Genetic conflict with a parasitic nematode disrupts the legume-rhizobia mutualism

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Abstract

Genetic variation for partner quality in mutualisms is an evolutionary paradox. One possible resolution to this puzzle is that there is a tradeoff between partner quality and other fitness-related traits. Here, we tested whether a susceptibility to parasitism is one such tradeoff in the mutualism between legumes and nitrogen-fixing bacteria (rhizobia). We performed two greenhouse experiments with the legume *Medicago truncatula*. In the first, we inoculated each plant with the rhizobia *Ensifer meliloti* and with one of 40 genotypes of the parasitic root-knot nematode *Meloidogyne hapla*. In the second experiment, we inoculated all plants with rhizobia and half of the plants with a genetically variable population of nematodes. Using the number of nematode galls as a proxy for infection severity, we found that plant genotypes differed in susceptibility to nematode infection, and nematode genotypes differed in infectivity. Second, we showed that there was a genetic correlation between the number of mutualistic structures formed by rhizobia (nodules) and the number of parasitic structures formed by nematodes (galls). Finally, we found that nematodes disrupt the rhizobia mutualism: nematode-infected plants formed fewer nodules and had less nodule biomass than uninfected plants. Our results demonstrate that there is genetic conflict between attracting rhizobia and repelling nematodes in *Medicago*. If genetic conflict with parasitism is a general feature of mutualism, it could account for the maintenance of genetic variation in partner quality and influence the evolutionary dynamics of positive species interactions.

Impact summary

Cooperative species interactions, known as mutualisms, are vital for organisms from plants to humans. For example, beneficial microbes in the human gut are a necessary component of digestive health. However, parasites often infect their hosts via mechanisms that are extraordinarily similar to those used by mutualists, which may create a tradeoff between attracting mutualists and resisting parasites. In this study, we investigated whether this tradeoff exists, and how parasites impact mutualism function in the barrelclover *Medicago truncatula*, a close relative of alfalfa. Legumes like *Medicago* depend on nitrogen provided by mutualistic bacteria (rhizobia) to grow, but they are also infected by parasitic worms called nematodes, which steal plant nutrients. Both microorganisms live in unique structures (nodules and galls) on plant roots. We showed that the benefits of mutualism and the costs of parasitism are predicted by the number of mutualistic structures (nodules) and the number of parasitic structures (galls), respectively. Second, we found that there is a genetic tradeoff between attracting mutualists and repelling parasites in *Medicago truncatula*: plant genotypes that formed more rhizobia nodules also formed more nematode galls. Finally, we found that nematodes disrupt the rhizobia mutualism. Nematode-infected plants formed fewer rhizobia nodules and less total nodule biomass than uninfected plants. Our research addresses an enduring evolutionary puzzle: why is there so much variation in the benefits provided by mutualists when natural selection should weed out low-quality partners? Tradeoffs between benefits provided by mutualists and their susceptibility to parasites could resolve this paradox.

Introduction

Nearly all species require mutualists to carry out crucial biological functions (Shapira 2016). Insects partner with mutualists for nutrition (Hansen and Moran 2014; Nygaard et al. 2016); most plants rely on mutualistic fungi or bacteria to grow (Friesen 2013; Busby et al. 2017), and on animal pollinators for reproduction (Johnson et al. 2015); and the gut microbiome is increasingly recognized as a key aspect of human physiology (Sachs et al. 2011; Shapira 2016). One common feature of most mutualisms is their abundant genetic variation in partner quality—the fitness benefits provided by one partner to another—despite the fact that natural selection is expected to erode variation in mutualism strategies over time (Heath and Stinchcombe 2014). Here we show that partner quality variation in the mutualism between plants and nitrogen-fixing bacteria may be maintained by a genetic tradeoff between attracting mutualistic bacteria and repelling parasitic nematodes.

The maintenance of genetic variation for partner quality in mutualisms is an evolutionary paradox (Heath and Stinchcombe 2014). As with other fitness-related traits, natural selection is expected to drive the highest-fitness partner strategy to fixation, eliminating low-fitness genotypes. Yet genetic variation in partner quality is ubiquitous (Smith and Goodman 1999; Ness et al. 2006; Heath 2010; Hoeksema 2010). Several hypotheses have been advanced to explain this pattern in mutualisms (Heath and Stinchcombe 2014), including the context dependence of partner quality (Barrett et al. 2012; Heath et al. 2012; Simonsen and Stinchcombe 2014a; Burghardt et al. 2017; Harrison et al. 2017b) and frequency-dependent selection balancing cooperative and uncooperative mutualist genotypes (Porter and Simms 2014; Jones et al. 2015). By contrast, the alternative hypothesis that selection favors poor-quality mutualists

83 over high-quality mutualists under some ecological conditions (Bronstein 2001a,b) remains
84 relatively understudied.

85 Parasites are one agent of selection with the potential to reverse selection on cooperative
86 traits in mutualisms. Parasites can induce major changes in the function and benefits of
87 mutualism, generally in two ways (Strauss and Irwin 2004). First, parasites disrupt the
88 occurrence (i.e., change the frequency) of mutualistic partnerships, typically causing infected
89 hosts to form fewer mutualistic associations (Strauss et al. 2002; De Román et al. 2011; Ballhorn
90 et al. 2014). Second, if the same trait attracts both mutualists and parasites—for example, flowers
91 that draw herbivores to plants along with pollinators—individuals experience a tradeoff between
92 the benefits of mutualism and the costs of parasitism (Gomez 2003; Irwin et al. 2004; Siepielski
93 and Benkman 2009; Ågren et al. 2013; Knauer and Schiestl 2017; Zust and Agrawal 2017).
94 Coupled with spatial or temporal variation in parasite abundance, conflicting selection imposed
95 by mutualists and parasites has been shown to maintain phenotypic variation in mutualism traits
96 (Siepielski and Benkman 2009; Ågren et al. 2013).

