



Genetic variation in mutualistic and antagonistic interactions in an invasive legume

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Abstract

Mutualists may play an important role in invasion success. The ability to take advantage of novel mutualists or survive and reproduce despite a lack of mutualists may facilitate invasion by those individuals with such traits. Here, we used two greenhouse studies to examine how soil microbial communities in general and mutualistic rhizobia in particular affect the performance of a legume species (*Medicago polymorpha*) that has invaded five continents. We performed two plant growth experiments with *Medicago polymorpha*, inoculating them with soil slurries in one experiment or rhizobial cultures in another experiment. For both experiments, we compared the growth of *Medicago* in competition with conspecific or heterospecific plants and examined variation among plant genotypes collected from the native and introduced ranges. We found that all genotypes experienced similar increases in biomass and formed more nodules that house rhizobia bacteria when inoculated with soil from a previously invaded site, compared to uninoculated plants or plants inoculated with soil from uninvaded and low invasion sites. In a second experiment, plants inoculated with rhizobia generally produced more biomass, had greater tolerance to interspecific competition, and had greater effects on competitor biomass than uninoculated plants. However, plant genotypes collected from the native range benefited more from rhizobia and were less tolerant of competition relative to genotypes collected from the introduced range. In the introduced range, compatible mutualists may not be readily available but competition is intense, causing *Medicago* to evolve to benefit less from interactions with rhizobia mutualists, while simultaneously becoming more tolerant of competition.

Keywords Competition · Genetic diversity · Invasive species · Mutualism · Rhizobia

Introduction

Most species on earth engage in interactions with mutualists, and the effects of mutualisms on communities and ecosystems are strong and far-reaching. Forming a successful

mutualism can expand species range limits (Afkhami et al. 2014), increase population growth rates (Ehrlén and Eriksson 1995; Rudgers et al. 2012), and protect species from antagonists such as predators or competitors (Strauss and Murch 2004; Aschehoug et al. 2012). Negative species interactions, such as competition, predation, and disease, are often hypothesized to provide biotic resistance to invasion (Levine et al. 2003, 2004), but the ability to recreate strong mutualistic interactions in the introduced range also may be key to invasion success. For example, obligate outcrossing plants that require specialist pollinators are unlikely to successfully invade habitats where the pollinator is absent (Parker 1997; Richardson et al. 2000). More generally, a recent meta-analysis showed that invasive plants benefited more from mutualism, including resource, pollination, and defense mutualisms, than exotic plants that failed to become invasive, suggesting that reestablishing mutualisms in the introduced range may be a key determinant of invasiveness (Schultheis et al., in revision).

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The soil microbiome can affect plant invasion success (Klironomos 2002; Callaway et al. 2004; Nijjer et al. 2007; Lau and Suwa 2016). In particular, the absence of mutualistic soil microbes, such as mycorrhizal fungi and rhizobia bacteria, may decrease the success of plant invaders (Parker et al. 2006; Rodriguez-Echeverria et al. 2007; 2009; Thiet and Boerner 2007; Nuñez et al. 2009; Dostal et al. 2013; Lau and Suwa 2016; Simonsen et al. 2017). For example, a review of 3500 legume species found that legumes that live symbiotically with nitrogen-fixing bacteria are less likely to invade new habitats than non-symbiotic legumes (Simonsen et al. 2017). Even intentionally introduced exotic Pinaceae can fail to establish when lacking compatible mycorrhizae (Nuñez et al. 2009).

Although a lack of mutualists can reduce the success of potential invaders, individuals can overcome this obstacle in two ways. First, individuals that co-invade with their mutualist partner (e.g., Porter et al. 2011) have traits that allow associations with novel mutualists in their new environment, or have a more generalist strategy for forming mutualisms (Mitchell et al. 2006; Batstone et al. 2018), are more likely to invade successfully than individuals that are unable to form mutualisms (Richardson et al. 2000; Baynes et al. 2012; Nuñez and Dickie 2014; Moeller et al. 2015). Second, invaders may be more likely to succeed in a novel environment where mutualists are absent if their performance is less dependent on mutualists (Richardson et al. 2000; Pringle et al. 2009; Waller et al. 2016). For example, autogamous plant species are common invaders because they do not require a pollinator for reproductive success (Baker 1974; Burns et al. 2011). Similarly, garlic mustard (*Alliaria petiolata*) is a widespread invader in the eastern United States, in part because it does not rely on mycorrhizal fungi for nutrients and actually inhibits mycorrhizae that are beneficial to competing native plants (Weir 2007; Callaway et al. 2008). Invaders that employ one of these two mechanisms are likely to overcome the potential negative effects of losing mutualists from their native range.

Plant species must contend with potential effects from competitors and other antagonists to invade successfully. Species with lower competitive responses or stronger competitive effects are more likely to overcome biotic resistance to invasion by heterospecific competitors. However, over time the type and intensity of competition is likely to change. Early in the invasion process, individuals are more likely to encounter heterospecific competitors, but at late stages of invasion, individuals will encounter more conspecifics. In some cases, the traits that make an individual competitive against conspecifics do not translate to heterospecific competitive ability (Lankau and Strauss 2007; Lankau 2011; Vasseur et al. 2011; Huang and Peng 2016).

However, interactions with antagonists occur in a community context, where interactions with other species result

in indirect effects among species that can play an important role in the outcome of species interactions (Vandermeer 1969; Lawler 1993; Miller 1994). Often mutualists play a direct role in reducing the effects of antagonists, such as when ant defenders reduce damage from herbivores (Rosumek et al. 2009), but the effect of mutualists on the effects of antagonists may also occur indirectly through effects on the plant (Marler et al. 1999; Borowicz 2001; Mitchell et al. 2006; Morris et al. 2007). For example, mycorrhizal fungi increased the competitive effect of invasive *Centaurea maculosa* on a native bunchgrass and enhanced the ability of the invader to overcome biotic resistance from native competitors (Marler et al. 1999). Mutualists may increase invasion success by altering competitive ability and the effectiveness of biotic resistance from antagonists.

