LETTER

Selection for cheating across disparate environments in the legume-rhizobium mutualism

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Abstract

The primary dilemma in evolutionarily stable mutualisms is that natural selection for cheating could overwhelm selection for cooperation. Cheating need not entail parasitism; selection favours cheating as a quantitative trait whenever less-cooperative partners are more fit than more-cooperative partners. Mutualisms might be stabilised by mechanisms that direct benefits to more-cooperative individuals, which counter selection for cheating; however, empirical evidence that natural selection favours cheating in mutualisms is sparse. We measured selection on cheating in single-partner pairings of wild legume and rhizobium lineages, which prevented legume choice. Across contrasting environments, selection consistently favoured cheating by rhizobia, but did not favour legumes that provided less benefit to rhizobium partners. This is the first simultaneous measurement of selection on cheating across both host and symbiont lineages from a natural population. We empirically confirm selection for cheating as a source of antagonistic coevolutionary pressure in mutualism and a biological dilemma for models of cooperation.

Keywords

Cheating, *Ensifer medicae*, legume-rhizobium, *Medicago polymorpha*, mutualism, natural selection, partner choice, partner fidelity feedback, sanctions, symbiosis.

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INTRODUCTION

Mutualisms, interspecific relationships that benefit all partners, are fundamentally important to ecology and evolution, yet little is known about natural selection on mutualist cooperation. Even within a cooperative interaction, bestowing limited resources upon a partner, at a cost to individual fitness, promotes conflict among partners (Sachs *et al.* 2004). Thus, when partners' interests are not coordinated by perfect vertical co-transmission, mutualists could experience a tragedy of the commons: partners that contribute resources could indirectly aid less-cooperative partners (Denison *et al.* 2003; Foster & Kokko 2006) which could selectively favour cheating strategies (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1991; Ferriere *et al.* 2002; West *et al.* 2002; Sachs *et al.* 2004; Foster & Kokko 2006; Kiers & Denison 2008). We consider cheating an adaptive uncooperative strategy: cheaters have higher fitness than more-cooperative genotypes yet reduce partner fitness relative to that of partners paired with more-cooperative genotypes (reviewed by Frederickson 2013). Thus, we treat cheating as a continuous, quantitative trait; selection favouring cheating occurs when less-cooperative mutualists are more fit than more-cooperative mutualists. However, despite the central role of such antagonistic selection in models of mutualism evolution, whether populations of cooperative partners experience selection for cheating in mutualism remains a critical frontier of evolutionary ecology (Heath 2010; Sachs *et al.* 2010a,b; Friesen 2012; Frederickson 2013; Kimbrel *et al.* 2013).

Selection for cheating might be difficult to detect if it is frequently countered by mechanisms that preferentially direct mutualism benefits to more-cooperative individuals (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1991; West *et al.* 2002; Sachs *et al.* 2004; Foster & Kokko 2006; Kiers & Denison 2008; Weyl *et al.* 2010). Such mechanisms, including partner choice (Bull & Rice 1991), sanctions (Kiers & Denison 2008) and partner fidelity feedback (Weyl *et al.* 2010), are thought to be evolutionarily maintained by selection for symbiont cheating (Foster & Kokko 2006; Weyl *et al.* 2010), yet would also conceal it. Experimentally restraining counter-selecting mechanisms might therefore reveal the potential for selection to favour cheating.

Detecting selection for cheating could also be complicated by context-dependence (Bronstein 1994). The magnitude or direction of selection for cheating could be altered by environmental shifts in the valuation of traded resources and levels of physiological stress (Bronstein 1994). Increasing environmental quality could decrease host dependence on mutualists, which might reduce rewards bestowed on beneficial symbionts and weaken selection against cheating (Hochberg *et al.* 2000; Neuhauser & Fargione 2004; Thrall *et al.* 2007). For example, plant benefit from mycorrhizal inoculation decreases when phosphorus is abundant (Hoeksema *et al.* 2010), which would favour less-cooperative strains if plants take up phosphorus directly and reduce rewards to cooperative mycorrhizae. Correspondingly, fertile habitats are often populated by less-beneficial symbionts (Thrall *et al.* 2007) and more stress-tolerant hosts can be less dependent on symbionts (Thrall *et al.* 2008).

