LETTER: Mutualist-Provided Resources Increase Susceptibility to Parasites

Running Title: Mutualist-Provided Resources and Parasitism

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Statement of authorship: EY and CWW designed the study, EY and NM ran the experiment and collected data, CWW and EY analyzed data, EY wrote the first draft of the manuscript, and all authors contributed to revisions.

Data accessibility statement: The data and code supporting results have been archived on Figshare. https://doi.org/10.6084/m9.figshare.24442738

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

Abstract: 150 words

Main Text: 4495 words

Reference Count: 83

Figures, tables, and text boxes: 6

Abstract

Mutualistic microbes often increase their host's susceptibility to parasite infection. We tested two competing hypotheses to identify the cause of this phenomenon. First, mutualist-provided resources could attract antagonists by making hosts more resource-rich. Second, mutualism establishment itself might increase host vulnerability to antagonists. To test which mechanism underlies increased parasite susceptibility, we experimentally decoupled mutualism establishment and mutualist-provided resources in the legume-rhizobia mutualism. We measured parasite load on *Medicago truncatula* plants infected with root-knot nematodes in a full-factorial design, in which we independently manipulated rhizobia nodulation (mutualism establishment) and nitrogen availability (mutualist-provided resources). We found little effect of mutualism establishment on susceptibility, as nodulation without nitrogen fixation did not significantly increase parasite infection. By contrast, nitrogen did increase parasite infection, but with non-linear effects not fully explained by nitrogen assimilation into tissues. Our results suggest that mutualist-provided resources are an important but complex driver of indirect ecological costs of mutualism.

Introduction

Mutualistic species interactions are ubiquitous (Janzen 1985). Mutualists provide fundamental biological functions for their partners and their ecosystems (Bronstein 2001; Traveset & Richardson 2014). These functions are diverse, spanning transportation, protection, reproduction, and nutrition (Klepzig *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 indirect costs can be highly significant, as mutualisms can strengthen competition, compromise other mutualisms, or increase susceptibility to various antagonists (Hartley & Gange 2009; Hoek *et al.* 2016; Johnson 2021; Johnson & Bronstein 2019; Ness 2006; Richardson *et al.* 2000; Schmitt & Holbrook 2003).

The phenomenon of mutualisms increasing susceptibility to antagonists is a significant and especially well-documented subset of indirect costs. Examples span many different fields under various terms such as priority effects, priming, and niche modification (Mauch-Mani *et al.* 2017; Mhlongo *et al.* 2018; Pickard *et al.* 2017; Tiwari *et al.* 2022). Host-associated mutualisms such as the plant-pollinator and legume-rhizobia mutualisms have been shown to open up opportunities for increased predation and herbivory on hosts (Knauer *et al.* 2018; Simonsen & Stinchcombe 2014). 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 (Frederickson & Gordon 2007; Herrera *et al.* 2002; Johnstone & Bshary 2002; Little & Currie 2009; Meehan *et al.* 2009).

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 & Stinchcombe 2014).

Alternatively, mutualism establishment in and of itself may increase 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 & 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 & 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 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.

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 (Cook 1999; Rose 2008). Rhizobia bacteria fix atmospheric nitrogen in exchange for plant carbon (Masson-Boivin & Sachs 2018; Shanmugam *et al.* 1978). 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 (Fischer 1994; Lindström & Mousavi 2020). 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).

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). Nitrogen, on the other hand, is a commonly limited resource and an essential nutrient for various biological processes; it is thus often sought after in species interactions such as herbivory (Chen *et al.* 2010; Van Alstyne *et al.* 2009).

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 (Rose 2008). Rhizobia form specialized nodule organs on roots through a tightly regulated process of signaling between the plant host and bacteria (Ferguson *et al.* 2019). 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*, a 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 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). Rhizobia strains Em1021 and Em1022 both nodulate and fix nitrogen on *M. truncatula*, although Em1022 is a 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 & 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 corresponding to its respective nitrogen treatment (Batstone *et al.* 2017). 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 the risk of cross-contamination across rhizobia strains. We used 12 inch 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 sieves with 75 and 25 μm pore size 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

We harvested all plants 7.5 weeks after the start of the experiment (53 days; May 28-July 20, 2021). Shoots were 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 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 *et al.* 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, 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. 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). Contamination was 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 (Figure S1D; Table S1). 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.