97 We lack direct evidence, however, that tradeoffs between mutualism and parasitism are
98 genetically based, a necessary criterion for selection imposed by parasites to contribute to the
99 maintenance of genetic variation in mutualism (Strauss and Irwin 2004; Heath and Stinchcombe
100 2014). Genetic trade-offs between mutualism and parasitism can preserve genetic variation in
101 mutualist quality in at least two complementary ways. First, if the genotypes that form the most
102 mutualistic associations (or provide the greatest benefit to their partners) necessarily suffer more
103 parasitism, this may reduce or eliminate their fitness advantage, preventing or slowing the
104 fixation of the 'best' mutualist genotypes in populations. Second, if mutualism and parasitism are
105 genetically linked, correlated evolutionary responses may lead to temporally variable selection

106 on mutualism- and parasitism-related traits. That is, if selection favoring effective mutualists
107 causes a correlated decrease in parasite resistance, eventually countervailing selection favoring
108 increased parasite resistance is likely to drive a correlated decrease in mutualist quality, thus
109 preserving variation in mutualism traits. In similar fashion, spatial variation in the abundance of
110 mutualists or parasites can create a mosaic of correlated responses to selection in mutualism- or
111 parasitism-related traits, preserving genetic variation at larger spatial scales. A genetic
112 relationship between mutualism and parasitism traits is one precondition for these evolutionary
113 forces contribute to the maintenance of genetic variation for partner quality in mutualisms.
114 Although we lack direct evidence for genetic tradeoffs between mutualism and parasitism
115 in most systems, several lines of indirect evidence raise the intriguing possibility that
116 susceptibility to parasites is a common pleiotropic genetic cost of mutualism. Many species are
117 attacked by parasites that bear remarkable resemblance to their mutualists (Adams et al. 2012;
118 Chomicki et al. 2015), and parasites and mutualists frequently use the same cues to infiltrate
119 their host (Sachs et al. 2011). Host genes that affect interactions with mutualists are often also
120 used in defense against parasites (Sachs et al. 2011; Damiani et al. 2012). Consistent with this
121 observation, some species suppress immune function when establishing mutualistic partnerships,
122 leaving them vulnerable to infection (Toth et al. 1990; Miller 1993; Salem et al. 2015).
123 Ultimately, it remains unclear whether these mechanistic tradeoffs create genetic conflict
124 between mutualism and parasitism at the population level, and whether there is genetic variation
125 for the extent to which parasites influence mutualism structure and function.
126 The keystone ecological and agricultural mutualism between leguminous plants and
127 nitrogen-fixing bacteria (rhizobia) is a promising system for testing for genetic tradeoffs between
128 mutualism and parasitism. In this mutualism, rhizobia provide their plant host with nitrogen, and

129 the plant trades carbohydrates in return. Plants house rhizobia in root organs called nodules
130 (Figure 1). However, many legumes are also infected by parasitic root-knot nematodes that steal
131 photosynthates (Dhandaydham et al. 2008; Goverse and Smant 2014). Nematodes form galls on
132 plant roots that are strikingly similar to the nodules formed by rhizobia (Figure 1). Molecular
133 genetic evidence suggests that genetic conflict between legume responses to mutualistic rhizobia
134 and parasitic nematodes is extensive. Nematodes infiltrate the plant via a stereotyped infection
135 process that mimics that of rhizobia (Goverse and Smant 2014). Many of the same legume genes
136 mediate the two interactions (e.g., receptor genes required to initiate infection) (Koltai et al.
137 2001; Weerasinghe et al. 2005; Dhandaydham et al. 2008; Damiani et al. 2012). Finally,
138 nematodes have acquired several parasitism genes via horizontal gene transfer from close
139 relatives of rhizobia (Danchin et al. 2010, 2016).

140 In this report we describe a genetic conflict between plant responses to mutualistic
141 rhizobia and parasitic nematodes in the model legume *Medicago truncatula*. Using two
142 greenhouse-based inoculation experiments, we addressed four questions: (1) How do rhizobia
143 and nematodes impact fitness in co-infected plants?; (2) Is there genetic variation in plant
144 susceptibility to nematodes?; (3) Is there genetic conflict between plant responses to mutualistic
145 rhizobia and parasitic nematodes?; and (4) How do parasitic nematodes impact the rhizobia
146 mutualism? We found a genetic tradeoff between attracting rhizobia and repelling nematodes,
147 and that nematodes disrupt the legume-rhizobia mutualism by deterring nodulation. Our results
148 suggest that genetic conflict with nematodes may maintain variation in partner quality in the
149 legume-rhizobia mutualism, and that genetic tradeoffs with parasitism may be an important
150 overlooked source of variation in positive species interactions.
151

Methods

Study species

Medicago truncatula is an annual plant native to the Mediterranean (Cook 1999). Because nodule number is correlated with rhizobia fitness in *M. truncatula* (Heath and Tiffin 2009), it can be used as a proxy for *M. truncatula*'s partner quality (i.e., the benefits it provides) in the rhizobia mutualism. The *M. truncatula* accessions used for this study came from the French National Institute for Agricultural Research (INRA), and the US National Plant Germplasm System (NPGS) Western Regional Plant Introduction Station. For rhizobia inoculations, we used the *E. meliloti* strain Em1022, a highly effective nitrogen-fixer, supplied by (Batstone et al. 2016). We obtained soil infected with the northern root-knot nematode *Meloidogyne hapla* from Dr. Benjamin Mimee (Agriculture and Agri-food Canada, Saint-Jean-sur-Richelieu, Quebec), and maintained these nematodes on tomato plants (cv. Rutgers) in growth chambers and greenhouses at the University of Toronto.

Greenhouse experiments

We performed two greenhouse experiments to investigate genetic conflict between *M. truncatula*'s response to mutualistic rhizobia and parasitic nematodes. In both experiments, we scarified *M. truncatula* seeds with a razor blade, sterilized them in bleach and ethanol, and stratified them in the dark at 4°C for 36 hours on sterile water agar plates (Garcia et al. 2006). We incubated seeds at room temperature for 16 hours before planting to initiate radicle elongation. We planted the seedlings into sand in 120ml autoclavable Cone-tainers, autoclaved twice at 121°C, and maintained seedlings in the greenhouse at the University of Toronto at 22ºC

