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LETTER

Multiple Mutualism Effects generate synergistic selection and strengthen fitness alignment in the interaction between legumes, rhizobia and mycorrhizal fungi

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Abstract

Nearly all organisms participate in multiple mutualisms, and complementarity within these complex interactions can result in synergistic fitness effects. However, it remains largely untested how multiple mutualisms impact eco-evolutionary dynamics in interacting species. We tested how multiple microbial mutualists—N-fixing bacteria and mycorrrhizal fungi—affected selection and heritability of traits in their shared host plant (*Medicago truncatula*), as well as fitness alignment between partners. Our results demonstrate for the first time that multiple mutualisms synergistically affect the selection and heritability of host traits and enhance fitness alignment between mutualists. Specifically, we found interaction with multiple microbial symbionts doubled the strength of natural selection on a plant architectural trait, resulted in 2- to 3-fold higher heritability of plant reproductive success, and more than doubled fitness alignment between N-fixing bacteria and plants. These findings show synergism generated by multiple mutualisms extends to key components of microevolutionary change, emphasising the importance of multiple mutualism effects on evolutionary trajectories.

KEYWORDS

fitness, fitness alignment, genotypic selection analysis, Medicago, multiple mutualism effects, multispecies interactions, mycorrhizal fungi, nitrogen-fixing bacteria, non-additive, symbiosis

INTRODUCTION

Mutualisms—associations in which all participants benefit—dramatically affect the interacting species as well as the dynamics of their populations, communities and ecosystems (Bronstein, 2015; Chomicki et al., 2019; David et al., 2019). Most organisms participate in many mutualisms throughout their lifetime or even simultaneously (Afkhami et al., 2014), including interactions of diverse mutualists within guilds and across them. For instance, plants may associate with multiple pollinators, seed dispersers, ant defenders, foliar endophytes and rhizosphere nutritional symbionts (e.g. Keller et al., 2018). Similarly, animals interact with a diversity of within and across guild mutualists (e.g. coral that depends on multiple 'guard' crustaceans or ants that cultivate fungal gardens for food and depend on bacterial symbionts to kill pathogens attacking these gardens; Currie, 2001; McKeon et al., 2012). Despite the ubiquity of these complex associations, our understanding of how interacting with multiple mutualisms affects the ecological and evolutionary dynamics of the interacting species remains limited. Here we examine how multiple symbiotic microbes—N-fixing bacteria and mycorrrhizal fungi—affect trait selection and heritability in their shared host plant (*Medicago truncatula*), as well as fitness alignment between partners.

Historically the study of mutualism has focused on 'pairwise interactions' (i.e. between pairs of species), although there has been a growing body of empirical and theoretical research emphasising that interactions with multiple mutualisms are common and exploring the ecological consequences of multiple mutualist species within and across mutualistic guilds (Afkhami et al., 2014; Keller et al., 2018; McKeon et al., 2012; Ossler et al., 2015; Stanton, 2003). The consequences of interacting with multiple mutualisms—Multiple Mutualism Effects (MMEs)—can be non-additive for performance or fitness of shared hosts (Afkhami et al., 2014) as well as for the molecular phenotypes underpinning these effects (Afkhami & Stinchcombe, 2016; Palakurty et al., 2018). In particular, multiple mutualisms can synergistically increase the fitness of shared hosts if each partner species provides distinct and complementary fitness benefits (e.g. pollination services and seed dispersal when both are required for reproductive success). For instance previous work has shown that two beneficial snails synergistically affect their shared seaweed host by protecting against different types of herbivores (Stachowicz and Whitlatch 2005), having both dominant honeybees and non-Apis bees can generate higher pollination rates than expected under additivity (Brittain et al., 2013), associating with multiple ant species increases lifetime fitness of acacia by providing protection against herbivores at different stages of tree development (Palmer et al 2010), and the Mucoromycotina and Glomeromycotina fungal symbionts provide complementary rewards to liverworts (primarily nitrogen and phosphorus respectively; Field et al., 2019).

One important way that multiple mutualisms could have significant consequences for the evolutionary trajectories of participating organisms is through non-additive selection (Bolstad, 2017; terHorst et al., 2017; TerHorst et al., 2015). For evolution to be driven solely by pairwise effects, traits involved in one interaction must evolve independently of other mutualistic partners, meaning that selection on those traits would be unaffected by multiple mutualisms (Iwao & Rausher, 1997). However, if there are emergent properties of multiple mutualisms, the selective effects of mutualists will be non-additive. In this case, selection exerted by the mutualist community on the focal species should not be predictable based simply on selection imposed by an individual mutualist partner species (Strauss & Irwin, 2004). Imagine, for example, that two separate mutualist species each provide a benefit (e.g. nutrients, defence, pollination) that improves plant fitness compared to not having them, but fitness is highest when interacting with both mutualists. In this instance, the traits or trait combinations leading to highest fitness when interacting with either mutualist alone, both, or neither are likely to differ. Surprisingly few studies have explicitly measured selection generated by multiple mutualisms (e.g. Sahli & Conner, 2011), especially among mutualists conferring different types of rewards or fitness benefits. Thus, it is difficult to assess the frequency and strength of non-additive selection resulting from these common but complex interactions.

