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For the Special Section: The Ecology, Genetics, and Coevolution of Intimate Mutualisms

Nitrogen addition does not influence pre-infection partner choice in the legume–rhizobium symbiosis[1]

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PREMISE OF THE STUDY: Resource mutualisms such as the symbiosis between legumes and nitrogen-fixing rhizobia are context dependent and are sensitive to various aspects of the environment, including nitrogen (N) addition. Mutualist hosts such as legumes are also thought to use mechanisms such as partner choice to discriminate among potential symbionts that vary in partner quality (fitness benefits conferred to hosts) and thus impose selection on rhizobium populations. Together, context dependency and partner choice might help explain why the legume–rhizobium mutualism responds evolutionarily to N addition, since plant-mediated selection that shifts in response to N might be expected to favor different rhizobium strains in different N environments.

METHODS: We test for the influence of context dependency on partner choice in the model legume, *Medicago truncatula,* using a factorial experiments with three plant families across three N levels with a mixed inoculation of three rhizobia strains.

KEY RESULTS: Neither the relative frequencies of rhizobium strains occupying host nodules, nor the size of those nodules, differed in response to N level.

CONCLUSIONS: Despite the lack of context dependence, plant genotypes respond very differently to mixed populations of rhizobia, suggesting that these traits are genetically variable and thus could evolve in response to longer-term increases in N.

KEY WORDS mutualism; context-dependency; Fabaceae; legume–rhizobium; nitrogen deposition; partner choice; nodulation; *Medicago truncatula*

Mutualistic interactions are fundamental components of every ecosystem on Earth, span all domains of life, and contribute immensely to ecosystem function. In resource mutualisms, partners trade in limiting resources such that both species enjoy higher fitness (Schwartz and Hoeksema, 1998; Hammerstein and Noe, 2016). Higher-quality mutualists provide more resources and thus increase the fitness of their partners over those of lower-quality mutualists; therefore, partner quality can be viewed as a quantitative trait of one mutualist, measured as the effects it has on its partner's fitness, and composed of genetic and environmental effects (Heath and Stinchcombe, 2014). Partner quality is quite variable in nature, but theoretical models of mutualism cannot easily explain how such variation is maintained in the face of selection (Foster and Kokko, 2006; McNamara and Leimar, 2010; Heath and Stinchcombe, 2014). One explanation for the maintenance of mutualism variation is context-dependency; if selection acting on mutualist populations shifts depending on the environment, then variable environments (biotic, abiotic) can maintain genetic variation in partner quality. Resource mutualisms might be particularly context dependent, as the availability and thus the value of traded commodities varies over spatial and temporal scales (Neuhauser and Fargione, 2004; Sachs and Simms, 2006; Thrall et al., 2007; Kiers et al., 2010; Chamberlain et al., 2014). Thus, understanding whether known agents of selection in mutualisms vary with the environment is part of a general understanding of how resource mutualisms (co)evolve. Here, we tested for environmental context dependence in plant rhizobial mutualisms by examining plasticity in host associations in response to external variation in nitrogen.

Plants in the legume family (Fabaceae) exchange photosynthate for plant-available forms of N generated by N-fixing bacteria (collectively termed rhizobia) that are housed in root nodules. The legume–rhizobium symbiosis is ecologically and economically significant, as it is the main source of nonanthropogenically fixed N in terrestrial systems (Vitousek et al., 1997; Cleveland et al., 1999). The Fabaceae is also one of the most speciose plant families (Doyle, 1998; Werner et al., 2014) and the second most important crop

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family behind the grasses (Graham and Vance, 2003). Since there is no vertical transmission of symbionts, plant hosts must acquire rhizobial partners anew with each generation, and these prospective plant hosts are faced with a diverse range of potential rhizobium species and genotypes (Smith and Goodman, 1999; Simms and Taylor, 2002). The rhizobium with which a plant associates is potentially very important, since rhizobia can vary considerably in terms of the fitness benefits they confer to the plant host (Burdon et al., 1999; Simms et al., 2006; Heath, 2010; Sachs et al., 2010a; Barrett et al., 2012; Porter and Simms, 2014).