174 during the day and 18ºC at night, on a 16:8 light:dark cycle. We top-watered seedlings with
175 distilled water for two weeks, and bottom-watered them for the remainder of the experiments.
176 Two weeks after germination, we inoculated seedlings with the rhizobium *E. meliloti* and
177 the nematode *M. hapla*. We cultured rhizobia strain Em1022 on solid tryptone yeast (TY) agar
178 media, re-plated colonies three times, and inoculated liquid TY media with these cultures. We
179 diluted liquid cultures to an OD600 reading of 0.1, following previous methods (Simonsen and
180 Stinchcombe 2014b), and inoculated each plant with 1mL of culture at two and three weeks post-
181 germination. We inoculated plants with nematode eggs at the same time. To harvest nematode
182 eggs from infected tomato plants for inoculation, we followed a bleach extraction protocol
183 (Eisenback 2000). Female nematodes lay several hundred eggs into a gelatinous matrix on the
184 outside of each gall (Maggenti and Allen 1960). We rinsed the roots of infected tomato plants
185 and incubated them in a shaker at room temperature for 5 minutes in 10% commercial bleach
186 (0.5% NaOCl) to dissolve the gelatinous matrix binding the eggs together. We poured the
187 solution through a series of mesh soil sieves, collected nematode eggs on a #500 mesh sieve
188 (25μm pore size), and stored collected eggs in distilled water in Falcon tubes. We inoculated
189 each plant with nematode eggs twice (at two and three weeks post-germination), on the same
190 schedule as the rhizobia inoculations.
191 *Experiment 1*: To test how rhizobia and nematodes impact fitness in co-infected plants,
192 and to measure genetic variation in nematode infectivity, we used a fractional factorial design
193 with a total of 400 *M. truncatula* plants from 10 genotypes across 10 blocks. We inoculated each
194 plant with 1 of 40 nematode genotypes. Each block included 4 replicates of each plant genotype
195 and 1 replicate of each nematode genotype, for a total of 40 replicates of each plant genotype and

196 10 replicates of each nematode genotype. Each nematode genotype inoculated 2 different plant
197 genotypes, for a total of 5 replicates per nematode genotype-plant genotype combination.
198 To culture individual nematode genotypes, we inoculated tomatoes with second-stage
199 juvenile (J2) nematodes from individual egg masses (Thies et al. 2002). This protocol ensured
200 that each tomato plant was infected by a single maternal family. After approximately three
201 months, we extracted nematode eggs from these tomatoes and used them to inoculate our
202 experimental plants. We inoculated each plant with ~200-400 nematode eggs, depending on
203 availability, and included number of eggs as a covariate in our statistical analyses. Nine plants
204 received >400 eggs; excluding these plants from the analysis did not qualitatively affect our
205 results. We harvested plants 3.5 months after planting.
206 *Experiment 2*: To measure genetic conflict between attracting rhizobia and repelling
207 nematodes, and to test how parasitic nematodes impact the rhizobia mutualism, we used a split-
208 plot randomized design. Each block contained two treatments: one in which we only inoculated
209 plants with rhizobia, and one in which we inoculated plants with both rhizobia and nematodes.
210 Plants received a total of 400 nematode eggs from a genetically variable nematode inoculum.
211 Each treatment in each block contained one *M. truncatula* individual from each of 50 genotypes.
212 In each block, we bottom-watered all plants in the same treatment from the same tray. We
213 replicated this design across 10 blocks (50 plants per treatment per block × 2 treatments × 10
214 blocks = 1000 plants). We did not include a nematode-only treatment because *Medicago* grows
215 poorly under nitrogen-poor conditions without rhizobia (Harrison et al. 2017a). We harvested
216 plants 4.5 months after planting.
217 We checked flowering and collected ripe fruit daily throughout both experiments. Upon
218 harvesting the plants, we stored the roots at 4°C in zip-top plastic bags until processing. We

219 dried the aboveground tissue in a drying oven for approximately 1 week and weighed it to the
220 nearest 1mg. We weighed all fruit each plant produced to measure total fruit mass. To verify that
221 fruit mass was an accurate measurement of reproductive success, we measured the correlation
222 between fruit mass and seed number for a subset of plants (N = 167) and found that fruit mass
223 and seed number were tightly correlated ($r = 0.76$, $P < 0.001$, $df = 165$). We counted the number
224 of nodules and galls on each root system under a dissecting microscope. To capture differences
225 in nodule size, we haphazardly harvested up to ten of the largest nodules on each plant. Nodules
226 were stored in 2mL tubes containing silica desiccant and synthetic polyester for a month until
227 they dried out, and we weighed the dried nodules collected from each plant to the nearest 1µg.
228 We estimated total nodule biomass for each plant by multiplying total nodule number by mean
229 nodule mass. After counting nodules and galls and harvesting nodules, we dried the roots in a
230 drying oven for approximately 1 week and weighed them to the nearest 1mg.
231
232 *Statistical analyses*
233 We performed all analyses in R 3.3.2 with deviation coding ("contr.sum") for categorical
234 variables (R Core Team 2016). Unless stated otherwise, we ran all analyses with the *(g)lmer*
235 function in the *lme4* package (Bates et al. 2015). We tested significance of fixed effects with type
236 III sums of squares using the *Anova* function in the *car* package (Fox and Weisberg 2011), and
237 used likelihood ratio tests to test significance of random effects (Bolker et al. 2009). We
238 confirmed that all models met the parametric statistical assumptions of normality,
239 homoscedasticity, and linearity by inspecting quantile-quantile plots, scale-location plots, and
240 plots of the residuals versus fitted values, respectively. We also checked for overdispersion by
241 testing whether the ratio of the residual variance to the residual degrees of freedom was equal to

242 1. We calculated least-squares treatment and genotype means using the *lsmeans* package (Lenth
243 2016) and created figures using *ggplot2* (Wickham 2009).
244
245 *Effect of rhizobia and nematodes on fitness in co-infected plants (Experiment 1)*
246 To test how rhizobia and nematodes impact fitness in co-infected plants, we analyzed two
247 fitness components, aboveground biomass and total fruit mass. These models included number of
248 nodules, number of galls, root mass, researcher (to control for differences among researchers in
249 nodule and gall counts), and the number of nematode eggs in the inoculum as fixed effects, and
250 block as a random effect. We log-transformed aboveground biomass for analysis. We included a
251 fixed effect of root mass in this and subsequent analyses to control for differences in overall root
252 system size and foraging ability, as well as differences in the root space available for the
253 formation of symbiotic structures (i.e., nodules and galls).
254
255 *Genetic variation in plant susceptibility to nematodes (Experiments 1 and 2)*
256 In Experiment 1, we tested for genetic variation in infectivity among nematode
257 genotypes, and for a plant genotype-by-nematode genotype interaction. A genotype-by-genotype
258 interaction for gall number would indicate that the number of galls formed depends on the
259 combination of plant and nematode genotypes. In this analysis, we included random effects of
260 plant genotype, nematode genotype, plant genotype × nematode genotype, and block. We
261 included fixed effects of root mass, researcher (to control for differences among researchers in
262 gall counts), and the number of nematode eggs in the inoculum. We log-transformed gall number
263 for this analysis because the log transformation met parametric statistical assumptions much
264 better than a Poisson or negative binomial GLMM. When testing for the genotype-by-genotype

interaction, we excluded plant genotype-nematode genotype combinations with fewer than three replicates.