Multiple mutualisms can also influence evolution in mutualisms by affecting fitness alignment, the correlation in fitness functions, between interacting mutualists (Jones et al., 2015). The interaction between species is designated a mutualism based on a comparison to when one partner is absent (Figure 1a). Given that an interaction is

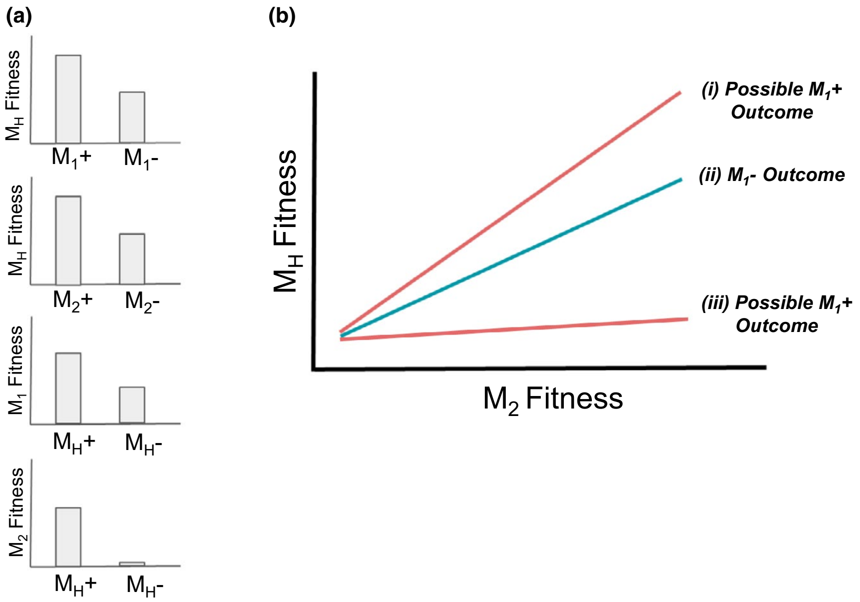

FIGURE 1 Schematic showing multiple mutualisms and fitness alignment. In (a) the host mutualist (M_H) has higher fitness with each partner mutualist (M_1 and M_2) than without, and M_1 and M_2 have higher fitness with their host than without (i.e. the relationships are mutualisms). In (b) we show two hypothetical cases where the presence of one mutualism, between M_1 and the host, alters the relationship between the fitness of another mutualist M_2 and the fitness of the host. (ii) shows the relationship in the absence of multiple mutualisms (i.e. when M_1 is absent; M_1−), (i) shows case where the presence of another mutualism (M_1+) strengthens fitness alignment between the host and M_2 via complementarity of mutualist fitness benefits for the host and (iii) shows case where conflict between partner mutualists (M_1 and M_2) over resource access leads to a weaker return for the host of increasing M_2 fitness

a mutualism, an important issue is the fitness alignment between partners. Does higher fitness for one partner result in higher fitness for the other in a straight-forward way (Figure 1b(ii))? It is also unknown how additional mutualisms (or species within the same mutualism) alter the fitness alignment between a pair of interacting mutualists (Figure 1b(i, iii)). For example, is the relationship between plant and N-fixing bacterial fitness altered by the presence or absence of mycorrhizal fungi, or the relationship between plants and pollinators affected by the presence or absence of ant bodyguards? The fitness alignment between mutualist partners will also affect positive feedbacks within the component interactions. In the hypothetical scenario shown in Figure 1a and 1b(ii), fitness in a pair of mutualist species is positively correlated in the absence of a third species, and this correlation is strengthened by the presence of an additional mutualist (Figure 1b(i)). All else being equal, we would expect genotypes of the first two species engaging in three-way interaction to have an advantage over those that do not, and the genotype combinations having the highest fitness under these conditions to spread. In contrast, in another hypothetical scenario, the addition of a third mutualist species weakens the correlation in fitness between the focal pair (Figure 1b(iii)); in this scenario, there is substantial variation in the fitness of the mutualist on X-axis, but little variation in fitness for the Y-axis. The potential for additional mutualists to modify the fitness alignment between a pair of mutualist partners is an essentially unaddressed empirical question.

To address the role of MMEs in the eco-evolutionary dynamics of mutualisms, we grew replicate plants from 213 genotypes of *Medicago truncatula* in a 2 × 2 factorial experiment manipulating the presence of rhizobia and mycorrhizal fungi. Specifically, we asked two related questions: (1) Do multiple mutualism effects enhance or diminish fitness alignment? and (2) Do multiple mutualisms alter heritability of and impose non-additive selection on host traits?