Current research suggests that legumes possess mechanisms that mitigate the potential impacts of less-beneficial rhizobia on host fitness (or conversely, reward better mutualist partners) and thus potentially act as agents of selection on rhizobium partner quality. There is evidence that plants can allocate fewer resources to nodules that generate less fixed N, resulting in smaller nodules containing fewer rhizobium offspring ("sanctions" sensu Denison, 2000; Kiers et al., 2003; Oono et al., 2009; but see Frederickson, 2013). Many legumes also seem to be able to discriminate among rhizobia and preferentially form nodules with strains that confer greater fitness benefits ("partner choice" sensu Lie et al., 1987; Bull and Rice, 1991; Heath and Tiffin, 2009; Sachs et al., 2010b; Ehinger et al., 2014). It is important to note, however, that there is likely not a single universally most beneficial strain of rhizobia; instead, the degree of fitness benefits provided by a rhizobial symbiont depends on the genotype of the host plant (G × G interactions) and the environmental context (G × E) (Heath and Tiffin, 2007; Heath, 2010). Furthermore, the ability to discriminate among rhizobium partners of varying quality appears to be genetically variable in natural populations of legumes (Heath and Tiffin, 2009; Simonsen and Stinchcombe, 2014). The dependence of rhizobium partner quality and host benefits on the environment suggests that host-mediated selection on rhizobium populations and resulting evolutionary effects on rhizobium partner quality will also be context dependent.

Modern human activity has elevated soil nitrogen (N) availability in both natural and agricultural systems through direct fertilization and atmospheric deposition (Canfield et al., 2010). If soil N is readily available, it presumably becomes less costly for a plant to simply acquire N from the environment rather than invest resources into symbiosis. Legumes typically decrease nodulation in response to N addition (Streeter, 1988; Caetanoanolles and Gresshoff, 1991; Glyan'ko et al., 2009), suggesting that symbiosis is costly to maintain. For these reasons, increases in soil N are expected to disrupt the legume–rhizobium mutualism by weakening the positive fitness feedbacks between partners that maintain mutualism (West et al., 2002; Sachs and Simms, 2006; Akcay and Simms, 2011; Sachs et al., 2011). Indeed, recent evidence indicates that N fertilization leads to changes in partner quality in rhizobium populations both in the short term (one season; Simonsen et al., 2015) and long term (22 yr; Weese et al., 2015; Klinger et al., 2016), though the exact way in which N addition altered selection on rhizobium populations remains unresolved.

Together, this previous knowledge of the legume–rhizobium symbiosis gives rise to the prediction that legumes should invest less in discriminating mechanisms when N is readily available. Regus and colleagues (2014) recently studied stabilizing mechanisms under N addition in *Lotus strigosus* and found no evidence that partner choice or sanctions changed in response to high N. We build on this work by studying these mechanisms in a different legume–rhizobium system, incorporating different N conditions and host genetic variation. Using a manipulative greenhouse experiment with three genotypes of the model legume *Medicago truncatula* and a mixed inoculum of three genotypes of its primary symbiont *Ensifer meliloti* across three N levels, we asked: (1) Do the frequencies of rhizobium strain occupancies (of host nodules) depend on the N level? (2) Do the sizes of host nodules inhabited by particular rhizobium strains vary across N levels? (3) How does context dependency in partner choice, or plant responses to mutualist quality, affect the maintenance and evolution of these traits?

MATERIALS AND METHODS

We used a factorial experiment, consisting of three plant maternal families, three N levels, and five replicates ($3 \times 3 \times 5 = 45$ pots total) grown in sterile Magenta box leonard jars (Heath et al., 2010) in the University of Toronto plant growth facilities in December of 2007. All plants received the same inoculum of an equal mixture of three strains (48e, 35c, 30a: *Chat c*, *Sals b*, and *Naut a,* respectively, from Heath et al. [2010]). Soil was autoclave-sterilized and wet with ~30 mL nutrient solution before inoculation with ~10^8 total rhizobium cells in 1 mL diluted culture broth. The soil surface was covered with a layer of sterile sand, and pots were bottom-watered with sterile nutrient solution throughout the experiment. We kept Magenta pots filled with 1/4 strength Fahreus solution (Somasegaran and Hoben, 1994), with intermediate and high N treatments supplemented with 0.1 mM or 1.0 mM KNO_3, respectively. N levels used were those chosen by Heath et al. (2010) to replicate the range of reported N levels near fertilized agricultural fields (Kanwar et al., 2006), while not eliminating nodulation by *Medicago* (Fei and Vessey, 2009).

Plants were harvested after 6 weeks. We clipped the aboveground tissue for drying and measuring plant aboveground biomass and counted total nodules on each plant. For each of 10 nodules from each plant, we assigned a size score (1–4; 1 = very small and white; 2 = small but pink; 3 = medium/average (~2 mm); 4 = large), then isolated and genotyped the rhizobium strain inhabiting the nodule using previously described procedures to assess nodule occupancy (Heath and Tiffin, 2009). Briefly, nodules were surface-sterilized, crushed, and plated before DNA extraction and genotyping using a post-PCR restriction digestion assay designed specifically to differentiate these three *E. meliloti* strains. Of 441 genotyped nodules, 11 nodules were obviously of mixed occupancy even after culturing and were excluded from further analysis.