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. Thirty-six 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, Table 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 (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 (Lenth 2023). 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 ($p<0.001$, Figure 1A, Table 1). Increasing nitrogen increased parasite load from no-N to low- and high-N (Figure 1A, Table S6). This effect was qualitatively similar across inoculated and uninoculated plants, and across rhizobia strains (Table 1, Table S5).

We found a significant effect of nitrogen on the intensity of parasite infection (size-adjusted parasite load) ($p<0.001$, Figure 1B, Table 1). Larger plants had more parasites overall,

such that size-adjusted parasite load peaked at low-N, with no-N and high-N plants having similarly low intensities of infection (Figure 1A, Table S6).

We did not find a significant effect of nodulation (mutualism establishment) on total parasite load (Figure 1A, Table 1). Inoculated and uninoculated plants did not significantly differ in total parasite load (Figure 1A, Table 1). 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). Rhizobia strain had no significant effect on total parasite load (Figure 1A, Table S5).

We found no significant effect of non-nitrogen-fixing nodule count on total parasite load (Figure 2, Table 2). 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 ($p=0.0123$, Figure 2, Table 2). Even accounting for the significant effect of N-fixing nodules, no-N plants had a lower parasite load than low- and high-N plants (Figure 2, Table S7).

We did not find a significant effect of rhizobia strain or symbiosis (nodulation) on intensity of infection (Figure 1B, Table 1, Table S5).

Mechanism of the nitrogen effect

We observed a significant effect of tissue nitrogen composition on intensity of parasite infection ($p<0.001$; Table 1). However, contrary to our initial expectation, higher tissue nitrogen composition led to a lower intensity of parasite infection (Table S8). Furthermore, significant

differences in parasite infection between nitrogen treatments remained even after accounting for plant size and tissue nitrogen composition ($p<0.001$, Table 3). There was no relationship between tissue carbon composition and the intensity of parasite infection ($p=0.181$, Table 3).

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 are known to downregulate their local immune response when forming a symbiosis with rhizobia (Cao *et al.* 2017; Grundy *et al.* 2023). 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).

Furthermore, 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 level of infection.

It is important to note that mutualist-provided resources increasing susceptibility to parasites does not necessarily mean that mutualists impose 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 actually decreased parasite load (Figure 3, Table 3). This was surprising, given the extensive literature on preferential feeding by herbivores on nitrogen-rich tissue due to its better nutritional quality, although research has also shown that nitrogen addition can decrease plant palatability for some contexts of species interactions (Chen *et al.* 2010; Tomas *et al.* 2011; Van Alstyne *et al.* 2009). 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 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 (Evans 1991; Zenk & Juenger 2007). This might also account for the observed peak in the intensity of parasite infection at intermediate nitrogen levels (Figure 1B). This intermediate peak could represent a balance between increased parasite infection on N-rich plants and resistance to parasites by some extremely N-rich plants. Plant hosts growing with high-N allocated their additional nitrogen to either growing larger in size or increasing density of nitrogen composition (Figure S5). Larger size could reduce relative density and costs of parasites, while N-rich compounds or forms of dense storage may have deterred parasites outright. 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 & Baldwin 2004). The peak in size-adjusted parasite load at intermediate nitrogen levels 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; Clay 1988; González Teuber *et al.* 2014; Jandér 2015; Wang *et al.* 2023). Nitrogen as a resource could both attract as well as defend against antagonists depending on the resource landscape, availability, and use.

Second, assimilated nitrogen does not fully explain the effect of nitrogen on parasite infection. Even after accounting for the significant effect of tissue nitrogen composition 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. It is 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 (Hancock 2020; Vidal *et al.* 2020; Xuan *et al.* 2017). Broadly, nitrogen can be utilized for size, photosynthesis, signaling, and defense.

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. 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. Resources can also be differentially allocated to different functions: for instance, increased use for photosynthesis can be inferred from a high leaf count, shoot:root ratio, and possibly shoot nitrogen composition (Figure S3, Figure S4). 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 (Dovrat *et al.* 2020; Zhang *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 statistically significant differences in parasite load between host plants inoculated and uninoculated with rhizobia. 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.