In Experiment 2, we tested for genetic variation in plant susceptibility to nematodes by testing for significant variation among plant genotypes in the number of galls they produced. This analysis included fixed effects of root mass and researcher (to control for differences among researchers in gall counts), and random effects of plant genotype and block. Gall number was zero-inflated and overdispersed, so we fit a zero-inflated negative binomial GLMM using the R package *glmmADMB* (Fournier et al. 2012).

Genetic conflict between attracting rhizobia and repelling nematodes (Experiment 2)

To test for genetic conflict between plant responses to mutualistic rhizobia and parasitic nematodes, we estimated the genetic correlation between nodule number and gall number. To estimate genotype means for gall number, we extracted the conditional modes of the genotype random effect from a model that included fixed effects of root mass and researcher, and random effects of genotype and block. Because we found evidence that nematodes disrupt the mutualism by inhibiting nodulation (see Results), we use estimates of nodulation from the rhizobia-only treatment to estimate the genetic correlation with gall formation. We estimated genotype means for nodule number using a model similar to the gall model, and specified a negative binomial error distribution and allowed for zero inflation in both models.

We also estimated the genetic correlation between gall number and the change in nodule number between the two treatments. We estimated genotype means for nodule number in nematode-infected plants using a similar model to the one used to estimate nodule number in the rhizobia-only treatment. We subtracted the genotype mean for nodule number in nematode-

infected plants from the genotype mean for nodule number in uninfected plants to calculate the change in nodule number for each genotype.

Effect of nematodes on the rhizobia mutualism (Experiment 2)

To test how parasitic nematodes impact the rhizobia mutualism, we compared nodule number, mean nodule mass, and total nodule biomass between nematode-infected and uninfected plants. These analyses included treatment (nematode presence or absence) and root mass as fixed effects, and random effects of genotype, block, treatment × genotype and treatment × block. The treatment × block interaction is necessary when analyzing split-plot experiments to allow the effect of nematode treatment to vary across blocks (Altman and Krzywinski 2015). We specified a negative binomial error distribution for nodule number and allowed for zero inflation using the function *glmmadmb* in the R package *glmmADMB* (Fournier et al. 2012), and log-transformed mean nodule mass and total nodule biomass for analysis. The nodule number analysis included a fixed effect of researcher to control for researcher differences in nodule counts.

We ran similar analyses to compare aboveground biomass, flowering time, and total fruit mass between nematode-infected and -uninfected plants. We log-transformed all three variables for analysis, and omitted the fixed effect of root mass. For flowering time and total fruit mass, we analyzed a subset of genotypes (N = 22) with at least three replicates that flowered and fruited in each treatment, to test for treatment × genotype interactions.

Results

Effect of rhizobia and nematodes on fitness in co-infected plants (Experiment 1)

Rhizobia and nematodes affected different fitness components in co-infected plants (Figure 2). Plants that formed more nodules had significantly greater aboveground biomass than plants with fewer nodules ($\chi^2_{df=1} = 33.918$, $P < 0.001$; Figure 2A). There was no corresponding effect of gall number on aboveground biomass ($\chi^2_{df=1} = 0.370$, $P = 0.543$; Figure 2B). By contrast, the number of nodules did not significantly affect the total fruit mass that plants produced ($\chi^2_{df=1} = 0.490$, $P = 0.484$; Figure 2C), but plants with more galls produced less total fruit mass than plants with fewer galls ($\chi^2_{df=1} = 9.394$, $P = 0.002$; Figure 2D).

Genetic variation in plant susceptibility to nematodes (Experiments 1 and 2)

In both experiments, there was significant variation among plant genotypes in the number of galls formed (controlling for root biomass) (Experiment 1: $N_{genotypes} = 10$, $P = 0.001$; Experiment 2: $N_{genotypes} = 48$, $P < 0.001$), indicating that there is genetic variation in plant susceptibility to nematode infection. In addition, there was significant variation in gall number among nematode genotypes in Experiment 1 ($N_{genotypes} = 40$, $P < 0.001$). There was no significant plant genotype × nematode genotype interaction ($N_{plant\text{-}nematode\ combinations} = 74$, $P = 0.539$).

Genetic conflict between attracting rhizobia and repelling nematodes (Experiment 2)

There was a significant positive correlation between gall number and the number of nodules produced in the absence of nematodes ($r = 0.30$, $P = 0.039$; Figure 3A). This correlation disappeared when the outlier genotype HM170, which formed 2.9 standard deviations more nodules than the mean in our experiment, was included in the analysis ($r = 0.06$, $P = 0.710$). In another study of nodulation in *M. truncatula*, this genotype also formed more nodules than 90%

of 250 accessions surveyed (Stanton-Geddes et al. 2013a,b). Together, our results and those of Stanton-Geddes et al. suggest that this genotype may be a biological outlier with respect to the rhizobia mutualism, so we ran subsequent analyses with and without this outlier genotype.

There was no significant genetic correlation between gall number and the number of nodules produced in the presence of nematodes, regardless of whether the outlier genotype HM170 was included in the analysis (with HM170: $r = -0.20$, $P = 0.153$; without HM170: $r = 0.04$, $P = 0.789$). However, there was a significant positive genetic correlation between gall number and the change in nodule number between the two treatments ($r = 0.31$, $P = 0.034$; Figure 3B), indicating that plant genotypes that were most susceptible to nematodes (i.e., formed the most galls) decreased the most in nodule number when infected with nematodes. Excluding HM170 did not qualitatively change this result ($r = 0.29$, $P = 0.052$).