We used fixed-effects ANOVA implemented in the program JMP (version 12, SAS Institute, Cary, North Carolina, USA) to test for the effects of plant family, N level, and their interaction on the two main response variables (nodule occupancy and nodule size) and nodule number and plant biomass. For nodule occupancy, we analyzed the frequency of each strain separately in three separate ANOVA models; then, we created a "nodule occupancy" variable by assigning each plant a 1, 2, or 3 based on which strain was dominant in its nodules (1 = strain 30a, 2 = strain 35c, or 3 = strain 48e). Similarly, for nodule size, we first analyzed all plant nodules together, then separately for each occupying strain. Results in the separate and combined analyses were similar for both occupancy and size; therefore, we present the latter analysis in the paper for

simplicity (see Appendix 1 for per-strain results). Least-squares means (lsmeans) for each plant family from the per-strain analyses for traits X and Y were regressed against the partner quality means (from a previous single-strain inoculation using the same families, strains, and N levels; Heath et al., 2010) to assess partner choice and plant allocation responses to partner quality in this experiment. Partner choice would be indicated by a positive relationship between strain partner quality in single strain inoculation and that strain's occupancy in mixed inoculation; sanctioning would be indicated by a positive relationship between strain partner quality in single inoculation and that strain's nodule size in mixed inoculation (Kiers et al., 2003; Simms et al., 2006; Heath and Tiffin, 2009; Regus et al., 2014).

RESULTS

As expected, plant biomass increased with elevated N levels and varied among plant families (Table 1; see means in Appendix 2). Nodule number ranged from 3 to 64 per plant and varied among plant families, but did not respond significantly to N level or the plant family × N interaction (Table 1; Appendix 2). Nevertheless, nodule number and biomass were positively correlated (R^2 = 0.1743, P = 0.0048; Table 1; Appendix S1, see Supplemental Data with the online version of this article), suggesting that even the highest N treatment (1 mM) was not enough to saturate the growth benefits of association with rhizobia.

Nodule occupancy varied significantly among plant families, but was not altered by N addition or the plant family by N interaction (Table 1; Appendix 1). Plant family Chat 1 associated most often with strains 30a and 48e, with mean occupancy of N treatments ranging from 54.3 to 59.3% and 33.8 to 42.5% respectively, and rarely associated with strain 35c (mean occupancy of N treatments ranging from 1.2 to 6.9%; Fig. 1, Appendix 2). Conversely, plant families Chat 3 and Sals 1 predominantly associated with strain 35c, with 68.4–80.3% and 71.1–91.5% of nodules being occupied with 35c among N treatments, respectively (Appendix 2). Nodule size also varied significantly among plant families, but did not respond to N level or the interaction (Table 1, Appendix 1). Overall, Chat 1 had larger nodules than the other plant families (Appendix 2). There was no evidence that nodule occupancy or size among the three rhizobium strains became more similar with increasing N level; in other words, plants did not become less choosy as N increased (Fig. 1; Appendix 2).

We tested for relationships between nodule occupancy and nodule size and partner quality as estimated from a previous single inoculation experiment (Heath et al., 2010). Overall, we found no significant association between either nodule occupancy (R^2 = 0.0355, P = 0.347) or nodule size (R^2 = 0.0087, P = 0.643) and rhizobium partner quality (Fig. 2).

DISCUSSION

Nitrogen is the main nutrient that limits primary productivity in terrestrial ecosystems (reviewed by Canfield et al., 2010). In their evolutionary history, plants in the legume family evolved to circumvent this constraint by forming mutualistic associations with rhizobia (Doyle, 2011; Werner et al., 2014); however, anthropogenic increases in nitrogen abundance threaten to disrupt this mutualism by altering the costs and benefits of resource exchange (Kiers et al., 2010; Weese et al., 2015). We tested whether environmental dependence of plant nodulation or nodule size with different rhizobium strains played a role in shifting natural selection on rhizobia by altering the strains that enjoy high fitness in high N environments. We found that (1) increased N did not alter nodule occupancy and that (2) increased N did not alter nodule size overall or the size of nodules containing particular rhizobium strains. Next, we explore the influence of context dependency—or its absence—on partner choice in terms of signaling and partner quality.