The ultimate effect of mutualists on invasion success may depend on individual-level variation in direct and indirect interactions with other species. Individuals within a species may vary in investment in and benefit derived from mutualism, either because of phenotypic plasticity (Bever 2015) or genetic variation in traits underlying mutualistic interactions (Yoder and Tiffin 2017). In the latter case, the traits underlying mutualistic interactions can be evolutionarily labile. Strong selection on traits during the invasion process can result in the rapid evolution of a wide variety of traits in invading species (Blossey and Notzold 1995; Lee 2002; Felker-Quinn et al. 2013; Bossdorf et al. 2004; Buswell et al. 2011), including traits that allow individuals to be generalists or take advantage of novel mutualists (Mitchell et al. 2006; Batstone et al. 2018) or traits that decrease reliance on mutualists (e.g., Seifert et al. 2009). Such genetic variation in traits mediating interactions with mutualists may lead to substantial genetic variation in invasion success. Previous work suggests that genotype-dependent invasion success arises from genetic variation in traits related to overcoming biotic resistance (e.g., herbivore tolerance, competitive ability; Lee and Petersen 2002; terHorst and Lau 2015; Bayliss et al. 2017), but genetic variation in mutualistic traits (Tawaraya 2003) or responses to indirect interactions (terHorst and Lau 2015) may also be relevant.

Legumes and rhizobia bacteria engage in a mutualism in which rhizobia fix atmospheric nitrogen in exchange for carbon fixed through photosynthesis. This mutualism is not obligate for legumes, and in higher nitrogen soils or in low-light environments, the benefits of association begin to degrade and can shift along a mutualism–parasitism continuum (Hirsch 2004; Denison and Kiers 2004; Sachs and Simms 2006; Lau et al. 2012; Shantz et al. 2016). To test whether this mutualism and soil biota more generally influence invasive plant growth and potentially invasion success, we performed two experiments with *Medicago polymorpha*, a species that has invaded five continents, inoculating plants with field soils from invaded and uninvaded sites in

one experiment and inoculating plants with pure cultures of rhizobia in the second experiment. In both experiments, we investigated effects of soil biota on several plant genotypes collected from the native and introduced ranges, and grown in the presence of conspecific or heterospecific competitors, to test for genetic variation in the benefits the introduced species received from rhizobia and how the introduced species responds to competitors. We found that genotypes collected from the introduced range tended to benefit less from rhizobia mutualists, and that rhizobia altered competitive interactions with other plants.

Methods

Study system

Medicago polymorpha (Fabaceae) is native to the Mediterranean region, but has invaded many other regions of the world. This annual legume, hereafter “*Medicago*”, typically invades grassland and edge habitats and can reach high densities in those areas. *Medicago* reproduces primarily through selfing (Vitale et al. 1998) and this strong inbreeding leads to homozygosity. Thus, maternal lines are effectively homozygous clones—hereafter “genotypes”. Previous work suggests that genotypes of *Medicago* differ in morphological traits, such as stem color, growth form, and fruit morphology (De Haan and Barnes 1998), as well as direct and indirect ecological interactions with herbivores and competitors (terHorst and Lau 2015; Bayliss et al. 2017; Getman-Pickering et al. *in review*).

In both the native and introduced range, *Medicago* associates with, and is largely specialized on, the rhizobium *Ensifer medicae* (Rome et al. 1996; Porter et al. 2011). *E. medicae* is presumed to have been introduced outside the native range concurrently with *Medicago* (Porter et al. 2011). At our California field sites, other legume species appear to use different rhizobia species, so competition for rhizobia between *Medicago* and other naturally occurring legumes is unlikely (Porter and Rice 2013). However, it is unclear whether *Medicago* genotypes from the native and introduced ranges differentially associate with rhizobia, whether associating with rhizobia is indirectly affected by the presence of other legume species, or whether associating with rhizobia affects competitive interactions with other legume species.

The National Plant Germplasm System (NPGS) at the United States Department of Agriculture maintains collections of *Medicago* from around the world. We haphazardly selected 17 accessions, or genotypes, from the NPGS collection (nine from the native range, eight from the introduced range). We used ten genotypes in each of our two experiments; three genotypes were used in both experiments (Appendix 1). We presume that all genotypes were grown

in similar environmental conditions at NPGS, but to further minimize any maternal effects caused by historical environment, we grew each genotype for one generation in a common garden greenhouse at the Kellogg Biological Station (Michigan, USA). Seeds collected from this common garden generation were used in two separate experiments described below.

Experiment 1: effects of soil microbial communities and competition on *Medicago* genotypes

We tested the effects of (a) inoculation with soils from three different locations plus an uninoculated control, (b) competitive environment (conspecific vs. heterospecific plant), and (c) *Medicago* genotype, on the biomass, nodulation, and shoot:root ratio of *Medicago* plants. We manipulated each of these three factors in a fully factorial design ($n=8$ replicates per inocula \times competition \times genotype treatment; $N=640$ total) in the Kellogg Biological Station greenhouse.

Due to space limitation, we limited the experiment to ten haphazardly chosen genotypes from our collection; six of these genotypes were originally collected from different regions in the native range (Ethiopia, Iran, Morocco, Portugal, Spain, Turkey), and four genotypes were originally collected from different regions of the introduced range [Gifu (Japan), Shimane (Japan), California (USA), Texas (USA)] (Appendix 1). We used the Invasive Species Compendium (www.cabi.org/isc) to determine the native/introduced status of each genotype. Seeds were initially germinated in petri dishes with DI water in September 2011, and after 3 days, seedlings were transferred to individual pots (164 mL containers, Stuewe and Sons, Tangent, OR, USA) filled with low nutrient potting media (LP5 Grower Mix from Sun Gro, Agawam, MA, USA: Canadian sphagnum peat moss, fine perlite, and dolomitic limestone, wetting agent, and RESILIENCE™). Competition and soil inocula treatments (see below) were randomly assigned to pots. During the first week, we replaced seedlings that died, although this was not always possible and resulted in the loss of a small number of replicates. We hand-watered pots in equal amounts as needed (typically 2 \times /week) to avoid rhizobia contamination among pots. We added fertilizer (1:100 dilution of Greencare Fertilizers Orchid RO Water Special, Kankakee, IL; 13:3:15 ratio of N:P:K) one time, 6 weeks after planting, because plants were showing signs of nutrient stress.