Effect of nematodes on the rhizobia mutualism (Experiment 2)

Nematode-infected plants produced fewer nodules and less total nodule biomass than uninfected plants, although mean nodule mass did not differ between infected and uninfected plants (Table 1, Figure 4A-C). There was a significant effect of plant genotype for all nodule traits (Table 1), indicating that genotypes differed in mutualism phenotypes. We detected a significant treatment × genotype interaction for nodule number and a marginally significant treatment × genotype interaction for total nodule biomass (Table 1, Figures 4D & 4F). These interactions indicate that plant genotypes differed in how nodule traits were impacted by nematode infection. There was no treatment × genotype interaction for mean nodule mass (Table 1, Figure 4E). Our results were qualitatively similar when we removed the outlier genotype HM170 (see Figure 4D).

356 Other plant traits were not strongly affected by nematode infection. Although there was
357 significant genetic variation for all plant traits, there was no difference between infected and
358 uninfected plants in aboveground biomass, flowering time, or total fruit mass (Table 2). There
359 was a marginally significant treatment × genotype interaction for aboveground biomass (Table
360 2).
361
362 **Discussion**
363 Here we showed that an ecologically relevant parasite disrupts the mutualism between
364 leguminous plants and nitrogen-fixing rhizobial bacteria. *Medicago truncatula* plants that were
365 infected by parasitic nematodes formed fewer rhizobia nodules and less nodule biomass per gram
366 of root tissue than uninfected plants. Strikingly, nematode infection impacted nodule traits more
367 strongly than other plant phenotypes, indicating that the parasite's effect on the legume-rhizobia
368 mutualism is not merely a byproduct of lower overall performance in infected plants. Moreover,
369 we found that a plant's affinity for rhizobia and susceptibility to nematodes were genetically
370 correlated: plants that formed more nodules with rhizobia were more heavily infected by
371 nematodes. Our results suggest that genetic conflict with parasitic nematodes is an important
372 factor shaping the *Medicago*-rhizobia mutualism. If genetic conflict with parasitism is a general
373 feature of many mutualisms, it may contribute to the maintenance of genetic variation for partner
374 quality and influence evolution in positive species interactions.
375
376 *Nematodes decrease associations between* Medicago *and mutualistic rhizobia*
377 Our work extends past research on the impact of antagonists on mutualism in two key
378 ways. First, we showed that mutualism traits were more strongly impacted by parasite infection

than other aspects of plant performance. In the presence of nematodes, plants formed fewer associations with mutualistic rhizobia. We found that nematode-infected *Medicago* plants formed 23% fewer nodules and 19% less total nodule biomass per gram of root than uninfected plants (Figure 4A-C). By contrast, nematode infection only weakly affected aboveground biomass, flowering time, and total fruit mass (Tables 1 and 2). Future work in other mutualisms should explore whether elevated sensitivity to parasites is a characteristic feature of mutualism traits.

Second, when ecological factors influence mutualistic associations, their evolutionary consequences depend on whether there is standing genetic variation for environmental responsiveness in the form of genotype-by-environment interactions. Although environmental effects on mutualism are common (Bronstein 1994; Bronstein et al. 2003; Kersch and Fonseca 2005; Afkhami et al. 2014), and antagonists often interfere with the fitness benefits of mutualists (Gomez 2005; Liere and Larsen 2010; Simonsen and Stinchcombe 2014a), these effects are rarely investigated from a genetic perspective (but see (Heath et al. 2010)).

Our results demonstrate that there is standing genetic variation for *Medicago*'s susceptibility to parasite infection, as well as in the degree to which the plant's mutualism was robust to parasite-mediated disruption (treatment × genotype interaction: Table 1 and Figure 4D-F). *Medicago truncatula* genotypes varied significantly in their susceptibility to nematode infection, with some genotypes forming dozens or hundreds of galls while others formed few or none. Moreover, while some plant genotypes formed substantially fewer nodules when infected by nematodes, others—including one hyper-nodulating outlier (Figure 4D)—were largely unaffected by the parasite. The degree to which the *Medicago*-rhizobia mutualism is impacted by parasitic nematodes therefore has the genetic capacity to evolve. There was also genetic variation

in infectivity in the nematode population (i.e., nematode genotypes differed in the number of galls they formed on plant roots), demonstrating that both the plant and the parasite have the genetic capacity to evolve in response to the other. However, we found no evidence for genotype-by-genotype interactions between plants and nematodes that would facilitate coevolution in the system.

Genetic tradeoff between attracting a mutualist and repelling a parasite

Medicago truncatula's susceptibility to nematode infection was genetically correlated with its affinity for mutualistic rhizobia (Figure 3A). Plant genotypes that formed the most rhizobia nodules also formed the most galls, while genotypes that formed few nodules were more resistant to nematode infection. One caveat to this result is that the genetic correlation was no longer significant when the hyper-nodulating outlier genotype was included (Figure 3A). This outlier appears to be behaving fundamentally differently with respect to the rhizobia mutualism, and may be an informative point of comparison for future work on the genomic underpinnings of the genetic correlation between nodulation and galling.

The genetic correlation between attracting rhizobia and repelling nematodes in *Medicago* is consistent with molecular genetic work in the legume *Lotus japonicus* showing that mutants that do not form nodules are also resistant to nematode infection (Weerasinghe et al. 2005). To our knowledge, only a handful of past studies have documented genetic conflict between mutualism and parasitism (Toth et al. 1990; Miller 1993). Both examined the symbiosis between plants and mycorrhizal fungi, and found pathogen-resistant genotypes formed fewer mycorrhizal associations. Genetic conflict may be a general feature of intimate symbioses like plant-microbe mutualisms, in which one partner lives inside the tissue of another.