Nodule occupancy is not context dependent—Here we found that nodule occupancy did not change with the N environment. Instead, nodule occupancy strongly depended on plant family, and these effects were consistent across N treatments. Such genetic variation among host genotypes in nodule occupancy agrees with our previous work (Heath and Tiffin, 2009), which examined nodule occupancy for 12 plant genotypes, including the three studied here (and without N manipulation); moreover, the dominant strain(s) occupying the nodules was largely consistent across experiments. Such consistency in occupancy patterns across studies, which invariably differed in experimental conditions (e.g., photoperiod, temperature), further suggests the robustness of nodule occupancy to environmental context. In a similar study in *Lotus*, Regus et al., (2014) also found that nodule occupancy did not vary with increased N availability, though they did not study the variation in occupancy among plant genotypes. Together these findings illustrate the lack of context dependency for nodule occupancy.

An absence of plasticity in nodule occupancy might be surprising given the potential costs of partner choice mechanisms and, by extension, selective pressure to downregulate them when N is readily abundant in the soil (West et al., 2002; Sachs and Simms, 2006). The notion that such mechanisms can be costly is supported by experiments documenting a fitness cost associated with pathogen defense mechanisms such as *R*-gene-mediated plant defense (Tian et al., 2003; van Hulten et al., 2006). Adaptive plasticity in plant responses to environmental cues (e.g., plant density, herbivores; Oldroyd et al., 2011) is well known in other traits (Dudley and Schmitt, 1996; Agrawal, 1998).

Nodule occupancy might be unresponsive to environmental conditions due to the underlying signaling mechanisms that

TABLE 1. ANOVA results of nodule occupancy, nodule size, nodule number, and plant biomass in this experiment with a mixed inoculation of three strains of *Ensifer meliloti* strains on each of three maternal plant families in three nitrogen treatments (0, 0.1, 1.0 mm KNO_3).

		Nodule occupancy		Nodule size		Nodule number		Plant biomass	
Source	df	*F* ratio	*P* value	*F* ratio	*P* value	*F* ratio	*P* value	*F* ratio	*P* value
Plant family	2	11.4655	<0.0001	35.579	<0.0001	9.5000	0.0005	3.5154	0.0406
Nitrogen level	2	0.9602	0.3277	2.3228	0.1160	2.3385	0.1114	4.6249	0.0163
Plant family × N level	4	0.6659	0.5144	1.3122	0.2888	0.9224	0.4620	0.6956	0.6001