METHODS

Study system

We used the annual legume *Medicago truncatula* (Barrel Medic), rhizobia (*Ensifer meliloti* Rm1021) and mycorrhizal fungi (*Rhizophagus irregularis* DAOM197198) to investigate multiple mutualism effects on selection and fitness alignment. This interaction represents a simple 'multiple mutualism' composed of a host interacting with two mutualist species, but also a common one as legumes (the third-largest plant family) often participate in and benefit from both component mutualisms (Wang & Qui, 2006; Sprent, 2001; Larimer et al., 2014; Afkhami *et al.* 2018). We chose *M. truncatula* because: (1) it interacts with two common plant mutualists (rhizobia and mycorrhizal fungi) that each primarily provides a distinct resource to host plants (fixed atmospheric nitrogen, and phosphorus and water from soil respectively) and are tractable to manipulate (Afkhami & Stinchcombe, 2016; Kafle et al., 2019), (2) plant genotypes vary in their response to multiple mutualisms (Franklin et al., 2020) and (3) it has >200 distinct plant genotypes available from across its range (http://www.medicagohapmap.org). These genotypes allow for the inclusion of a wide range of host genetic diversity and thus more rigorous genotypic selection and fitness alignment analyses.

Experiment

Experimental design

We grew 213 *M. truncatula* genotypes (Table S1) in four microbial environments: no microbes (M−R−), rhizobia alone (M−R+), mycorrhizal fungi alone (M+R−) and both microbes (R+M+) using a completely randomised block design with five spatial blocks. For each genotype, we mechanically scarified and surface-sterilised ~25 seeds in a bleach solution, rinsed with sterile water and germinated them on sterile 0.8% water agar plates for ~36 h at 4°C in the dark followed by ~18 h at 22°C (Afkhami & Stinchcombe, 2016; Garcia et al., 2006). We planted germinants into 164 ml cone-tainers (Stuewe and Sons, Oregon) filled with sand. Before planting, we sterilised all pots and sand at 121°C three times (45-min cycle) and then inoculated pots at 1 and 2 weeks post-planting to encourage colonisation and nodulation. Bacterial inoculum was grown for 36 h in TY media and diluted to ~10^6 cells/ml (OD600 = 0.1) with ddH2O (Simonsen & Stinchcombe, 2014). Germinants in R+ pots received 1 ml of inoculant and those in R− pots received the same 'inoculant' without rhizobia. Mycorrhizal inoculum for M+ germinants was ~300 spores in sterile water (Premier Tech, Riviere-du-Loup, Quebec, Canada; Antunes et al., 2008; Powell et al., 2009), whereas pots with M-germinants received inviable inoculum (achieved by autoclaving spores four times for 45 min at 121°C). We grew plants in the greenhouse for ~8 months with supplemental lighting to reflect daylength in *M. truncatula's* native range and fertiliser supplied at 3-month intervals (1:1:1 ppm N:P:K). In total, we planted ~4260 seeds into pots (=4 treatments × 213 genotypes × 5 replicates).

Data collection and harvest

We collected and counted mature pods (fruits) as they were produced. In *Medicago truncatula*, pods are commonly used to estimate fitness (e.g. Friesen et al., 2014) and are a good estimate because the species is largely selfing with cleistogamous flowers (such that pods account for male and female fitness), although *sensu strictu* they are a fitness component rather than true fitness. At harvest, we counted branches (Moreau, 2006) and collected above-ground biomass, which we dried at 60°C, and then weighed. Data on belowground-biomass and rhizobia nodule number were collected for a subset of plants in each genotype-microbial environment combination (n = 323.5 ± 24.3 and n = 314.5 ± 10.5 per treatment respectively; recall that we cannot count nodules in R- treatments). We calculated the ratio of root to shoot mass for each plant (to examine allocation) and genotype means for branches, root:shoot mass, pod number and nodule number in each microbial environment separately. These traits collectively describe size, above and belowground allocation, architecture/shape and reproductive success. We assigned a fitness of zero to individuals who did not produce mature pods.

Analyses

Analyses were conducted in R (version 3.6.1) using the car and boot packages for AN(C)OVA and bootstrapping (Fox & Weisberg, 2018; Canty & Ripley 2019).

Main effects and genetic variation

To examine whether plant genetic variation and microbial mutualisms affected plant fitness, we first used an ANOVA with pod number as a response variable and explanatory variables of mycorrhizal fungi (presence/absence), rhizobia (presence/absence), plant genotype and all interactions among these variables. We square-root+1 transformed pod number prior to analysis to improve normality. We conducted the same analysis for a measure of plant allocation and plant architecture: root:shoot ratio (square root+1 transformed) and branch number respectively. These two traits were selected based on preliminary analysis that found other plant size traits were strongly correlated with branch number, whereas root:shoot was uncorrelated with these traits (Table S2). For plants inoculated with rhizobia (M+R+ and M−R+ treatments), we also examined whether plant genetic variation and the presence of mycorrhizal fungi impacted nodule number (proxy for rhizobia fitness) using an ANOVA with explanatory variables of mycorrhizal fungi presence/absence, plant genotype and their interaction. In all models, we included a block term to account for spatial variation and used type III sums of squares. We considered block and genotype to be fixed effects because they did not represent random samples of spatial or genetic variation, respectively, about which we wished to generalise (Newman et al., 1997).