Soil inoculation treatment

We collected four soil cores (2 cm \times 15 cm) from each of three sites on the McLaughlin Natural Reserve in northern California (USA); cores from the same site were combined together. These sites were used in a previous study that measured survival and reproduction of > 700 *Medicago*

seedlings at each site (terHorst and Lau 2015). At one site (High Invasion), *Medicago* was abundant and seedlings transplanted into this site had higher survival (6.3%) than other sites. At the second site (Low Invasion), *Medicago* was less abundant and experimental transplants had lower survival (2.4%). We did not observe any *Medicago* at the third site (Uninvaded), and experimental transplants had very low survival (0.05%). For each of the three soils, we mixed 5 g of soil in 45 mL of DI water to create soil slurries. We then inoculated each pot with 2 mL of one of the three soil slurries or a water control 8 days after transplanting seedlings.

Competition treatment

Each container received either a second *Medicago* seedling of the same genotype, or a single *Acmispon wrangelianus* (Fabaceae) seedling, transplanted on the same day as the focal plant. We chose *Acmispon* because it commonly co-occurs with *Medicago* at our California field sites and because the two species are functionally and phenologically similar. *Acmispon* seeds were collected from many individual plants within the same site; seedlings were germinated and transplanted in the same manner as *Medicago* seedlings.

Response variables

Ten weeks after transplantation, we harvested the plants and counted the number of nodules on *Medicago* and *Acmispon* roots. We separated above- and belowground *Medicago* and *Acmispon* biomass and dried at 60 °C for 3 days before weighing. We used total biomass as a measure of plant performance, nodule number as a relative measure of plant investment in rhizobial mutualists, and shoot:root ratio as a relative measure of plant investment in resources towards above- or belowground growth. We also analyzed nodule mass as a measure of plant investment in rhizobia, and the results were qualitatively similar.

Data analyses

We used generalized linear mixed models (GLMM) to analyze the fixed effects of soil inoculant, competition treatment (con- or heterospecific), range (introduced or native), and their interactions on *Medicago* total biomass, shoot:root ratio, and nodule number. We included genotype in the model as a random effect, nested within range. We tested for the best-fit error distribution using Akaike's Information Criterion (gamma distribution for biomass and log-normal distribution for shoot:root and nodule mass). Models were fit using lmer and glmer in the R Statistical Environment (v. 3.3.1). Factors or interactions that did not significantly improve the model fit ($\Delta\text{AIC} > -2$) were not included in the final model. We tested the significance of each factor using Likelihood

Ratio Tests and “Anova” in the “car” package. The effects of genotype and interactions between genotype and main effects were tested in a separate set of fixed effect models.

Similarly, we also used a GLMM to test the effects of soil inoculant and *Medicago* collection range on *Acmispon* biomass (log-normal distribution) and nodule number (gamma distribution). We analyzed models with and without *Medicago* biomass as a covariate to account for effects of soil inoculant or collection range via effects on competitor size.

Experiment 2: effects of rhizobia and competitors on *Medicago* genotypes

To isolate effects mediated by rhizobia from other components of the microbial community, we conducted a second experiment that directly manipulated the presence of rhizobia, the competitive environment, and *Medicago* genotype in a fully factorial design. This experiment was conducted outside, adjacent to the greenhouse at California State University, Northridge in an uncovered area that received late afternoon shade. Replicate containers were randomly assigned to one of the 60 treatment combinations: two rhizobia treatments (presence/absence), three competitive environments (no competition, conspecific, or heterospecific), and ten *Medicago* genotypes. Because of limited availability of seedlings of some genotypes due to differential germination success, we used 2–5 replicates of each treatment combination ($\bar{n} \approx 4$), resulting in 254 replicate pots. One genotype (Belgium) did not have any surviving seedlings in the conspecific competition treatment.

To ensure that we were testing the effects of collection range (native vs. introduced) and not just a particular set of genotypes, we again haphazardly chose *Medicago* genotypes from the NPGS collection (three of these were also used in Experiment 1; Appendix 1). Four of the genotypes were originally collected from different regions in the native range (Cyprus, Egypt, France, and Morocco), and six genotypes were originally collected from different regions in the introduced range [Belgium, Bolivia (2 populations), California (USA), Japan, and Peru]. Seedlings from these ten genotypes were planted into 164-mL containers filled with Miracle-Gro™ potting soil in March 2014. Although we did not quantify soil nutrients, this was almost certainly a higher nutrient soil than that used in Experiment 1. Seeds were initially planted in flats, and seedlings were transferred to pots after 1 week. We hand-watered pots in equal amounts every 2 days.

Rhizobia inoculation treatment

We harvested nodules from three *Medicago* plants growing in the botanical garden at California State University, Northridge. We sterilized the surface of nodules using sequential rinses of bleach, ethanol, and sterile water. We squashed the collective

nodules in 0.2 mL of sterile water in a microcentrifuge tube and then spread this solution on Tryptone Yeast (TY) agar plates. After 48 h, we picked ten individual colonies from the plate, and used them to inoculate 1000 mL of sterile TY media to establish a rhizobia culture. We simultaneously maintained 1000 mL of sterile TY media without any inoculant. We incubated the rhizobia and sterile media at 30 °C with daily swirling for 1 week prior to use in the experiment. We added 2 mL of either rhizobia culture or sterile media to appropriate pots 2 weeks after seedlings were transplanted.

Competition treatment

Each seedling received one of three competition treatments: no additional plant, one additional *Medicago* seedling of the same genotype, or one *Acmispon* seedling. *Acmispon* seedlings were also germinated in flats and transplanted 1 day after *Medicago* seedlings.

Response variables

After 7 weeks, we harvested the plants and counted the number of nodules on *Medicago* and *Acmispon* roots. We separated above- and belowground *Medicago* and *Acmispon* biomass and dried plants at 60 °C for 3 days before weighing.

Data analyses

As above, we used GLMMs to analyze the fixed effects of rhizobia, competition treatment, and range (introduced or native) on *Medicago* total biomass (log-normal distribution), nodule number (gamma distribution), and shoot:root ratio (log-normal distribution). We included genotype, nested within collection range, as a random effect. The effects of genotype and genotype interactions with main effects were tested in a separate set of fixed effects models. Because plants in uninoculated pots produced very few nodules (~1% of the nodules in inoculated pots), we excluded the control plants when analyzing nodule number. We also used a GLMM to examine the effects of rhizobia and *Medicago* collection range on *Acmispon* biomass (log-normal distribution), with and without *Medicago* biomass included as a covariate.