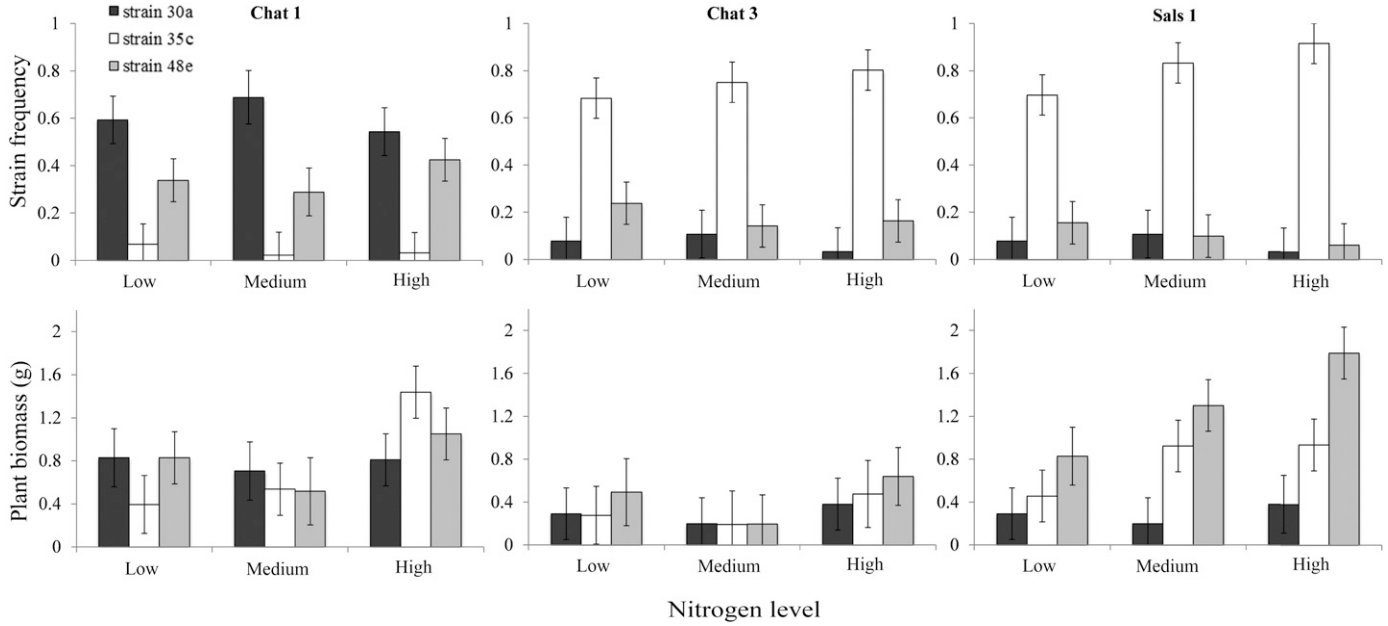

FIGURE 1 Components of plant partner choice. The top panel shows nodule occupancy as represented by the frequency of three *Ensifer meliloti* strains occupying nodules across three N levels for the three plant families in a mixed inoculation experiment. The lower panel provides partner quality data from Heath et al. (2010) for single-strain inoculations in the corresponding nitrogen levels and plant families. Error bars represent standard error.

control it (Oldroyd et al., 2011; Oldroyd, 2013). Initially, plants secrete unique flavonoids that are recognized by narrow a range of rhizobium (Peck et al., 2006), inducing the production of bacterial Nod factors that can be received by plant receptors and thus causing the root tip to curl and the initiation of nodule formation. Nod factors share a common core backbone, but can be highly modified among rhizobium species, altering plant perception (Lerouge et al., 1990; Peck et al., 2006). Similarly, plant Nod factor receptors can be diverse, which can cause symbiotic specificity (Perret et al., 2000; Wang et al., 2012). Beyond Nod factors, plants recognize surface polysaccharides and secreted proteins from rhizobia (Parniske et al., 1994; Simsek et al., 2007; Oldroyd et al., 2011; Wang et al., 2012), which has been shown to regulate host specificity in surface polysaccharide mutants (Hotter and Scott, 1991; Parniske et al., 1994) as well as between *Medicago* and *Ensifer* (Simsek et al., 2007). The plant receptors that recognize surface polysaccharides remain unknown (Wang et al., 2012), although lectins are suspected to function in this role (Bohlool and Schmidt, 1974; Laus et al., 2006). Additionally, a class of *R* genes, *Rj2*/*Rfg1*, have been shown to control host specificity in soybean (Yang et al., 2010). Taken together, these levels of signal exchange provide an array of "lock and key" type interactions that could govern specificity between legumes and rhizobia and are likely to be constitutively expressed and potentially unable to respond plastically to the environment. It is

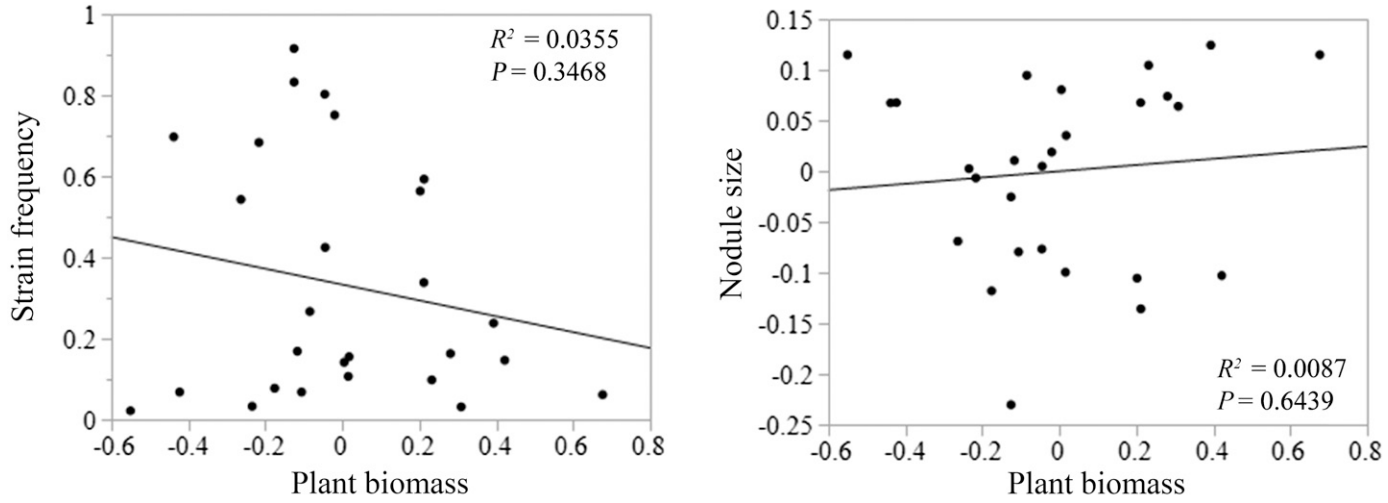

FIGURE 2 Correlations between nodule occupancy and nodule size on plant biomass. Nodule occupancy and size were measured in a mixed inoculation experiment with three *Ensifer meliloti* strains. Biomass data are from Heath et al. (2010) from single-strain inoculations of the three same strains. Biomass data are standardized relative to the plant family mean.

important to note, however, that the majority of these signaling mechanisms have been characterized in laboratory-generated mutants and crop plants; thus, we have only scanty knowledge of the genetics of natural variation in nodule occupancy within natural populations of hosts and symbionts and of their environmental sensitivity.