As a complement to our tests of the main effects of treatments and genotypes, we also estimated and compared heritabilities of plant traits in each microbial environment. Briefly, and only for estimating heritability, we fit mixed-model ANOVAs for traits with block as a fixed effect and line as a random effect for each microbial environment separately, using restricted maximum likelihood. We estimated broad sense heritability, H^2, as $V_{line}/(V_{line} + V_{residual})$. To characterise uncertainty in heritabilities, we adapted a procedure described by Houle and Meyer (2015) for G matrices. We considered REML estimates of V_{line} and $V_{residual}$ as means of a bivariate normal distribution, with a covariance matrix equal to the asymptotic variance matrix of the REML estimates. We drew 10,000 values from these bivariate normal distributions, and estimated confidence limits from the 2.5th and 97.5th percentiles of these values. We estimated heritability and its corresponding uncertainty in SAS v9.4, using Proc Mixed and IML.

Do multiple mutualism effects enhance or diminish fitness alignment?

To assess whether mycorrhizal fungi affected fitness alignment between rhizobia and host plants, we used an ANCOVA with a response variable of mean pod number (square root +1 transformed) and a fixed effect of mycorrhizal fungi (presence/absence), a continuous predictor variable of nodule number, and their interaction. A significant interaction between fungi presence and rhizobia fitness indicates multiple mutualism effects on fitness alignment of the rhizobia–plant mutualism. We followed up on a significant interaction with separate regressions for M+R+ and M-R+ plants to assess fitness relationships in each microbial environment.

Do multiple mutualisms impose non-additive selection on host traits?

To determine how multiple mutualisms select on their shared host, we conducted genotypic selection analysis (Rausher, 1992). We used ANCOVA to determine how each microbe individually and jointly altered the relationship between plant traits and fitness (i.e. altered the strength and/or direction of selection gradients). We modelled relative plant fitness using fixed factors of rhizobia (R+/R−) and mycorrhizal fungi (M+/M−), two traits (branching and root-to-shoot mass ratio), the interaction between microbes and the interactions between microbes and traits. We calculated plant relative fitness for this analysis by dividing the mean fitness for each genotype by the overall mean fitness across all environments. While doing so implicitly invokes a hard selection model (De Lisle and Svennson 2017), we made this choice so that differences in mean fitness and trait values, which were of *a priori* interest, were preserved in our ANCOVA models (see Batstone et al. 2020). To verify that any significant interaction terms were not driven solely by differences in mean fitness between treatments, we repeated this analysis with plant relative fitness calculated within a treatment, and found similar statistical results for this ANCOVA (Table S7), including the same significance for the three-way interactions. A significant three-way interaction between rhizobia, fungi and a trait in this analysis indicates non-additive selection on that trait. We followed up on significant three-way interactions with multiple linear regression of relative plant fitness (calculated across treatments) on the traits in each microbial environment separately to determine the selection gradient in each environment. Bias corrected 95% CIs around each selection gradient were calculated using 10,000 bootstraps.

RESULTS

Treatment effects and genetic variation

Pod number (an estimate of host plant fitness) varied significantly among genotypes (genotype main effect: $F_{212,3397} = 4.55$, $p << 0.00001$; Table S3), indicating genetic variation for fitness. Microbial interactions also altered host fitness, but the strength, direction, and non-addivitity of these effects depended to some extent on host genotype (e.g. rhizobia × genotype interaction: $F_{212,3397} = 3.50$, $p << 0.00001$, fungi × genotype: $F_{212,3397} = 1.29$, $p = 0.004$, marginally significant rhizobia × fungi × genotype interaction: $F_{212,3397} = 1.163$, $p = 0.059$; Table S3). Nodule number (an estimate of rhizobial fitness that was strongly associated with nodule mass in our study; $r = 0.884$, $F_{1,48} = 172$, $p << 0.00001$; Fig. S1) varied significantly by plant genotype ($F_{155,251} = 2.84$, $p << 0.00001$; Table S3). Mycorrhizal fungi also affected nodule production with genotypes varying in their response to the presence of the fungal mutualism, indicating genetic variation among hosts mediate mycorrhizal fungi's effect on nodulation (fungi × genotype interaction: $F_{155,251} = 1.33$, $p = 0.024$; Table S3). We found that host genotype ($F_{138,880} = 5.10$, $p << 0.00001$; Table S3) and the interaction between mycorrhizal fungi, rhizobia and host genotype ($F_{138,880} = 1.229$, $p = 0.0482$) significantly affected the branch number trait, and that investment in roots versus shoots (root:shoot ratio) differed among plant genotypes ($F_{112,398} = 1.72$, $p = 0.00008$; Table S3) and depended somewhat on microbial environment (rhizobia × fungi: $F_{1,398} = 3.57$, $p = 0.0595$).

Microbial treatments affected broad sense heritability of plant traits, but not in a consistent way across traits (Table S4). For example broad sense heritability of pod number, was highest in the treatment with both mutualists ($H^2 = 0.35$), and lower in the three remaining treatments (H^2 ranges 0.12–0.16). For branch number, heritability was highest with both mutualists ($H^2 = 0.51$), followed by rhizobia only ($H^2 = 0.42$), followed by the treatments lacking rhizobia ($H^2 = 0.18$ and 0.26 for with and without fungi respectively). In contrast for nodules (which require the presence of rhizobia), heritability was higher in the absence of mycorrhizal fungi ($H^2 = 0.48$) than in their presence ($H^2 = 0.29$), though the uncertainty estimates of these heritabilities overlap.