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 & 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 this particular parasite 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. 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 & 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 (Defossez *et al.* 2011; Pinkalski *et al.* 2018; Whelan *et al.* 2015). 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 (Berman-Frank *et al.* 2003; Guo *et al.* 2023). Nitrogen thus has effects on nutrient cycling, species interactions, and broader ecosystem functioning (Wiedenmann *et al.* 2023). Nitrogen provisioning may be a common mechanism that increases susceptibility to antagonists across many different mutualisms.

In addition to mutualist-provided nitrogen, 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, which are known to lead to potential breakdown of nitrogen-provisioning mutualisms (Johnson 1993; Weese *et al*. 2015). Moving forward, our data suggests that it would be worth investigating how nitrogen can cascade to other species interactions. How might nitrogen be affecting susceptibility to pathogens? Could increased susceptibility be an unexpected byproduct of resource input? It has been proposed that the ecophysiology of carbon resource allocation can explain the ecology and evolution of mutualisms (Pringle 2016). 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.

Future research should explore the relative roles of mutualism establishment and mutualist-provided resources in driving susceptibility to parasites in other systems. Their relative importance likely varies across systems, because the host mounts a slightly different physiological response to each microorganism, and because different resources will likely differ in their cascading consequences for ecological interactions. Nevertheless, our study highlights the potential wide-ranging significance of nitrogen—with its massive biotic and anthropogenic inputs—for multi-species interactions and their outcomes. Second, our results underscore the importance of integrating indirect costs into studies of mutualism to determine the conditions under which the net benefits of mutualism are highest or lowest. These questions should be

tested in the future; to do so will require drawing upon and synthesizing various subfields of biology.

Acknowledgements

We would like to thank Chigozie Ibe and Yoon Chang for data collection and their help in maintaining the experiment. We thank Ben Glass for his work on a previous version of the experiment. We also thank McCall Calvert, Addison Buxton-Martin, Chang-Yu Chang, Anisa Robinson, 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, the USDA for Em1021 and Em1022 rhizobia strains, and David Vann and the Instrumentation Core at the University of Pennsylvania's Earth and Environmental Science Department for their 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 the National Science Foundation's Division of Environmental Biology (grant no. 2118397) to Corlett Wood and a Penn Undergraduate Research Mentoring Program (PURM) fellowship award to Nova Meng.