For each genotype, we calculated the magnitude by which rhizobia altered the effect of conspecific or heterospecific competition on *Medicago* biomass. As in terHorst and Lau (2015), we calculated the indirect effect (IE) using *Medicago* biomass (B) in each treatment as:

$$\text{IE}_{\text{Conspecific}} = B_{\text{Con,Rhizobia}} - B_{\text{Con,NoRhizobia}} - B_{\text{Alone,Rhizobia}} + B_{\text{Alone,NoRhizobia}}$$

$$\text{IE}_{\text{Heterospecific}} = B_{\text{Hetero,Rhizobia}} - B_{\text{Hetero,NoRhizobia}} - B_{\text{Alone,Rhizobia}} + B_{\text{Alone,NoRhizobia}}$$

Positive values indicate that rhizobia and competitors decreased the effect of each other on *Medicago* biomass, and negative values indicate that rhizobia and competitors increased the effect of each other. Values closer to zero suggest that rhizobia and competitors had similar effects on *Medicago* biomass in the presence and absence of each other.

Data accessibility Data from this manuscript will be archived at the Dryad Repository prior to publication (<https://doi.org/10.5061/dryad.bq3k83n>).

Results

Experiment 1: Effects of soil type and competition on *Medicago* genotypes

The source of the soil inoculant significantly affected nodulation and *Medicago* biomass (Table 1). *Medicago* inoculated with soils from the High Invasion site produced at least 35% more biomass relative to *Medicago* inoculated with soils from other sites or uninoculated controls (Fig. 1a). *Medicago* inoculated with soil from the Low Invasion and Uninvaded sites produced similar biomass to uninoculated control plants (Fig. 1a). Plants inoculated with soil from the High Invasion site produced significantly more nodules than plants inoculated with soils from Low Invasion and Uninvaded sites (Fig. 1b). Nearly all uninoculated control plants produced no nodules (157/160 plants; 2 of the remaining plants only produced 1 nodule each). *Medicago* inoculated with High Invasion soil also displayed greater shoot:root ratios, relative to other soils and the uninoculated treatment (Fig. 1c, Table 1).

Medicago produced more biomass when grown with *Acmispon* than with a conspecific, regardless of the soil inoculation treatment (Fig. 1a, Table 1). The competition treatment influenced nodulation; *Medicago* grown next to *Acmispon* produced more nodules than plants grown next to other *Medicago*, but only in soils from the Low Invasion or Uninvaded sites, where nodulation was low overall (Fig. 1b, Table 1). The presence of *Acmispon* tended to increase shoot:root ratios of *Medicago* (Fig. 1c, Table 1).

In Experiment 1, we found no significant difference in biomass, nodule number, or shoot:root ratios among *Medicago* genotypes, nor did genotypes respond differently to *Acmispon* or soil inoculant (Table 1). However, we found significant differences in shoot:root ratios of *Medicago* genotypes (Table 1).

The soil inoculation treatments also affected *Acmispon* biomass (Table 2); *Acmispon* produced significantly more

Table 1 Results of effects on *Medicago* performance from best-fit generalized linear mixed models associated with Experiment 1

Effects	Total biomass			Nodule number			Shoot:root ratio		
	<i>df</i>	<i>G</i>	<i>P</i>	<i>df</i>	<i>G</i>	<i>P</i>	<i>df</i>	<i>G</i>	<i>P</i>
Soil source	3	14.7	0.002	3	117	< 0.001	3	11.8	0.008
Competitor	1	4.98	0.026	1	5.15	0.024	1	7.23	0.007
Range	1	0.203	0.653	1	2.01	0.157	1	0.173	0.678
Soil × comp	3	3.42	0.331	3	25.36	< 0.001	3	2.21	0.530
Range × comp	1	< 0.001	0.978	–	–	–	1	0.009	0.924
Soil × range	3	3.55	0.314	–	–	–	3	1.78	0.619
Soil × comp × range	3	1.01	0.799	–	–	–	3	0.557	0.906
Genotype	9	5.76	0.057	9	4.25	0.317	9	1.91	0.993
Geno × soil	–	–	–	27	12.7	0.266	–	–	–
Geno × comp	–	–	–	9	3.63	0.445	1	0.221	0.638
Geno × soil × comp	–	–	–	27	16.2	0.053	–	–	–

Significant effects are shown in bold

Marginally non-significant effects are shown in italics

Dashes indicate factors that were not part of the best-fit model

biomass when inoculated with soils from the Uninvaded site compared to inoculation with soils from the High Invasion site or uninoculated controls (Fig. 2). There was no significant effect of *Medicago* collection range on *Acmispon* biomass, although genotypes differed in their effect on *Acmispon* biomass (Table 2). Although increased *Medicago* biomass was significantly associated with reduced *Acmispon* biomass, this effect was independent of soil inoculant or range (interactions not part of the best-fit model; Table 2). Exclusion of *Medicago* biomass as a covariate produced qualitatively similar results, suggesting that the observed effect of soil inoculation was not driven by effects of soils on *Medicago* size. Soil source significantly affected *Acmispon* nodule number (Table 2), with more nodules produced on plants inoculated with Low Invasion or Uninvaded soil than with High Invasion or uninoculated soil.

Experiment 2: Effects of rhizobia and competitors on *Medicago*

The effects of rhizobia on *Medicago* were dependent on the collection range of the genotypes and the competition treatment in which they were grown (Table 3). Rhizobia increased the biomass of native range *Medicago* genotypes, but decreased the biomass of introduced range genotypes (except when introduced genotypes were grown with *Acmispon*) (Fig. 3a). These effects were not driven by differences in nodule number; native and introduced range *Medicago* genotypes produced similar nodule numbers (Table 3; Fig. 3b).

The effects of competition on *Medicago* biomass were dependent on genotype and the presence of rhizobia (Table 3). Without rhizobia, competition with a conspecific reduced the biomass of genotypes from the native range, but

competition had limited effects on genotypes from the introduced range (Fig. 3a). With rhizobia, native range genotypes still experienced a reduction in biomass when grown with conspecifics, but introduced range genotypes experienced an increase in biomass when grown with *Acmispon* (Fig. 3a).

The effects of competition on nodulation depended on *Medicago* genotype (Table 3). Native range *Medicago* genotypes grown with conspecifics produced fewer nodules per plant than those grown alone or with heterospecifics (Fig. 3b). Competition had no effect on nodulation of introduced range genotypes (Fig. 3b).