425 A genetic correlation underlying the tradeoff between attracting mutualists and repelling
426 parasites, like the one we documented in *M. truncatula,* is one mechanism that can contribute to
427 the maintenance of genetic variation for partner quality in mutualisms (Heath and Stinchcombe
428 2014) and alter evolutionary trajectories (Nuismer and Doebeli 2004; Strauss and Irwin 2004).
429 The genetic tradeoff between mutualism and parasitism has distinct evolutionary consequences
430 for mutualism at different spatial and temporal scales. First, within a single population, as more
431 mutualistic genotypes spread, susceptibility to parasites is also spreading: eventually, this should
432 erode, or eliminate the fitness advantage gained by being a better mutualist partner, slowing or
433 preventing their fixation. Second, variation in mutualist and parasite abundance among sites or
434 years is likely to create a selection mosaic that favors high-quality partners where parasites are
435 absent and low-quality partners where parasites are absent. Such spatial and temporal variation in
436 the direction of selection and could maintain genetic variation for partner quality in mutualism
437 (Thompson 2005; Huang et al. 2015). To directly assess how the genetic tradeoffs we report
438 influences selection on, and variation in, partner quality in the legume-rhizobia mutualism, future
439 work should characterize spatial and temporal variation in nematode and rhizobia abundance in
440 wild *Medicago* populations.
441 Although there are surprisingly few estimates of the genetic tradeoff between mutualism
442 and antagonism, widespread tradeoffs at the phenotypic level suggest that genetic conflict
443 between positive and negative species interactions may be extremely common. For example,
444 trypanosomatid parasites of firebugs mimic the vertical transmission mechanisms of their host's
445 bacterial symbionts, such that symbiont transmission is associated with a risk of parasite
446 infection (Salem et al. 2015). In the seed dispersal mutualism between Clark's nutcracker and
447 pine trees, selection exerted by a seed predator opposes mutualist-mediated selection (Siepielski

448 and Benkman 2009). Pollinators and herbivores often cue in on the same plant signals, imposing
449 conflicting selection on floral displays that weakens the overall strength of pollinator-mediated
450 selection (Rey et al. 2002; Gomez 2003; Schiestl et al. 2011, 2014; Ågren et al. 2013; Knauer
451 and Schiestl 2017). If these phenotypic tradeoffs are underpinned by genetic correlations like the
452 correlation we report in the legume-rhizobia mutualism, genetic conflict with parasitic
453 interactions is likely an important source of genetic variation in diverse mutualistic systems
454 (Bronstein 2001a; Strauss and Irwin 2004).
455
456 *Implications for mutualism evolution*
457 Our study joins a number of others demonstrating that an evolutionary genetic approach
458 to mutualism can yield meaningful new insights about these positive species interactions (Heath
459 2010; Heath et al. 2012; Afkhami and Stinchcombe 2016; Burghardt et al. 2017). A recent
460 transcriptomic study of the legume-rhizobia mutualism, for example, showed that genotype-by-
461 genotype interactions between plants and rhizobia impact carbon and nitrogen exchange, the
462 central function of the symbiosis (Burghardt et al. 2017). Intriguingly, Burghardt et al. (2017)
463 also found significant variation among plant genotypes in the expression of defense genes in
464 nodules. Together, our study and theirs raise the possibility that conflict with plant immunity is a
465 key feature of the legume-rhizobia mutualism whose evolutionary significance has been largely
466 overlooked.
467 Genetic conflict with parasites could significantly alter the rate and trajectory of
468 evolution in mutualisms. The impact of this conflict on mutualism evolution depends on three
469 factors about which little is known in any system: the degree of overlap in the genetic pathways
470 controlling the two symbioses; how parasites disrupt mutualistic partnerships; and the ecological

471 factors that mediate conflict. All three of these factors warrant further study in the legume-
472 rhizobia mutualism, and a diverse array of other positive species interactions.
473
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483
484 **Author contributions**
485 CW and JS conceived of the study, and CW, BP, PV and JS designed the experiments. CW, BP,
486 PV, and CB performed the experiments and collected data. All authors contributed to data
487 analysis and interpretation. CW wrote the initial draft of the manuscript, and all authors
488 contributed to manuscript revisions.
489
490 **Data accessibility**
491 Data from both experiments will be available on Dryad.
492

493 **References**

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682

Tables and Figures

Table 1. Effect of treatment (nematode presence or absence), plant genotype, and the treatment × genotype interaction on nodule traits.

	Nodule number		Mean nodule mass		Total nodule mass	
	$\chi^2_{df=1}$	*P*	$\chi^2_{df=1}$	*P*	$\chi^2_{df=1}$	*P*
Treatment	14.77	**<0.001**	2.66	0.103	19.48	**<0.001**
Genotype	-	**<0.001**	24.67	**<0.001**	13.39	**<0.001**
Trt × Geno	-	**0.022**	0	1.000	3.51	0.060

We do not report χ^2 values for the genotype and the treatment × genotype interaction for nodule number because glmmADMB models do not return χ^2 values for random effects. We used glmmADMB for the nodule number analysis to accommodate zero-inflation and overdispersion (see Methods).

Table 2. Effect of treatment (nematode presence or absence), plant genotype, and the treatment × genotype interaction on plant traits. Top: Aboveground biomass, flowering time, and total fruit mass.

	Aboveground biomass		Flowering time		Total fruit mass	
	$\chi^2_{df=1}$	*P*	$\chi^2_{df=1}$	*P*	$\chi^2_{df=1}$	*P*
Treatment	0.47	0.495	0.66	0.417	2.76	0.096
Genotype	56.33	**<0.001**	17.90	**<0.001**	5.65	**<0.001**
Trt × Geno	3.55	0.059	0	1.000	0.03	0.859

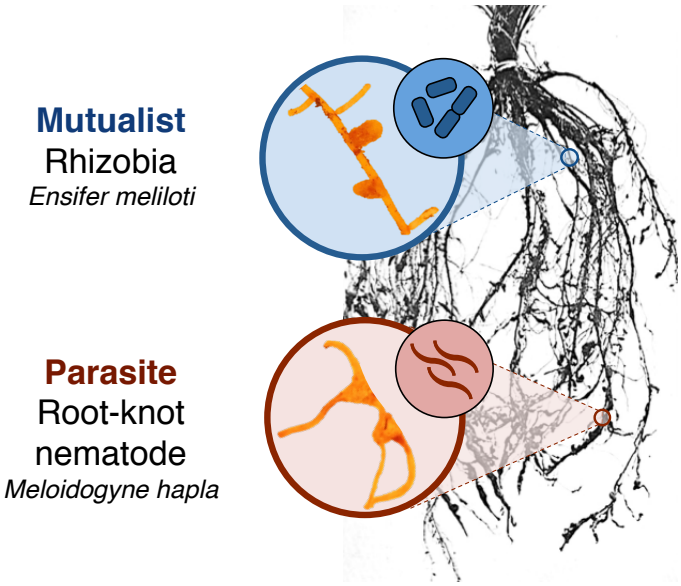


Figure 1. Nodules formed by mutualistic rhizobia (top) and galls formed by parasitic nematodes (bottom) on legume roots. Each gall contains one female nematode. Root image adapted from an image by L.T. Leonard (Fred et al. 1932).