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Figures & Tables

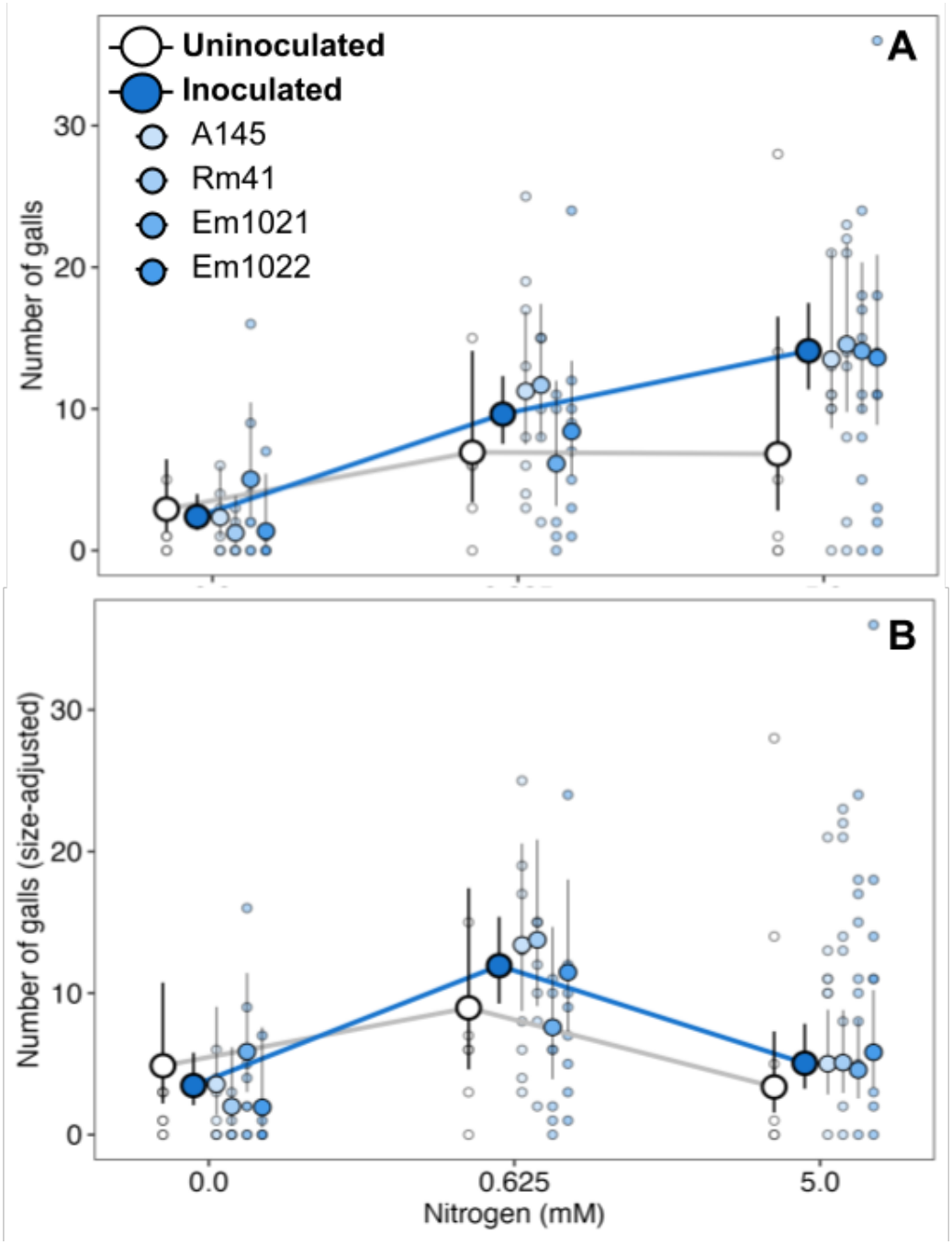

Figure 1: The effect of nitrogen and nodulation treatments on A) total parasite load and B) size-adjusted parasite load (infection intensity). For panels, smaller transparent points are raw data, while the larger opaque points are the estimated marginal means. Error bars on the estimated marginal means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

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

The effect of nodulation (mutualism establishment) and nitrogen on total parasite load			
Nodulation	1.27	1	0.259
Nitrogen	18.9	2	**<0.001**
Block	5.51	3	0.138
Nodulation:Nitrogen	1.95	2	0.377
The effect of nodulation and nitrogen on size-adjusted parasite load (intensity of infection)			
Nodulation	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**
Nodulation:Nitrogen	1.71	2	0.425

