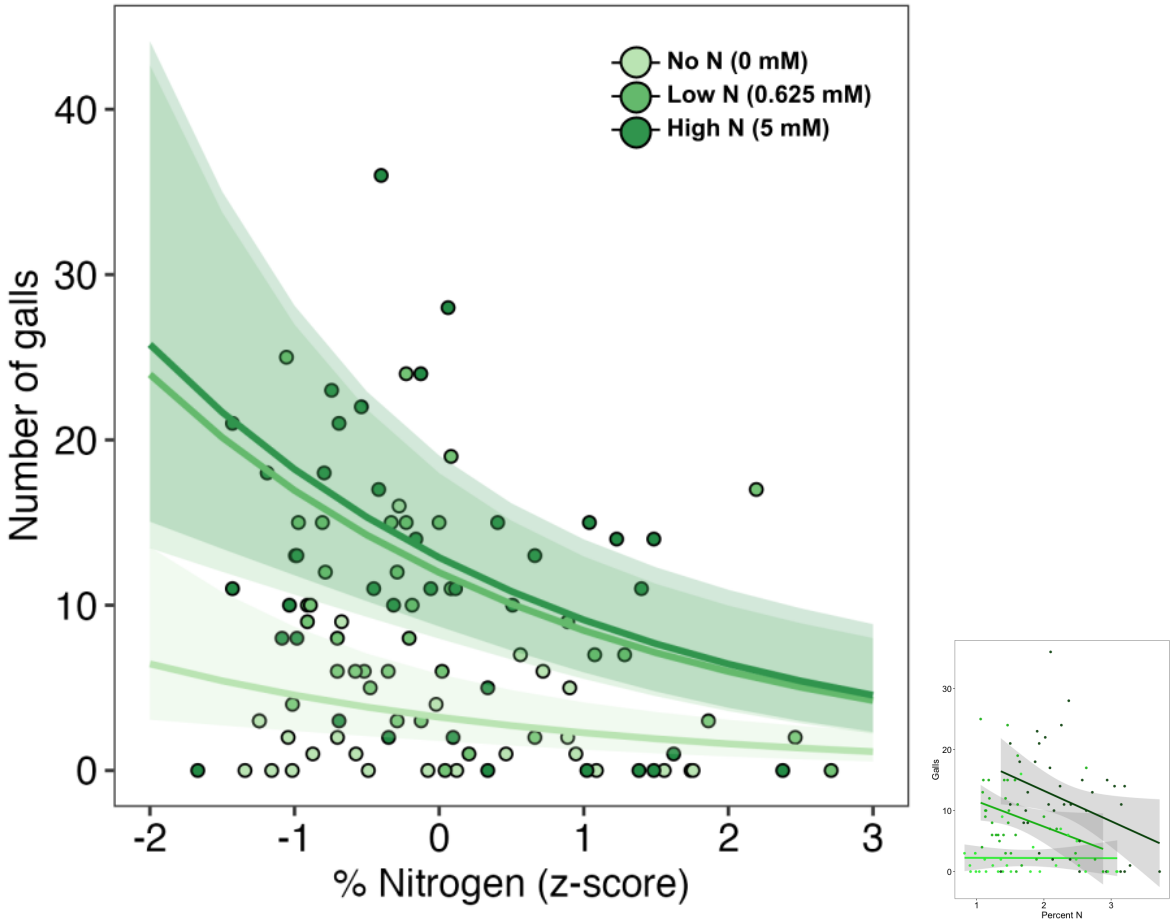

Figure 3: The relationship between percent composition of nitrogen and total parasite load. Each point consists of one plant sample, while lines of best fit are for plotted variables, not adjusted for the contribution of model covariates. Additional model covariates included carbon composition, root biomass, rhizobia strain, and block.

Table 3: The relationship between percent composition of nitrogen and total parasite load.

	χ^2	**Df**	**P**
Nitrogen	26.0	2	<0.001
Nitrogen Composition	7.86	1	0.00507
Carbon Composition	0.559	1	0.455
Root biomass	12.3	1	0.000451
Rhizobia	0.806	4	0.938
Block	5.62	3	0.132