The effects of rhizobia on shoot:root ratio were dependent on *Medicago* genotype (Table 3). Rhizobia tended to decrease shoot:root ratios in native range genotypes, but rhizobia increased shoot:root ratios in introduced range genotypes (Fig. 3c).

We also found significant variation among *Medicago* genotypes in how rhizobia affected the response to competition (Table 3). For three genotypes, rhizobia and conspecific competitors decreased the effect of each other, but for another genotype, rhizobia and conspecific competition acted synergistically to increase the effect of each other (Fig. 4). Another four genotypes experienced additive effects of rhizobia and conspecific competition. With respect to the competitive effects of *Acmispon*, rhizobia and competitors reduced the effects of each other on the biomass of six genotypes, acted synergistically to increase the effects of each other on one genotype, and acted additively on four genotypes.

The effect of the collection range of *Medicago* genotypes on *Acmispon* biomass was dependent on the presence of rhizobia (Table 4, Fig. 5). Rhizobia had little effect on how *Medicago* from the introduced range affected *Acmispon* biomass (Fig. 5). However, *Acmispon* growing in the presence of *Medicago* from the native range grew larger when

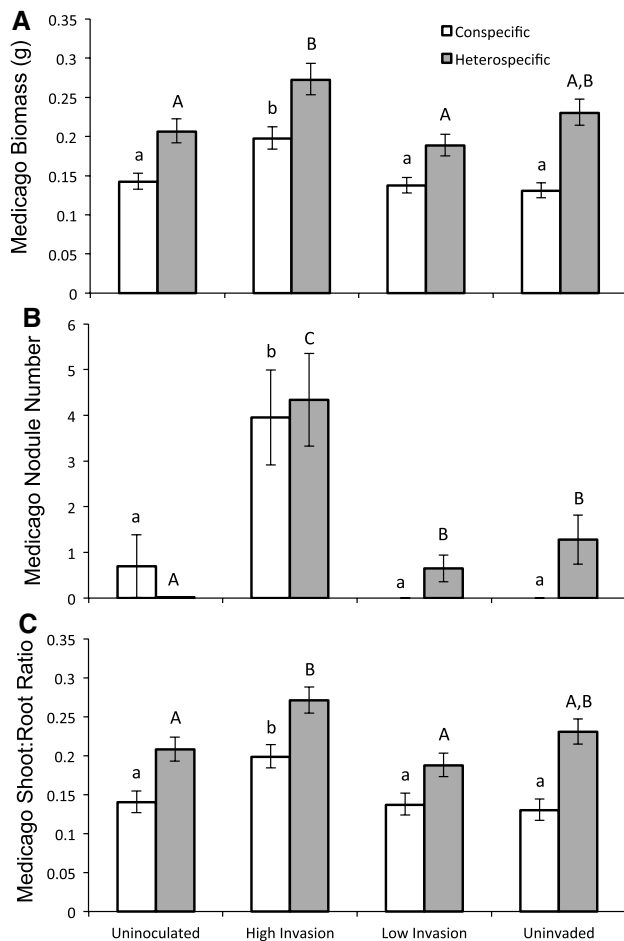


Fig. 1 Least square means (\pm s.e.) of **a** total biomass, **b** nodule number, and **c** shoot:root ratio of *Medicago* grown in the presence of either a conspecific or heterospecific (*Acmispon*) in uninoculated soil or soil inoculated from one of three sites. Letters above bars indicate Tukey-adjusted pairwise differences among soil types, conducted separately for each type of competition

rhizobia were present (Fig. 5). These effects were no longer significant when *Medicago* biomass was excluded from the model, suggesting that the effects of *Medicago* biomass counteract the effects of collection range and rhizobia on *Acmispon* biomass. Few *Acmispon* plants showed signs of nodulation.

Discussion

Our results suggest that rhizobia may be limiting to an invasive legume in some areas of the introduced range, that rhizobia alter competitive interactions between an invasive legume and a commonly co-occurring native competitor, and that there is substantial genetic variation, including differences between native and introduced range genotypes, in the benefits the legume receives from rhizobia and how it

responds to competitors. These findings indicate that evolutionary changes in how *Medicago* interacts with rhizobia mutualists may have occurred at some point during or after introduction, and that some *Medicago* genotypes may be able to overcome biotic resistance imposed by native competitors by associating with rhizobia in the local soil.

Evidence for rhizobia limitation in natural field soils

Inoculation with soils from the site that had the highest *Medicago* abundance and that supported the greatest survival of *Medicago* seedlings in the field (terHorst and Lau 2015) dramatically increased nodulation and increased *Medicago* biomass (Fig. 1). Inoculation with soil from sites with lower abundances of *Medicago* that supported lower survival of seedlings produced very few nodules and smaller *Medicago* (Fig. 1). These soil differences also influenced the biomass of competing *Acmispon*. This may have been due to decreased availability of rhizobia mutualists for *Acmispon* in High Invasion soils relative to other soils, as *Acmispon* formed fewer nodules in High Invasion soil. The relatively small amount of inoculant (2 mL of a soil slurry) suggests that abiotic differences in soils were unlikely to drive effects on *Acmispon* or *Medicago* biomass, but other members of the soil microbiome (bacteria, fungi, viruses, etc.) may have played important roles. However, our second experiment, in which we inoculated pots directly with rhizobia, suggests that rhizobia play an important role in determining *Medicago* biomass, but that the magnitude of this effect depends on the competitive and genetic environment in which the interaction with rhizobia occurs.

Although soil inoculant had a significant effect on *Acmispon* biomass and nodule mass in the first experiment, this effect occurred independently of *Medicago* biomass, suggesting that the positive effects of heavily invaded soils on *Medicago* biomass do not contribute to these patterns. In the second experiment, the effects of *Medicago* biomass may have counteracted some of the effect of rhizobia on *Acmispon* biomass. The rhizobia addition treatment increased the biomass of *Acmispon*, especially when grown with native range genotypes of *Medicago*. Because *Acmispon* and *Medicago* use different rhizobia species, they are unlikely to compete for or increase the local pool of rhizobia for each other. Instead, rhizobia effects on *Acmispon* may be the result of reduced competition for soil nitrogen, either because *Medicago* receives more nitrogen from rhizobia and relies less on soil nitrogen, or because nitrogen leaks from *Medicago* into the soil and is available for use by *Acmispon*. If so, the decreased reliance on soil nitrogen or higher nitrogen leakage would have to be greater for native range genotypes than introduced range genotypes, which may be likely given that native range genotypes benefit more from rhizobia.