Understanding selection on cheating could be hampered when inference is drawn from artificial trait space, which may not reflect available natural variation. Artificial mutants, physical or physiological manipulation of cooperation phenotypes, and combining host and symbiont genotypes that have not co-evolved (e.g: plant-microbe: Kiers & Denison 2008; Friesen 2012; squid-vibrio: Nyholm & McFall-Ngai 2004; ant-plant: Frederickson 2009; fig-wasp: Jandér *et al.* 2012) might elicit unviable phenotypes or unnatural selection pressures.

The empirical focus on symbiont, not host, cheating, also reduces the likelihood of detecting selection for cheating. Host cheating, though rarely explored empirically, is predicted in costly, asymmetric mutualisms when symbionts are unable to terminate the interaction (Johnstone & Bshary 2002; Frean & Abraham 2004; Raven 2010). Ample evidence suggests plants can cheat microbial symbionts (Douglas 2008): over 400 species of non-photosynthetic and 30,000 species of partially photosynthetic mycoheterotrophic plants parasitize mycorrhizal networks for nutrients and carbon (Douglas 2008). Therefore, it is critical to evaluate selection for cheating in both host and symbiont partners (West *et al.* 2002).

We investigated selection on cheating in the legume-rhizobium symbiosis. Host legumes trade photosynthetically derived carbon for nitrogen fixed by endosymbiotic rhizobia housed in root nodules. Rhizobia are environmentally acquired by legumes. Each plant hosts multiple nodules; each nodule can contain a different rhizobium genotype. Resource exchange appears to entail pleiotropic costs for both partners (Kiers & Denison 2008). For example, when rhizobia expend energy to fix nitrogen for the host, they benefit from enhancing host fitness, but sacrifice the opportunity to hoard high-energy storage compounds, such as polyhydroxybutyrate (PHB) and rhizopines (Kiers & Denison 2008), which can improve survival of their progeny (Oono *et al.* 2009). Similarly, when plants allocate photosynthetically derived sugars to rhizobia, they benefit from enhancing rhizobium productivity, but sacrifice opportunities to allocate sugars to their own growth and progeny.

We paired legumes and rhizobia collected from a natural *Medicago polymorpha-Ensifer medicae* population to examine whether host or symbiont genotypes can experience selection for cheating when interacting with native partners. Legumes exposed to multiple rhizobia can employ relative counterselection mechanisms, such as partner choice, to preferentially associate with higher quality partners (Bull & Rice 1991; Heath & Tiffin 2009; Sachs *et al.* 2010a,b). Such mechanisms could conceal pleiotropic costs of cooperation that would otherwise favour less-cooperative strategies ['potential' cheats, (Ghoul *et al.* 2013)]. To experimentally restrain such relative mechanisms, each legume in our study was inoculated with a single rhizobium strain and thus formed nodules with a uniform population of symbionts. In this design, counterselection mechanisms such as absolute sanctions or partner fidelity feedback, whereby hosts reward and/or punish a symbiont based upon its individual cooperation phenotype (Sachs *et al.* 2004; Kiers & Denison 2008; Ghoul *et al.* 2013), could continue to favour more-cooperative strategies. Thus, our design restrains some, but not all, potential counter-selection mechanisms that could favour cooperation.

With this design, we investigated the following: (1) Does genotypic selection favour host or symbiont cheating in the absence of relative counter-selection mechanisms? (2) Do host or symbiont populations exhibit genetic variation in the cooperative benefits they gain and bestow? and (3) Do both partners derive fitness benefits from cooperating? We examined these questions in two natural, adjacent soil contexts: a low-quality environment, i.e., physiologically harsh, low-nitrogen serpentine soil, and a high-quality environment, i.e., physiologically benign, higher nitrogen non-serpentine soil. The availability of traded resources and physiological stress imposed by the environment could alter selection on cheating (Bronstein 1994; Thrall *et al.* 2007, 2008); examining selection in environmental conditions that differ along both of these axes provides generality to our findings.