Do multiple mutualism effects enhance or diminish fitness alignment?

Surprisingly, mycorrhizal fungi had a strong impact on fitness alignment between the plants and the rhizobia (fungi presence × rhizobia fitness: $F_{1,355} = 4.94$, $p = 0.0269$; Figure 2, Table S5a). In absence of mycorrhizal fungi, there was a weak, but significant, positive relationship between rhizobia relative fitness (calculated from nodule number) and plant relative fitness (calculated from fruit number) ($F_{1,173} = 6.9$, $p = 0.0094$; Figure 2, Supplementary Table S5b). In the presence of mycorrhizal fungi, rhizobia and plant fitness were substantially more positively aligned ($F_{1,182} = 21.45$, $p < 0.00001$; Figure 2, Table S5b) with a slope that is 2.5 times greater than when the fungal mutualist was absent.

Do multiple mutualisms impose non-additive selection on host traits?

Rhizobia and mycorrhizal fungi exerted significant non-additive selection on branching of their host plant (fungi × rhizobia × branch number: $F_{1,664} = 5.71$, $p = 0.0171$; Figure 3, Table S6–7). In the absence of all microbes or in the presence of just mycorrhizal fungi, selection on plant branch number was very weak ($\beta_{M-R-} = 0.031$ and $\beta_{M+R-} = 0.034$; Figure 3, Table 1, S8). In the presence of rhizobia (alone), we observed a positive relationship between branch number and relative plant fitness, indicating selection for increased branching of host plants occurs in the presence of rhizobia ($\beta_{M\text{-}R+} = 0.278$, Table 1, S8). Interestingly, the strength of selection on branching doubled in the presence of multiple mutualisms ($\beta_{M+R+} = 0.557$) compared to plants grown with rhizobia alone and was >16x stronger than with mycorrhizal fungi alone. The selection gradient in the presence of both partners (0.557) was nearly double the expected additive selection gradient of 0.281; calculated following TerHorst et al., (2015)($\beta_{\text{additive}} = 0.034+0.278–0.031$). The non-additive selection gradient, which quantifies how much selection is modified by indirect ecological interactions, was 0.276 (calculated as $\beta_{M+R+} - \beta_{\text{additive}}$). Selection gradients reported here are from the multiple linear regression (Table 1), but univariate analyses (where each trait's relationship with plant fitness is analysed separately) showed nearly identical values (Table S8).

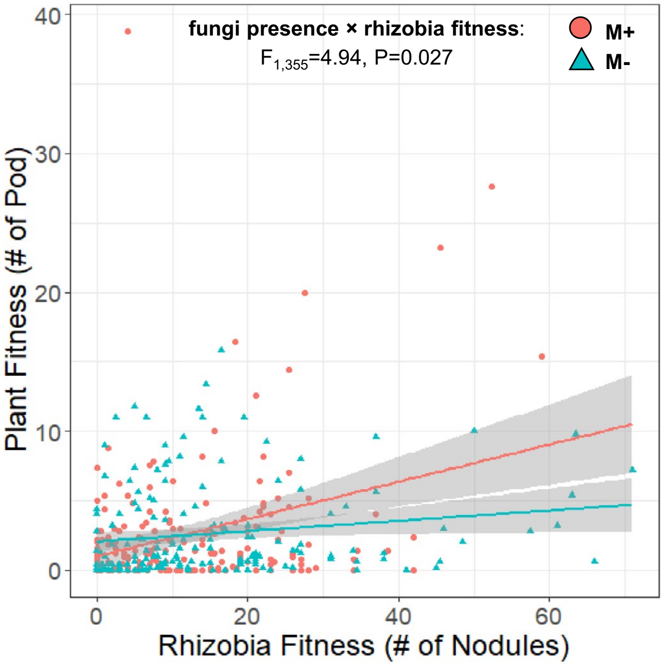

FIGURE 2 Fitness alignment between the rhizobia and the host plant was stronger in the presence of mycorrhizal fungi. Each point represents an individual plant genotype, lines indicate relationship between plant and rhizobia fitness in each mycorrhizal treatment environment, and the shaded grey areas represent the 95% confidence interval around each best fit line. Y-axis value is the mean fitness of a plant genotype as indicated by fruit production (pod number), and x-axis value is the mean fitness of the rhizobia associating with that plant genotype as indicated by nodule number. The red circles and line represent the treatment with mycorrhizal fungi and blue triangles and line represent the treatment without mycorrhizal fungi