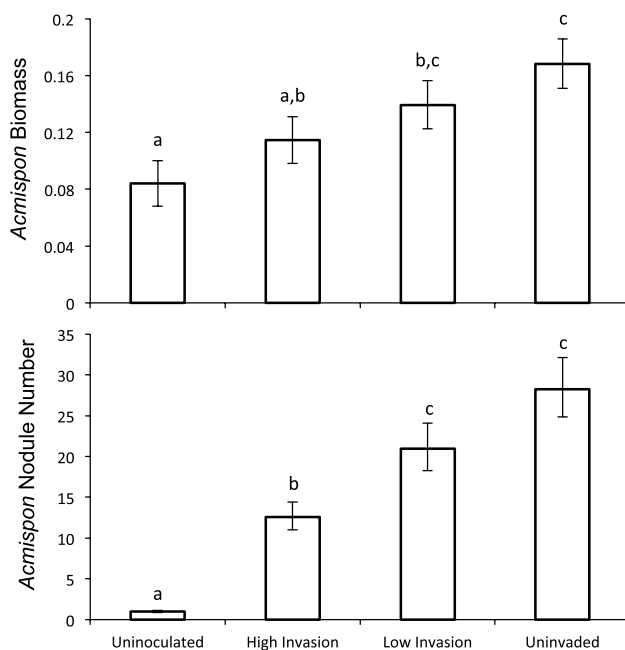
Table 2 Results of effects on *Acmispon* performance from best-fit generalized linear mixed models associated with Experiment 1

Fixed effects	Total biomass			Nodule number		
	<i>df</i>	<i>G</i>	<i>P</i>	<i>df</i>	<i>G</i>	<i>P</i>
Soil source	3	14.5	0.002	3	44.8	< 0.001
Range	1	0.043	0.835	1	2.05	0.152
Soil × range	3	1.35	0.718	3	8.18	0.043
Medicago biomass	1	10.8	< 0.001	1	0.018	0.895
Soil × biomass	–	–	–	3	3.43	0.329
Range × biomass	–	–	–	<i>1</i>	<i>2.91</i>	<i>0.088</i>
Soil × range × biomass	–	–	–	3	8.02	0.046
Genotype (range)	9	36.1	< 0.001	9	10.6	0.507
Geno × soil	–	–	–	–	–	–

Significant effects are shown in bold

Marginally non-significant effects are shown in italics

Dashes indicate factors that were not part of the best-fit model

**Fig. 2** Least square means (\pm s.e.) of *Acmispon* biomass and nodule number of plants grown in uninoculated soil or soil inoculated from one of three sites. Letters above bars indicate Tukey-adjusted pairwise differences among soil types

Effects of rhizobia on competitive interactions

The extent to which genotypes benefited from rhizobia was dependent on whether plants were grown with a conspecific or heterospecific. On average, plants realized more benefit from rhizobia in the presence of heterospecific than conspecific competitors. In competition with conspecifics, the benefits of associating with rhizobia likely provided less competitive advantage because the conspecific also benefited from rhizobia. In contrast, *Acmispon* associates with

different rhizobia species (*Bradyrhizobium* spp.) than *Medicago* (Porter and Rice 2013), so in our rhizobia addition treatment, *Medicago* were more likely to benefit in competitive interactions because they were supplied with compatible rhizobia while their competitor was not.

In the first experiment, plants inoculated with soil from the High Invasion site had significantly higher shoot:root ratios than uninoculated plants or plants inoculated with other soils (Fig. 1c). Plants that readily associated with rhizobia may have invested less in root biomass because they did not need to further explore the soil in search of mutualists or nitrogen, whereas plants that were unable to quickly associate with rhizobia may have had to reduce or delay investment in aboveground biomass. In that case, the benefits of rhizobia for potential invaders may be twofold—rhizobia provide more nutrients, but also allow plants to alter their allocation strategy towards aboveground competition.

Our results of rhizobia altering competitive effects are consistent with previous results showing that an invasive grass had increased competitive effects on a native plant when soil microbes were present (Hodge and Fitter 2013; Emam et al. 2014). However, another study found that in competitive environments, invaders benefit from lower investment in mutualisms with mycorrhizal fungi, allowing them to divert limited resources to competitive interactions (Waller et al. 2016). Our second experiment demonstrated that rhizobia could reduce or enhance the competitive response of *Medicago* to *Acmispon* (Fig. 4), depending on the particular genotype of *Medicago*. Rhizobia may ameliorate biotic resistance against some genotypes and aid in legume invasion success by allowing plants to grow larger and overcome biotic resistance imposed by antagonistic interactions such as competition or herbivory, possibly allowing further successful invasion by other genotypes or species (a.k.a. invasional meltdown; Simberloff and von Holle 1999; Prior et al. 2015). Alternatively, for one of the genotypes

Table 3 Results of best-fit generalized linear mixed models associated with Experiment 2

Fixed effects	Total biomass			Nodule number			Shoot:root ratio		
	<i>df</i>	<i>G</i>	<i>P</i>	<i>df</i>	<i>G</i>	<i>P</i>	<i>df</i>	<i>G</i>	<i>P</i>
Rhizobia	1	18.5	< 0.001	–	–	–	1	4.48	0.034
Competitor	2	17.3	< 0.001	2	120	< 0.001	2	4.78	0.092
Range	1	1.31	0.252	1	4.62	0.032	1	0.657	0.418
Rhiz × comp	2	171	< 0.001	–	–	–	–	–	–
Range × comp	2	114	< 0.001	2	1920	< 0.001	–	–	–
Rhiz × range	1	26.1	< 0.001	–	–	–	1	5.61	0.018
Rhiz × comp × range	2	73.5	< 0.001	–	–	–	–	–	–
Genotype (range)	9	0.270	0.062	9	14.3	0.004	9	34.4	0.012
Geno × rhiz	–	–	–	–	–	–	–	–	–
Geno × comp	–	–	–	–	–	–	–	–	–
Geno × rhiz × comp	9	8.92	0.003	–	–	–	–	–	–

Significant effects are shown in bold

Marginally non-significant effects are shown in italics

Dashes indicate factors that were not part of the best-fit model

included in this experiment, rhizobia increased the negative fitness consequences of competition on the potential invader, and therefore, increased biotic resistance in the community.