Despite the absence of plastic responses, nodule occupancy can still evolve, particularly if an environmental change alters the relative fitness benefits of associating with different partners. Abundant evidence from our work and others (Caetano-Anolles and Gresshoff, 1991; Heath and Tiffin, 2009; Simonsen and Stinchcombe, 2014) demonstrates standing genetic variation for nodule occupancy in natural plant populations. Moreover, the diversity in host–symbiont specificity among species within the Fabaceae (Wang et al., 2012; Oldroyd, 2013) implicates coevolution with rhizobia in shaping the signaling mechanisms of extant legumes. Additionally, there is molecular population genetic evidence for natural selection targeting known signaling genes within the genus *Medicago* (De Mita et al., 2006, 2007). For the most part, however, molecular population genetics/genomics approaches have not been robustly associated with phenotypic differences in nodule occupancy or with environmental shifts (e.g., N availability) that might serve as agents of selection (but see Stanton-Geddes et al., 2013); this is a promising avenue for continued future work.

Partner choice as a context-dependent, composite trait—Nodule occupancy alone is not partner choice. For nodule occupancy to be adaptive for plants and impose selection for beneficial rhizobia, i.e., to qualify as partner choice (Bull and Rice, 1991; Foster and Kokko, 2006), plants must selectively associate with symbionts that confer high fitness benefits. Thus, the assessment of adaptive partner choice is contingent upon two other traits—occupancy due to signaling interactions and partner quality (Heath and Tiffin, 2009; Sachs et al., 2010; Regus et al., 2014). We are able to assess partner choice by comparing the nodule occupancy results presented here with our previous work (Heath et al., 2010) examining partner quality conferred by each individual rhizobium strain. Unlike Heath and Tiffin (2009), who studied these plant genotypes and nine more, here we found no relationship between plant biomass after a single-strain inoculation and the occupancy of the three strains in our mixed-strain inoculation experiment—not supporting partner choice in the context of this experiment.

Because the outcome of partner choice depends on the match between partner quality and signaling, changes in either of these traits could lead to a change in outcome. If neither trait displays context dependency (Fig. 3A), or if both traits change in a coordinated manner that maintains the match between partner quality and signaling (Fig. 3B), then partner choice would not change across environments, i.e., would be context independent. In response to an environmental change, however, shifts in signaling without a