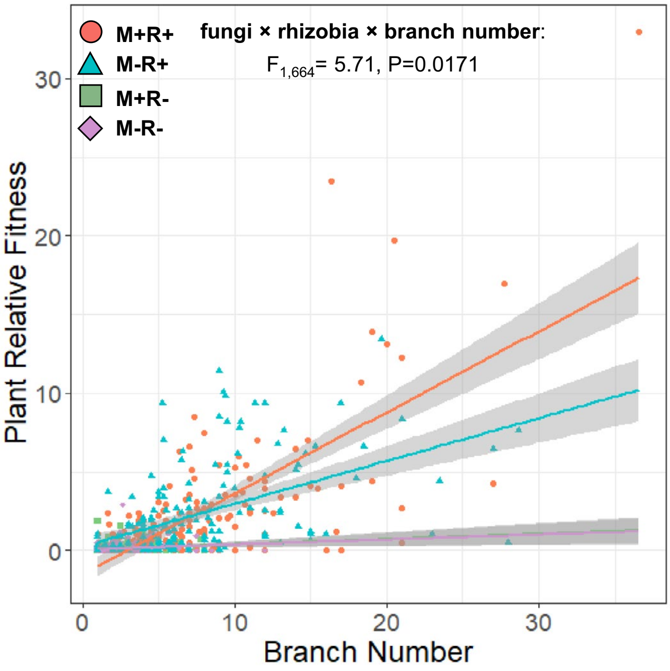

FIGURE 3 Mycorrhizal fungi and rhizobia select non-additively on their host plant's branching. Each point represents an individual plant genotype, lines indicate relationships between mean branch number and plant relative fitness in the four microbial treatment environments, and the shaded grey areas represent the 95% confidence interval around each best fit line. The purple diamonds and line represent the no microbe treatment, green squares/line are for the mycorrhizal fungi only treatment, blue triangles/line are for the rhizobia only treatment, and red circles/line are for the multiple mutualisms (both microbe) treatment. Note that the green line is not visible because the slopes of M−R− and M+R− lines (purple and green) are nearly identical

TABLE 1 Results of analyses to determine selection gradients for microbial effects on plant traits

Treatment	Trait	*β*	SE	*p*-value	95% CIs	Trait mean	Trait SD	Mean fitness
M+R+	**Branching**	**0.557**	**0.041**	**<2E−16**	**(0.38, 0.76)**	**7.25**	**5.58**	2.27
	Root:Shoot	−0.496	0.496	0.3190	(−1.11, −0.04)	0.46	0.47	
M−R+	**Branching**	**0.278**	**0.037**	**7.40E−12**	**(0.17, 0.38)**	**7.06**	**5.38**	2.16
	Root:Shoot	−0.438	0.381	0.2530	(−1.08, −0.001)	0.52	0.52	
M+R−	**Branching**	**0.034**	**0.013**	**0.0102**	**(0.01, 0.06)**	**2.78**	**1.69**	0.14
	Root:Shoot	0.023	0.031	0.4695	(−0.03, 0.08)	0.84	0.70	
M− R−	**Branching**	**0.031**	**0.013**	**0.0167**	**(0.01, 0.06)**	**2.62**	**1.93**	0.13
	Root:Shoot	0.004	0.038	0.9167	(−0.04, 0.04)	0.71	0.66	

Note: Selection gradient (β) were calculated in each of the four microbial environments. M and R indicate mycorrhizal fungi and rhizobia, and + and − indicate presence or absence of that microbe. Significance of the selection gradient (denoted under '*p*-value') was determined using multiple linear regression analyses within each of the microbial treatments, and bias corrected 95% CIs around each selection gradient were calculated using 10,000 bootstraps. Reported trait means and standard deviations for each treatment were calculated across the genotype means within that treatment. Significant selection on a trait is indicated by bold text.

DISCUSSION

A large gap remains in our understanding of how multiple mutualisms impact evolutionary trajectories of species. Our study showed for the first time that (1) multiple mutualisms not only can have non-additive effects on host performance, but also on selection and heritability of host traits and (2) multiple mutualisms can enhance fitness alignment between mutualist species. Below, we first discuss the implications of non-additivity of fitness, heritability and selection in multiple mutualisms. We then discuss synergism and conflict in the direction of selection in these interactions and fitness alignment between mutualists.

Non-additivity of performance, fitness, heritability and selection

In past studies, we detected non-additive effects on plant performance (biomass), on gene expression profiles, and on coexpression network structure in *Medicago truncatula* (Afkhami & Stinchcombe, 2016; Hernandez et al., 2020; Palakurty et al., 2018). Here, we detected non-additive effects of multiple mutualisms on plant fitness and an architectural trait, branch number. Non-additive effects thus have the potential to affect plant population dynamics, through fecundity and the interaction between plants (as branchier plants may shade each other more). Collectively, the pervasive multiple mutualism effects that we have detected suggest that understanding plant traits—including gene expression, size, shape, fecundity—requires embracing the effects of multiple mutualisms, rather than pairs of species. Whether MMEs have evolutionary consequences, however, had been relatively unexplored.