Differences between the two experiments

We found variable effects of soil inoculant or rhizobia on *Medicago* shoot:root ratios between experiments. Soil inoculation from the High Invasion site increased shoot:root ratios, however, inoculation with rhizobia produced a more complicated result. Although the magnitude of the effect of rhizobia was dependent on competition treatment, rhizobia on average increased shoot:root ratios in *Medicago* genotypes from the introduced range, but had the opposite effect on genotypes from the native range (Fig. 3c). Genotypes from the native range apparently invested more heavily in belowground biomass when rhizobia were present. The difference between experiments may be due to different genotypes used in the two experiments, as there was little overlap in genotypes between experiments (Appendix 1). More likely, the differences between these two experiments could be due to environmental or treatment differences (inoculation with soil versus rhizobia). Whole soil inoculations include diverse communities of bacteria and fungi, and both mutualists and pathogens. Differences in how introduced and native range genotypes interact with these other taxa could yield the divergent response in shoot:root ratios observed for introduced genotypes.

Resource availability also differed across experiments; the first experiment was conducted in low nitrogen soil, where plants were more likely to be dependent on rhizobia as a source of nitrogen. Nitrogen levels, at least initially, were higher in the second experiment. The increased availability of nitrogen may make plants less reliant on rhizobia and shift

this association from mutualism towards parasitism (Johnson et al. 1997; Sachs and Simms 2006), although if this is the case, this phenomenon only occurred for plant genotypes from the introduced range. Further, rhizobia population densities likely differed between experiments. Although we did not quantify cell density, nodule numbers were almost an order of magnitude lower in experiment 1 than 2, likely because soil slurries had several orders of magnitude fewer bacteria than the dense cultures that were used to inoculate pots in the second experiment. Indeed, the three genotypes used in both experiments produced 4, 21, and 28 times more nodules in Experiment 2 than in Experiment 1. The ability of plants to choose or sanction certain bacteria may be affected by the abundance or diversity of available rhizobia (Burghardt et al. 2018), and these associations with different rhizobia strains may yield different shoot:root responses (Ogutcu et al. 2010).

Plant genetic variation in response to rhizobia and competitors

The extent to which rhizobia increased *Medicago* biomass was dependent on plant genotype. Although we did not observe any interaction between soil inoculant and plant genotype in the first experiment (Table 1), we found strong rhizobia × range and rhizobia × competition × genotype interactions on plant biomass in the second experiment (Table 3). Genotypes from the native range showed large increases in size when rhizobia were present, whereas genotypes from the introduced range showed considerably less benefit, or even decreases in biomass, from rhizobia. These results suggest that native genotypes benefit more from rhizobia, while genotypes from the introduced range have

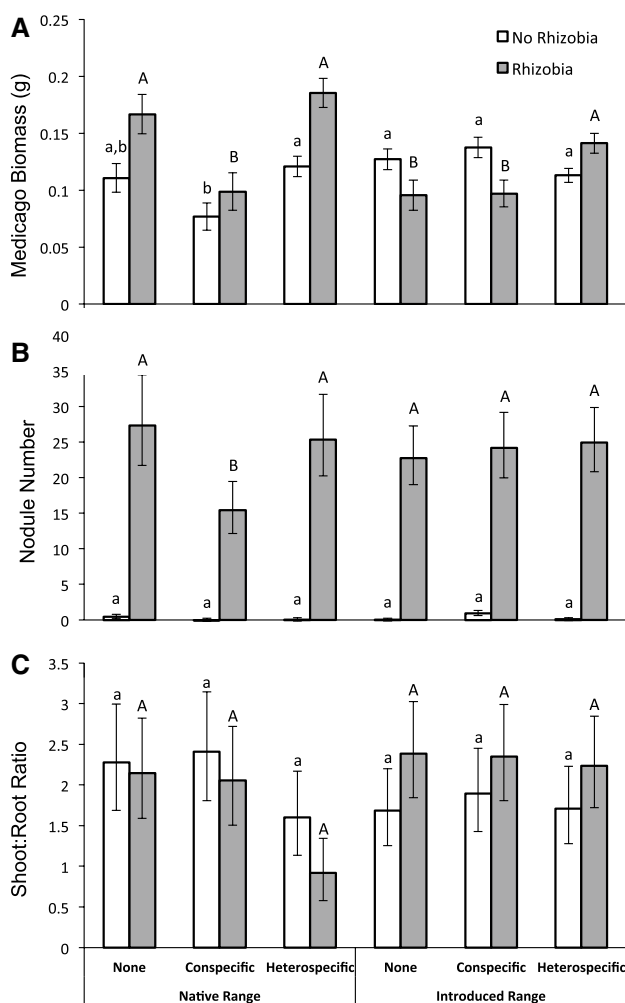


Fig. 3 Least square means (\pm s.e.) of **a** total biomass, **b** nodule number, and **c** shoot:root ratio of *Medicago* grown either alone, in the presence a conspecific, or in the presence of a heterospecific (*Acmispon*) in soils with and without rhizobia. Letters above bars indicate Tukey-adjusted pairwise differences among competition*range treatment combinations, conducted separately for each rhizobia treatment

evolved to benefit less from rhizobia. Similarly, other studies have shown genetic variation in dependence on mycorrhizae (Tawaraya 2003), another type of resource mutualism, and reduced dependence on mycorrhizae of invasive populations compared to native populations (Seifert et al. 2009).

These results suggest that evolutionary changes in how *Medicago* interacts with rhizobia mutualists may have occurred at some point during or after introduction. These evolutionary changes not only resulted in reduced benefits, but also high costs from associating with rhizobia, at least when heterospecific competitors are absent, as evidenced by the reduction in biomass of introduced genotypes when rhizobia were present. Introduced range genotypes may have shifted allocation to traits related to nitrogen acquisition or resource use efficiency, so that plants rely less on rhizobia to

provide fixed nitrogen. Alternatively, native genotypes may be better at sanctioning non-beneficial rhizobia, relative to introduced range genotypes. Recent evidence suggests that plants can sanction ineffective rhizobia, even within nodules (Regus et al. 2017), providing a mechanism to precisely control which rhizobia strains with which the legume associates; standing genetic variation within *Medicago lupulina* for reducing associations with exploitative rhizobia strains suggests there is potential for such sanction-related traits to evolve within populations (Simonsen and Stinchcombe 2014).