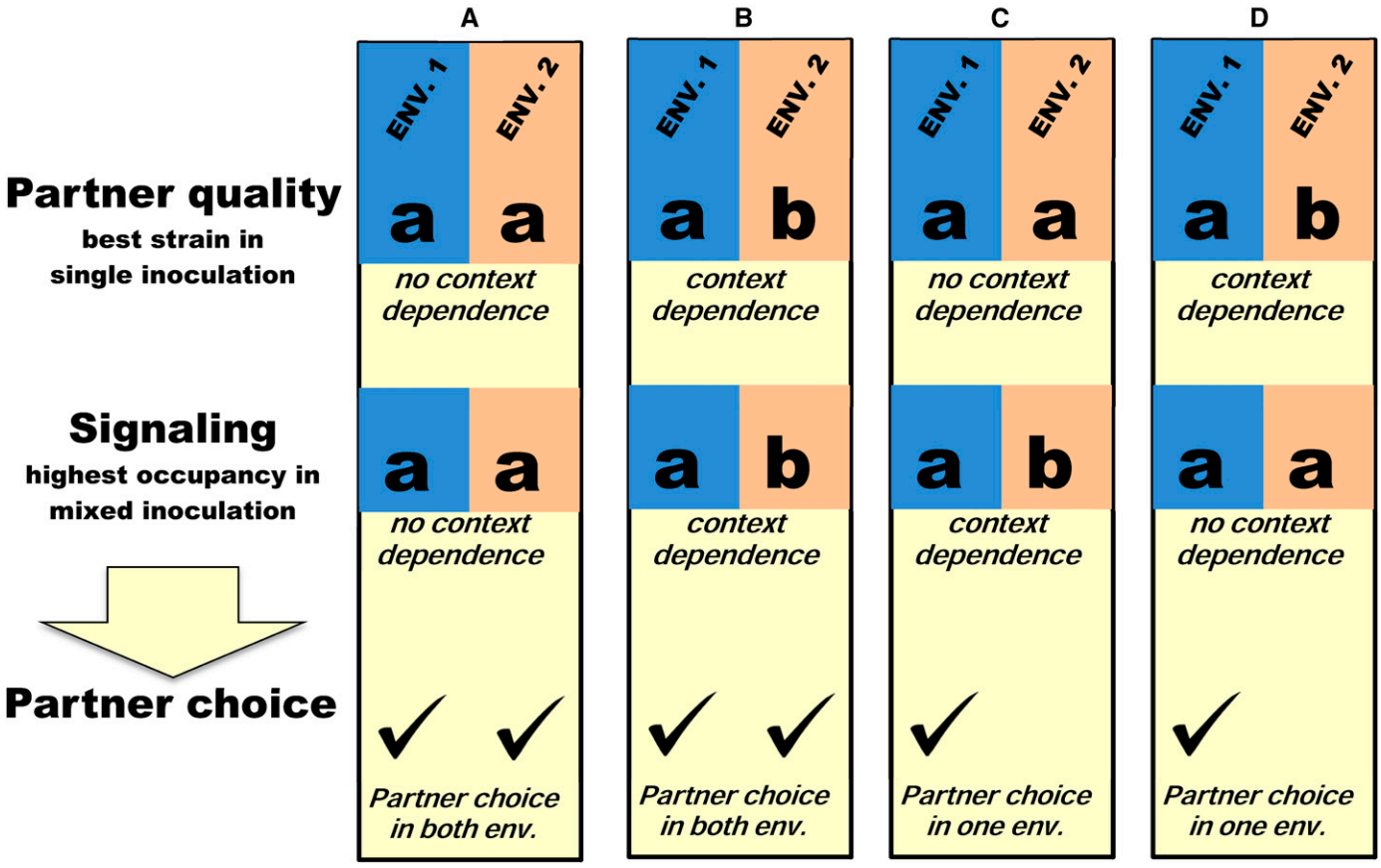

FIGURE 3 Context dependency in partner choice. Partner choice occurs when the same strain (a or b) confers the highest fitness benefits (partner quality) and also occupies the most nodules (signaling). This outcome, therefore, can either be context independent (scenarios A and B), or context dependent (scenarios C and D), as a result of how partner quality and signaling change across environments (env.).

concomitant shift in partner quality (Fig. 3C), or conversely shifts in partner quality without a concomitant shift in signaling (Fig. 3D), would make the outcome of partner choice context dependent as well.

Unlike our previous study (Heath and Tiffin, 2009), only one family (Chat 1) and in one N environment (low N) "matched" occupancy to partner quality (by forming the most nodules with 30a or 48e and few with 35c; Fig. 1). The other treatment combinations (plant genotypes × N environments) were mismatched as a result in shifts in partner quality (i.e., scenario Fig. 3D). For example, Chat 3 made the vast majority of nodules with 35c in both mixed experiments, but partner quality as estimated in single inoculation differed; this plant family had highest biomass with 48e here, but previously had highest fitness with 35c (Heath and Tiffin, 2009). Unlike occupancy resulting from signaling interactions, which appears to remain constant across environments (see discussion above), abundant evidence has documented context dependency in partner quality in legume–rhizobium mutualism, with the fitness benefits of particular mutualists changing with the nutrient environment (Heath and Tiffin, 2007; Heath et al., 2010; Lau et al., 2012), light environment (Lau et al., 2012), and herbivory (Simonsen and Stinchcombe, 2014). Context dependency in mutualism benefits appears to be widespread (Thompson, 1988; Bronstein, 1994; Johnson et al., 1997; Chamberlain et al., 2014). To our knowledge, context dependency in the signaling/occupancy component of mutualisms is much less studied, though it is quite possible that in other systems with different mechanisms, such as choice of cleaner fish (Bshary and Grutter, 2005) or nectar production (Frederickson et al., 2012), could be much more plastic.

While our main result, that nodule occupancy was consistent across N environments, is quite consistent with that of Regus et al. (2014), they did not find context dependency in partner choice. In their study, plants formed fewer nodules with strain 2 (vs. three higher quality strains) in both low and high N treatments. Although Regus et al. (2014) did not explicitly decompose partner choice into partner quality and occupancy components, their data on plant growth after single inoculation indicated that partner quality did not shift between N environments (i.e., strain 2 was always the least-beneficial strain). Thus their least-beneficial rhizobium strain appears to be a universally poor performer (Sachs et al., 2010a, b), consistent with Fig. 3A. We have found no such naturally occurring, universally poor strains of *E. meliloti* (Heath, 2010).