Our data convincingly show that MME and non-additivity extend to key components of microevolutionary change, genetic variation and natural selection. Pod number, a fitness component in selfing annual plants, showed the highest heritability in the presence of multiple mutualisms, with a value two to three-fold higher than with single mutualisms ($H^2 = 0.35$, vs. 0.12 and 0.16 for mycorrhizal and rhizobia-only treatments). We also observed substantial differences in genetic variance components and coefficients of genetic variation (Table S4), indicating that increased H^2 in the presence of multiple mutualisms was neither driven by reduced environmental variance nor solely by increases in mean pod number. Mechanistically, increased heritability could be due to more variability in expression of the same genes because of microbial treatments, or the expression of novel genes; for either to increase heritability, these effects must differ between genotypes/lines. Almost certainly both mechanisms contribute, but determining their relative magnitude would be daunting. On a practical level, higher heritabilities suggest artificial selection and planned breeding programs for legumes (of which there are many important food crops; Graham & Vance, 2003) would expose more genetic variation and lead to larger responses to selection in the presence of multiple mutualisms. On a conceptual level, these data also suggest substantially more genetic variation for fitness components in the presence of multiple mutualisms. While acknowledging that pod number may not represent *true fitness* and that our study was carried out in the greenhouse, these results nonetheless suggest that MMEs could affect the rate of adaptation, as determined by genetic variation for fitness (Fisher, 1930). Empirically testing this in the field would be challenging, though recent work (Kulbaba et al., 2019; Sheth et al., 2018) suggests some promise.

Genetic variance in and selection on branch number was affected by MMEs, which could impact evolution of this trait. Heritability for branch number was significantly elevated when both microbial mutualists were present (as were genetic variances and coefficients of genetic variation), and branch number was subject to significant non-additive selection in the presence of both mutualisms. Because of increased heritability, genetic variance and stronger selection, any predicted evolutionary response of this trait is much greater in conditions with both mutualisms than with one or no mutualisms. The evolutionary response of a single trait can be predicted from the univariate version of Lande's equation, $R = V_g\beta$, where R is the response to selection, V_g is the genetic variance and β represents the selection gradient for a trait. Solving this equation for branch number suggests negligible changes in branch number in the absence of rhizobia (0.03–0.04 branches), moderate changes in the M−R+ treatment (~4.4 branches) and nearly a three-fold larger evolutionary response in the M+R+ treatment (~12.7 branches). While these are almost certainly over-estimates (mean branch number in these treatments ranged from 2.6 ± 0.11 to 7.3 ± 0.33; $\bar{x}$ ± 1 SE), they nonetheless illustrate that MMEs have substantial quantitative effects on predicted microevolutionary responses. Much effort has focused on the ecological conditions that can produce non-additive selection (Haloin & Strauss, 2008; TerHorst et al., 2015), and how ecological interactions in complex communities can alter the strength of selection in ways that cannot be predicted from pairwise experiments. Our results suggest that this holds for the expression of genetic variation, and that combined with non-additive selection, could dramatically affect microevolutionary trajectories.

One potential caveat to our investigation of how MMEs affect heritability and genetic variances is that we performed univariate analyses, which preclude estimates of genetic covariances between traits. (We note that our estimates of selection are from multiple regression, and thus account for indirect selection on other included traits). A multivariate genetic approach would provide information about genetic covariances between traits (as in Ossler & Heath, 2018), for example between branch number and root:shoot ratio, and whether multiple mutualisms affected **G** matrices. Given evidence of MMEs affecting plant performance, phenotypes, molecular traits, genetic variances and selection, it seems likely that they can also affect **G**, although this remains untested.

Selection in multiple mutualisms

Much of the work on selection in multispecies interactions has focused on conflicting selection, especially between antagonistic and mutualistic interactions (Gómez, 2003; Kessler & Halitschke, 2009; Pérez-Barrales et al., 2013) and among multiple antagonistic interactions (e.g. Miller et al., 2014). However, concordant selection (agreement in the direction of selection) has also been observed. For instance, Sletvold et al., (2015) found that the same pollinator and herbivore that imposed conflicting selection on flowering time, imposed concordant selection on nectar spurs; the genetic covariances between these traits will determine how the conflicting and concordant selection in this system affects evolutionary responses. The few previous multiple mutualism selection studies have also typically been motivated by identifying conflicting selection generated by different partners, as this could either constrain evolution in the shared partner or lead to adaptive differentiation, depending on the trait's genetic architecture (Assis et al., 2020). These studies also predominantly focused on within-guild interactions where partner species provide largely the same resource and may compete to interact with a shared host. In particular, studies of plant–pollinator interactions have often documented conflicting selection. For instance Kulbaba and Worley (2013) found conflicting selection on corolla diameter by hummingbirds and hawkmoths. Similarly, floral corolla flare was predicted to be under selection to be narrower with hummingbirds but predicted selection was neutral with bumblebees (Aigner, 2004). However, multiple pollinator mutualists have been shown to impose concordant selection on some traits; Sahli and Conner (2011; re-analysis by TerHost et al., 2015), for example documented concordant selection on the degree of anther exertion by sweat bees and larger bees. Since floral phenotypes typically determine the ability of mutualists to interact via matching, these traits may be predicted to more often be under conflicting selection than traits involved in resource-exchange mutualisms, where concordant selection—and even synergistic concordant selection—may play a more important role.

We know of no other cases of synergistic (concordant) selection in multiple mutualisms, raising the questions of when it should be expected and why it has not been documented previously. Synergistic selection may not have been previously shown because most studies of multiple mutualisms simply do not measure selection (Mack & Rudgers, 2008; Orivel et al., 2017; Palmer et al., 2010) and those that do are usually measure selection in a pairwise fashion (i.e. selection imposed by each species individually, but not collectively, or as a group but not individually) and thus are not designed to test non-additivity (TerHorst et al., 2015). A notable exception is Sahli and Conner (2011), who estimated how pollinators individually and as a group imposed selection on floral morphology. However, unlike our study which demonstrated synergistic selection on branch number, re-analysis of their data by TerHorst et al., (2015) found evidence for concordant, but not synergistic, selection.