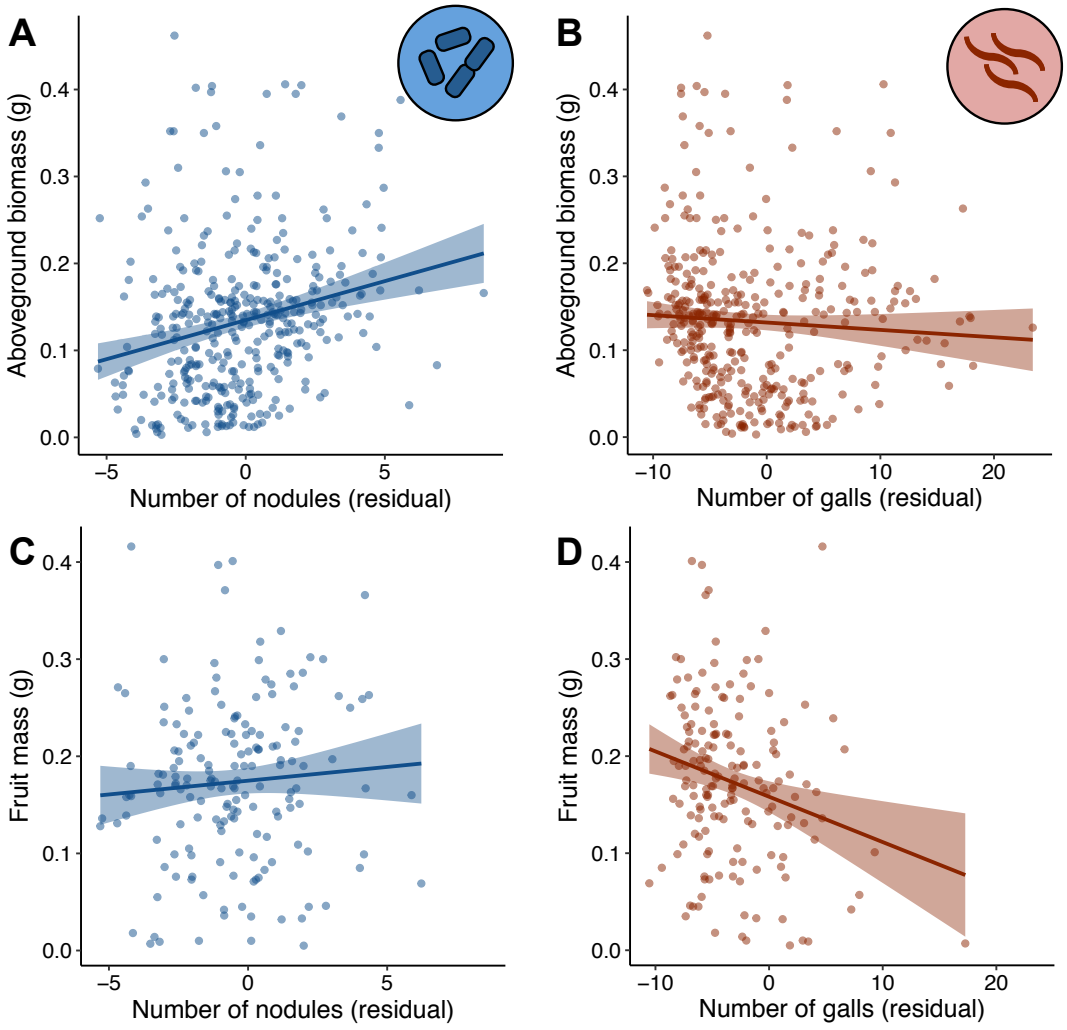


Figure 2. Rhizobia and nematodes affect different plant fitness components in co-infected plants. (A and C) The relationship between nodule number and aboveground biomass (A), and nodule number and fruit mass (C). (B and D) The relationship between gall number aboveground biomass (B), and gall number and fruit mass (D). Bands are standard errors. The negative relationship in (D) remained significant when the point in the lower right-hand corner was removed.