No evidence for plant allocation responses to partner quality—We found no evidence of a relationship between a strain's partner quality and the size of the nodules it occupied. This result is consistent with our previous work (Heath and Tiffin, 2009) and that of Gubry-Rangin et al., (2010) both in *Medicago truncatula*. In contrast, other legume species (including *Medicago sativa*) have been found to respond to rhizobia that are either prevented from fixing N (Kiers et al., 2003, 2006; Oono et al., 2009) or are naturally of lower-quality (Simms et al., 2006; Sachs et al., 2010b) by forming smaller nodules that harbor fewer rhizobium offspring. Unlike partner choice, the underlying genetic and physiological mechanisms underlying these responses are largely unknown (Oono et al., 2009; Regus et al., 2014). Moreover, given the many developmental parallels between nodules and lateral roots (Hirsch and LaRue, 1997), whether a change in nodule size in response to rhizobia of different N-fixation abilities represents a legume adaptation to deal with rhizobium partner quality per se, vs. a generalized root response to soil nutrient availability, remains to be tested (Frederickson, 2013).

CONCLUSIONS

Our work and that of others shows that relative nodule occupancy and nodule size among rhizobium strains does not differ among N environments. These results suggest that plastic responses in these traits are likely not a main driver of the evolutionary effects of N addition on this symbiosis (e.g., Weese et al., 2015). Nor would we expect plasticity in these plant traits to mediate any context-dependent selection acting to maintain partner quality variation in nature—other explanations are required. Nevertheless, plant genetic variation is an important and under-appreciated factor determining which rhizobium strains succeed in establishing nodules. Thus, nodule occupancy, and its composite trait partner choice, can evolve in plant populations in response to biotic and abiotic selective agents, including N levels or rhizobium partner choice.

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APPENDIX 1 **ANOVA results of nodule occupancy and nodule size for specific *Ensifer meliloti* strains on each of three maternal plant families in three nitrogen treatments (0, 0.1, 1.0 mm KNO_3).**

		Strain 30a		Strain 35c		Strain 48e	
Source	df	*F* ratio	*P* value	*F* ratio	*P* value	*F* ratio	*P* value
Nodule size							
Plant family	2	8.4806	0.0023	29.552	<0.0001	21.414	<0.0001
N level	2	1.2422	0.3112	1.1224	0.3420	2.8588	0.0770
Plant family × N level	4	0.7077	0.5966	1.4594	0.2455	0.4770	0.7522
Nodule occupancy							
Plant family	2	43.291	<0.0001	147.166	<0.0001	9.687	<0.0001
N level	2	0.8054	0.4450	2.3278	0.1124	0.1233	0.8844
Plant family × N level	4	0.2004	0.9364	1.1919	0.3315	0.4608	0.7639

APPENDIX 2 **Mean trait values for each maternal plant family and nitrogen treatment in this experiment. Nodule occupancy is the proportion of nodules occupied by a specific *Ensifer meliloti* strain. Standard errors are presented in parentheses.**

					Nodule occupancy		
Plant family	N level	Biomass (g)	Nodule number	Nodule size (mm)	Strain 30a	Strain 35c	Strain 48e
Chat1	High	0.248 (0.044)	17.4 (1.98)	2.72 (0.09)	0.543 (0.0735)	0.032 (0.0595)	0.425 (0.0795)
Chat1	Medium	0.118 (0.041)	18.2 (7.45)	2.56 (0.09)	0.575 (0.0822)	0.012 (0.0665)	0.413 (0.0889)
Chat1	Low	0.148 (0.025)	14.4 (2.56)	2.65 (0.11)	0.593 (0.0735)	0.069 (0.0595)	0.338 (0.0795)
Chat3	High	0.309 (0.058)	37.2 (8.75)	1.89 (0.06)	0.033 (0.0735)	0.803 (0.0595)	0.163 (0.0795)
Chat3	Medium	0.249 (0.025)	42.6 (2.08)	1.88 (0.08)	0.107 (0.0735)	0.751 (0.0595)	0.142 (0.0795)
Chat3	Low	0.167 (0.053)	40 (10.17)	2.02 (0.08)	0.078 (0.0735)	0.684 (0.0595)	0.238 (0.0795)
Sals1	High	0.184 (0.037)	25.2 (9.91)	1.43 (0.11)	0.022 (0.0735)	0.915 (0.0595)	0.062 (0.0795)
Sals1	Medium	0.159 (0.016)	51.6 (4.11)	1.43 (0.09)	0.068 (0.0735)	0.833 (0.0595)	0.096 (0.0795)
Sals1	Low	0.143 (0.029)	40.6 (7.39)	1.87 (0.07)	0.141 (0.0735)	0.711 (0.0595)	0.148 (0.0795)