We posit two factors may lead to synergistic selection: the importance of the trait in acquiring resources later traded to mutualist partners and complementarity of partner-provided benefits (Afkhami et al., 2014, 2020). For host resource-acquisition traits, we expect that they will be subject to synergistic selection when the same resource is provided to all partners: in these cases, selection can favour trait values that increase the size of the resource ‘pie’. For instance, selection favouring plant traits involved in increased carbon acquisition can increase the overall level of C-based benefits available to pollinators, ant-defenders, microbial partners and other mutualists. In these cases, environments with multiple mutualisms may lead to an increase in the selection of those traits. The non-additivity of selection in our study likely resulted from complementarity of the resources provided to host plants by microbial partners. In the absence of rhizobia, plants in our experiment did not perform well and produced very few pods and relatively few branches, regardless of whether mycorrhizal fungi were present. Without rhizobia-fixed N, plant growth and reproduction were limited, such that increased branching could not improve plant fitness through increased carbon acquisition or allocation to mycorrhizal fungi. In the presence of both partners, the selection was stronger than what was observed when rhizobia were present alone, likely because rhizobia and mycorrhizal fungi alleviated different limitations on growth and reproduction. While complementarity of partner-conferred resources increases the probability of synergistic selection, even functionally redundant mutualists that provide identical benefits (e.g. multiple insects with identical pollination characteristics) may result in synergistic selection on traits if each partner alone would not provide sufficient resources to allow host survival and reproduction. Alternatively, in the case of saturating effects of the benefit received on the trait (or fitness), we would predict that multiple mutualisms would result in non-additive selection, where both partners still produce selection in the same direction (i.e. there is no conflicting selection) but the total strength of selection with multiple mutualisms is lower than additive predictions.

Fitness alignment in mutualisms

A classic expectation in mutualisms is that they should be subject to ‘cheating’ that could threaten their persistence and stability (Douglas, 2008; Ferriere et al., 2002). This has led to a large literature on mechanisms that can maintain mutualisms in the face of cheating (e.g. partner choice, partner fidelity feedback, screening and sanctions), definitions of cheating (Ghoul et al., 2014; Jones et al., 2015), and whether there is any evidence of cheating (Frederickson, 2013). One important mechanism capable of stabilising mutualisms is partner fidelity feedback, where mutualisms persist because of positive feedbacks between partners: symbionts and hosts benefit by improving each other's fitness (Sachs et al., 2004; Weyl et al., 2010). A key prediction of this view is a positive correlation between host and symbiont fitnesses, exactly as we observed (Figure 2). Our data show that plant genotypes with high fitness also produce more nodules, a fitness component of their rhizobial partners. Interestingly, while plant and rhizobia fitness is positively and significantly correlated when mycorrhizal fungi are absent, the correlation is appreciably stronger in their presence. In other words, the presence of multiple mutualisms leads to scenario Figure 1(i) by enhancing the fitness alignment within a pair of interacting species. If this is a general finding, it may be that multiple mutualisms can increase the persistence of the component interactions by more tightly aligning the fitness of the interactors.

We suggest that synergistic effects of multiple mutualisms are likely to facilitate partner fidelity feedback. The logic is straightforward: if participating in multiple mutualisms improves host fitness beyond what would be expected from participating in a single mutualism, hosts would be in better than expected conditions to provide fitness benefits to mutualists. While positive feedbacks are also expected in the additive case, the synergistic effects of multiple mutualisms would lead to the strongest feedbacks, while only conflicting selection would reduce them. Evaluating the generality of these results requires more fully characterising the fitness alignment of multiple interacting species.

Future directions

In our opinion, there are at least three types of studies that will be valuable for furthering our understanding of multiple mutualisms’ roles in eco-evolutionary dynamics. First, future work should include the effects of multiple mutualisms on traits in all partners (e.g. do rhizobia affect mycorrhizal fungi's allocation to intra- vs extraradical hyphae; van Aarle & Olsson, 2008), rather than remaining host-centric. Second, more studies testing how multispecies beneficial interactions impact selection and heritability of host traits across a wide range of systems is crucial to determine the generality of synergistic and conflicting selection as well as which types of mutualisms exhibit each type of selection. Field common garden-style studies with tractable partners would be especially valuable. Third, to link complementarity of partner mutualists with synergistic selection, studies factorially manipulating within- versus across-guild multiple mutualisms and measuring selection and complementarity are needed. Collectively, studies like these will make meaningful progress in understanding the eco-evolutionary consequences of multiple mutualism effects.

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AUTHORSHIP

All authors conceived the overall project, contributed to the development of experimental plans and the manuscript draft. MEA conducted the experiment and collected the data with advice from JRS. MEA and JRS analysed the data; funding to MEA and JRS supported experimental work, with funding to all authors supporting manuscript writing and analysis.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository at https://doi.org/10.5061/dryad.vq83bk3sb

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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