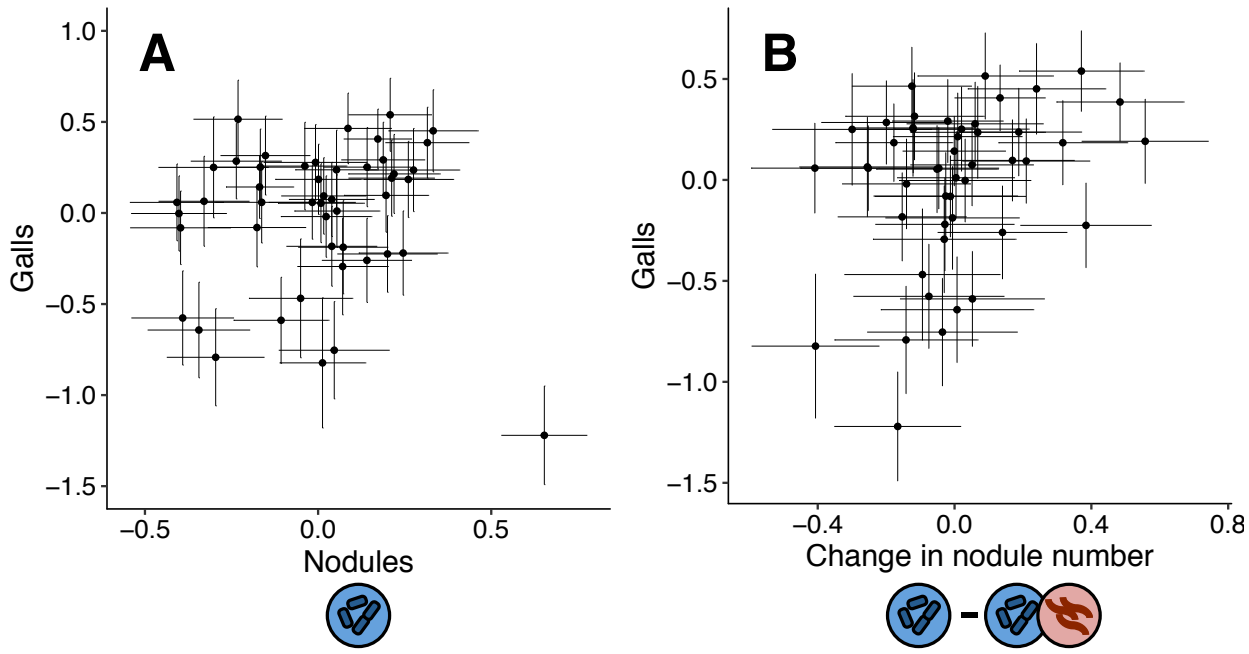
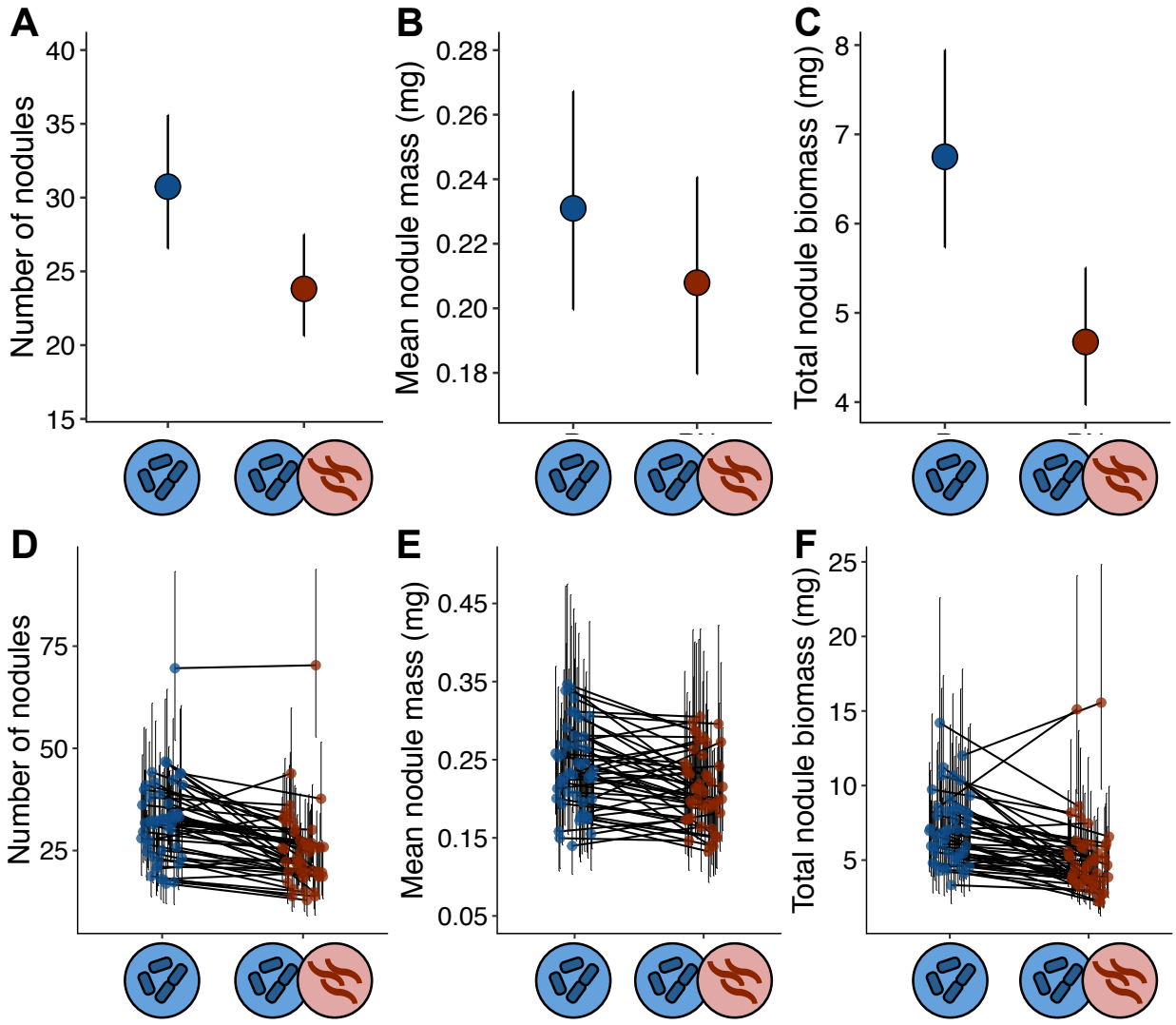


Figure 3. Genetic correlation between the number of galls and nodules that plants produce. Points are conditional modes (BLUPs) for each plant genotype ± SE. (A) Genetic correlation between gall number and the number of nodules produced by plants in the absence of nematodes. There is a significant positive correlation when the outlier in the lower right-hand corner is excluded (r = 0.30, P = 0.039), but not when it is included (r = 0.06, P = 0.710). (B) Genetic correlation between gall number and the change in nodule number in the absence and presence of nematodes (r = 0.31, P = 0.034). Excluding HM170 did not qualitatively change this result (r = 0.29, P = 0.052). We used a resampling approach to generate the standard errors on the change in nodule number in panel B.



721

722 Figure 4. Nematodes affect the nodule phenotypes. Number of nodules (A), mean nodule mass
723 (B), and total nodule biomass (number of nodules × mean nodule mass) in nematode-infected
724 and -uninfected plants. In A-C, points are least-squares treatment means ±95% CIs. (D-F)
725 Genotype-by treatment interactions for number of nodules (D), mean nodule mass (E), and total
726 nodule biomass (F). In each treatment, points are least-squares genotype means ±95% CIs; lines
727 connect the same genotype in the two treatments.

728