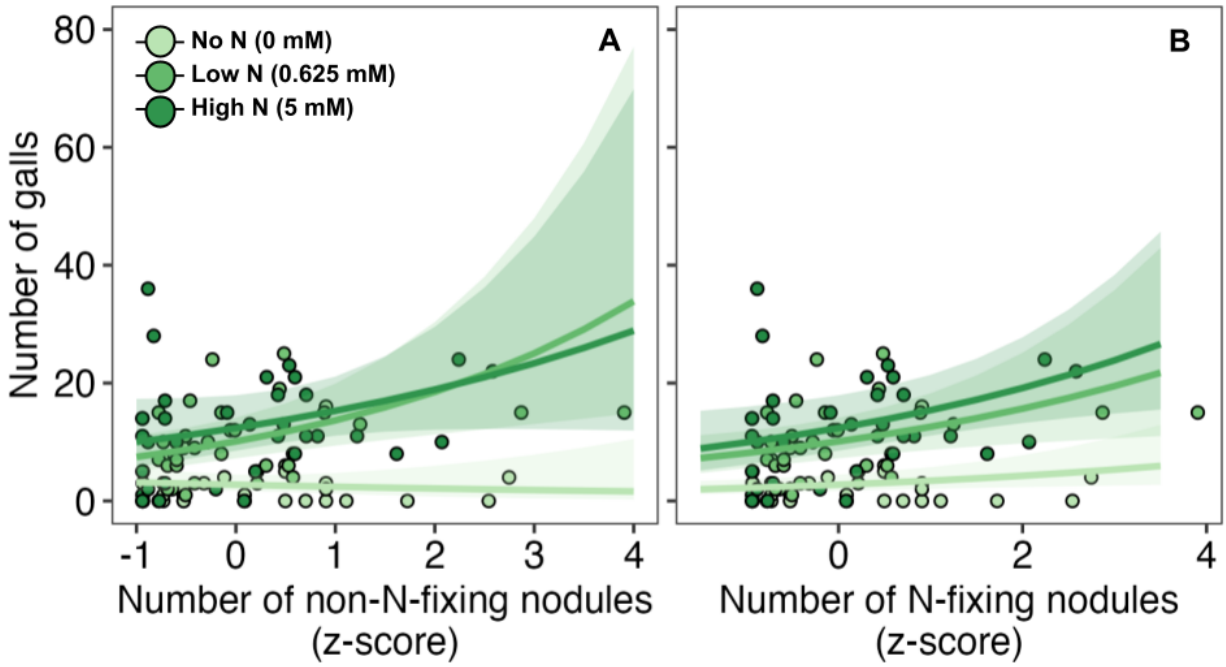

Figure 2: The relationship between nodulation and gall number, for A) non-N-fixing nodules and B) N-fixing nodules. Each point consists of a single plant sample, while lines of best fit are for plotted variables, not adjusted for the contribution of model covariates. Additional model covariates included 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:non-N-fixing (white) nodules	3.12	3	0.210

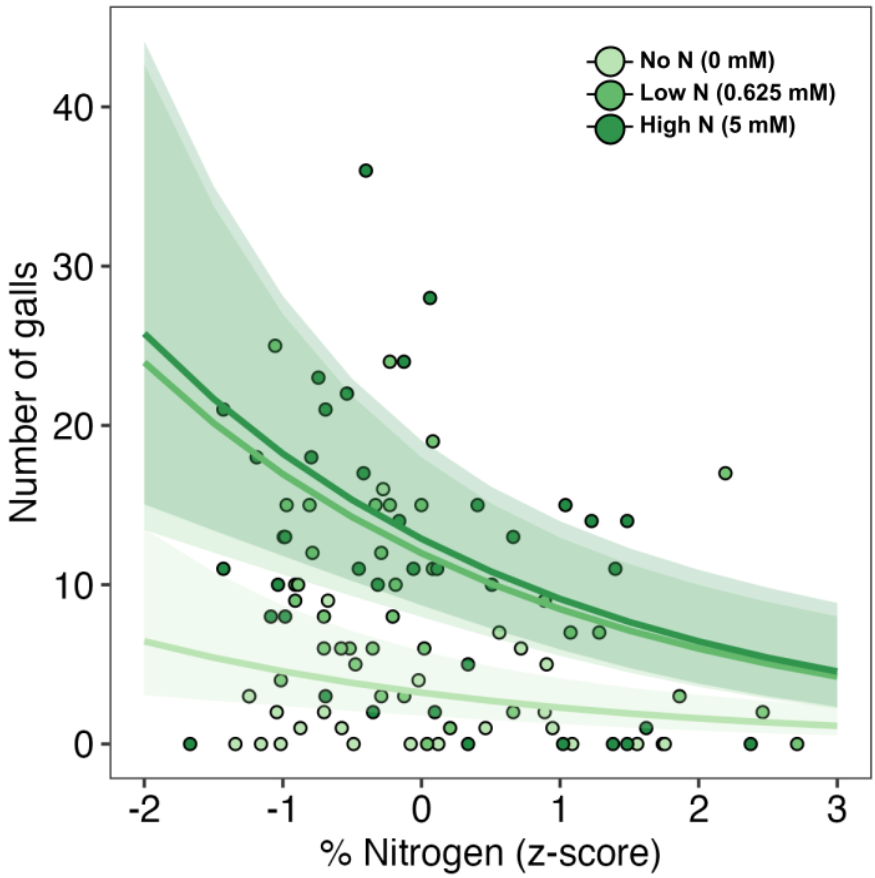

Figure 3: The relationship between percent composition of nitrogen and total parasite load. Each point consists of one plant sample, while lines of best fit are for plotted variables, not adjusted for the contribution of model covariates. Additional model covariates included carbon composition, root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

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

	χ^2	**Df**	**P**
Nitrogen	28.2	2	**<0.001**
Nitrogen composition	14.1	1	**<0.001**
Carbon composition	1.79	1	0.181
Root biomass	11.4	1	**<0.001**
Rhizobia	1.14	4	0.888
Block	6.35	3	0.0960