MATERIALS AND METHODS

Collections

The McLaughlin Natural Reserve, USA, contains patches of harsh serpentine soil, which is deficient in nitrogen and enriched in toxic heavy metals, embedded in a matrix of relatively benign non-serpentine soils. We collected random samples of *Medicago polymorpha* L. nodules, fruits and soils along a 15-25 m transect spanning a high density patch of *M. polymorpha* at each of six randomly selected focal sites: three serpentine outcrops and three non-serpentine areas, classified previously by soil chemistry (Porter & Rice 2013).

Experiment 1: *Measuring symbiotic traits*

Rhizobium genotypes
Medicago polymorpha associates nearly ubiquitously with *Ensifer medicae* at the Reserve (Porter & Rice 2013). To broadly sample the population of wild *E. medicae* at the Reserve, three strains were randomly selected from the population sample collected at each focal site (Table S1).

Plant genotypes
To broadly sample the population of wild *M. polymorpha* at the Reserve a single seed was obtained from each of three randomly selected *M. polymorpha* plants, at least 30 cm apart, at each focal site. Seeds were cultivated in the greenhouse and allowed to self-fertilise, generating abundant seed for 18 inbred lineages (Table S2). *Medicago polymorpha* is primarily self-pollinating (Porter *et al.* 2011).

Field soils
Soil from each of the six focal sites was collected, air-dried and sifted to 1 cm². Serpentine and non-serpentine soil mixes were generated by combining equal parts by volume of soil from the three replicate sites within a soil type. Each soil mix was then mixed 1 : 1 by volume with inert silica sand, to prevent compaction, and steam pasteurised twice to kill all nodulating rhizobia (Porter *et al.* 2011).

Experimental design
To determine plant and rhizobium fitness in symbiosis, in both serpentine and non-serpentine soil, 18 rhizobium genotypes and a no-rhizobium control were inoculated onto each of the 18 plant genotypes, and grown on both soil types, in a full factorial design. One rhizobium genotype was applied incorrectly; data related to this strain were removed from the analysis (Table S1). Therefore, 306 G × G combinations plus a negative rhizobium control were assessed on both soil types (648 treatment combinations). These were replicated twice, once in each of two complete randomised spatial blocks (1296 experimental units). No negative rhizobium control plant formed nodules.

Greenhouse cultivation

Seeds were scarified, surface-sterilised in bleach, rinsed with sterile water, and vernalized in darkness at 4°C for 8 days. Germinants were planted into 66-mL cylindrical pots containing steam-pasteurised soil. At the appearance of the first trifoliate leaf, plants received 0.5 mL of either inoculum or a water control.

Strains of *E. medicae* were grown in TY broth for 48 hours at 30°C at 300 rpm. Immediately before inoculation, rhizobia were centrifuged and re-suspended to 4×10^5 cells mL^{-1} in water (based on OD_{600}). To avoid cross-contamination, plants were spaced > 12 cm apart and watered twice-daily with ultra-fine mist. Soil and rhizobia were the sole sources of plant nutrition.

Plants grew to reproductive maturity (90 days) during the natural California winter growing season in a microbially controlled greenhouse (Fig. S1). At harvest, belowground tissue was washed and frozen. Samples were subsequently thawed and the nodules on each plant were counted, separated from root tissue, dried to constant weight at 60°C and weighed (mg per plant). Mean nodule weight, (total nodule number)/(total nodule weight), was calculated for each plant. Seeds were counted and weighed (mg per plant). Mean seed weight, (total seed number)/(total seed weight), was calculated for each plant.