Trait evolution may also allow introduced populations to overcome biotic resistance to invasion imposed by antagonistic interactions in the native community. In a previous study in this system, the strength of biotic resistance imposed by herbivores was strongest on native range genotypes, suggesting that introduced populations had evolved to overcome the negative effects of herbivory (terHorst and Lau 2015). In this study, rhizobia influenced the outcome of antagonistic competitive interactions, and these effects were greater on introduced range genotypes than on native range genotypes, although the direction of those strong indirect effects varied among specific genotypes (Fig. 4). Together, these results suggest that some genotypes may be able to overcome biotic resistance imposed by herbivores and competitors. Other genotypes may only be able to overcome biotic resistance imposed by competitors when compatible rhizobia mutualists are present.

These observed differences between native and introduced range populations may represent divergent evolutionary histories. For example, populations in the native range that are associated with human disturbance may be more likely to invade than populations in less disturbed habitats because such populations are already adapted to the human disturbed habitats they are likely to face in the introduced range (Hufbauer et al. 2012). Human disturbance (both physical and nutrient enrichment) is likely to select for plants that receive less benefit from rhizobia as well as a suite of other traits, including increased growth rates in low competition, high nutrient environments (Sachs and Simms 2006, Kiers et al. 2010). This hypothesis is consistent with the observation that introduced range genotypes are less dependent on rhizobia and also with the observation that introduced and native range *Medicago* genotypes perform equally well in the absence of rhizobia and heterospecific competitors. Thus, the differences between native and introduced range genotypes may reflect historical differences in the habitat types from which they were collected, whether they have experienced those environments for long evolutionary time scales (native range) or shorter, but still substantial, time scales (introduced range). Native range genotypes may be adapted to less disturbed, nutrient poor conditions, while introduced range genotypes may be adapted to highly

Fig. 4 The magnitude and direction of indirect effects of rhizobia and con- or heterospecific competition on *Medicago* biomass for each genotype. Positive numbers indicate that rhizobia decreased the effect of competitors, or competitors decreased the effect of rhizobia, on *Medicago* biomass; negative numbers indicate the rhizobia increased the effect of competitors, or competitors increased the effect of rhizobia. Effects for the Belgium genotype could not be calculated because no individuals of that genotype survived in the conspecific treatment

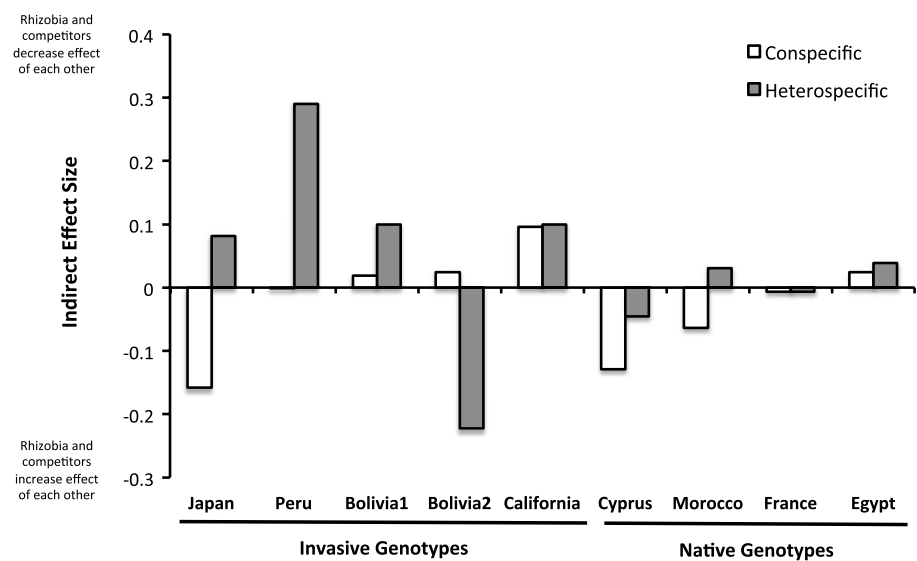


Table 4 Results of effects on *Acmispon* performance from best-fit generalized linear mixed models associated with Experiment 2

Fixed effects	Total biomass		
	<i>df</i>	<i>G</i>	<i>P</i>
Rhizobia	1	5.34	0.021
Range	1	0.538	0.463
Rhizobia × range	1	4.00	0.046
<i>Medicago</i> biomass	1	2.62	0.106
Rhizobia × biomass	1	14.6	< 0.001
Range × biomass	–	–	–
Rhizobia × range × biomass	1	11.32	< 0.001
Genotype (range)	9	0.121	0.839
Genotype × rhizobia	–	–	–

Significant effects are shown in bold

Dashes indicate factors that were not part of the best-fit model

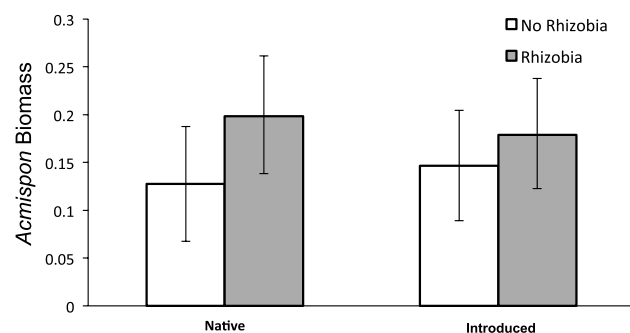


Fig. 5 Least square means (\pm s.e.) of *Acmispon* biomass grown with *Medicago* (after accounting for effect of *Medicago* biomass) collected from its native and introduced range and with and without rhizobia

disturbed, nutrient rich sites regardless of whether this adaptation occurred pre- or post-introduction.

Conclusions

Our results suggest that mutualists, and genetic variation in how much benefit is gained from mutualists, can influence plant performance and competitive outcomes and may play a role in determining invasion success. Rhizobia alter competitive outcomes between introduced and native legumes, and genotypes differ in their response to both rhizobia and competition and the extent to which rhizobia influence competitive interactions. Because introduced range genotypes benefitted less from the presence of rhizobia and were less negatively affected by competition with conspecifics, evolutionary shifts in traits mediating both mutualistic and antagonistic species interactions may contribute to invasion success in this system.

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Author contribution statement CPT and JAL conceived and designed the experiments, analyzed the data, and wrote the manuscript. CT performed the first experiment and CT and CW performed the second experiment.

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