In the *Medicago-Ensifer* symbiosis, nodule number and weight are important components of rhizobium fitness (Heath & Tiffin 2009). Because a nodule is founded by a single (or few) cell(s), nodule weight was interpreted as a fitness component that indicates the number of progeny a founding rhizobium cell obtains from a nodule. This assumption was tested in Experiment 2. Because each pot started with a standard isoclonal rhizobium density, nodule number was cautiously interpreted to indicate the total number of progeny gained by a rhizobium strain. We consider nodule size superior to nodule number as a measure of a rhizobium genotype's fitness, as nodule size more directly indexes a founding cell's fecundity. Under natural conditions nodule number may represent a common good that could be exploited unless rhizobium genotypes are spatially structured, whereas the size of a singly-infected nodule represents a private good. Seed number and mean seed weight were interpreted as plant fitness components indicating the number and condition of offspring, respectively.

Symbiotic investment by each plant or rhizobium lineage was measured as the mean fitness of partners interacting with that lineage. Thus, a rhizobium strain's symbiotic investment is the mean seed number and average seed weight produced by all plants inoculated with that strain; a plant lineage's symbiotic investment is the mean nodule weight and nodule number produced by all rhizobium strains inoculated onto that plant lineage.

Experiment 1: *Analyses*

Genotypic selection analysis

Natural selection on each plant and rhizobium symbiotic investment trait was estimated using standard genetic selection analysis (Rausher 1992; Smith & Rauscher 2008), which is similar to that of Lande & Arnold (1983), but analyses genotypic values of lineages rather than phenotypic values of individuals. This procedure reveals selection on genotypes by accounting for biases due to environmentally induced covariances between fitness and the measured traits (Smith & Rauscher 2008). We first calculated the least square lineage and strain means for response variables, with the effects of block removed, in ANOVAs with block and lineage or strain as fixed effects (lm, R Development Core Team). As is standard (Rutter & Rauscher 2004; Smith & Rauscher 2008), each fitness component was relativised by dividing lineage or strain least square means by the population mean within a soil type and each symbiotic investment trait was standardised to a mean of zero and a standard deviation of one for each lineage or strain within a soil type.

Directional selection gradients, which measure total directional selection (β) on a trait, were estimated separately for plants and rhizobia with first-order linear models containing only a single focal symbiotic investment trait (plants: mean nodule weight or nodule number; rhizobia: seed number or mean seed weight). Each total directional selection gradient was then partitioned into direct and indirect components using a first-order linear model containing both symbiotic investment traits. The linear partial-regression coefficients from this analysis estimate the magnitude of directional selection acting directly on each trait (β'), with the indirect effects of directional selection on the other symbiotic investment trait removed. Sample size (17–18 genotypes) limited power to detect quadratic and correlational selection gradients (γ); hence, we only present estimates of linear selection gradients (Zuur *et al.* 2009). All models of selection gradients utilised fixed effect ANOVAs with type III sums of squares (lm, R Development Core Team).

To test if selection differed between soil types, we used fixed effect ANCOVA models (car package; Fox & Weisberg 2011). Separate analyses on plants and rhizobia explored the effect of soil context on total directional and direct directional selection gradients (Rutter & Rauscher 2004; Smith & Rauscher 2008) (lm package, R Development Core Team 2013). A significant interaction of soil type with a trait would have indicated that selection differed with soil environment, but none were significant (Table S3). Therefore, selection gradient surfaces were visualised (mcGraph3 in rockchalk package, Johnson 2013) and interpreted by averaging across the two soil types.

Genetic variation in symbiotic traits

To determine whether the measured traits were genetically variable within the wild populations, we performed separate mixed model ANOVAs (lme4 package, Bates *et al.* 2013) for seed number, mean seed weight, nodule number and mean nodule weight as responses in factorial models of the main and interactive effects of plant genotype, rhizobium genotype, and greenhouse soil environment; block was included without considering its interactions. Plant and rhizobium genotype were random factors with 18 and 17 lineages (i.e., levels), respectively. Soil type (serpentine or non-serpentine) and block (1 or 2) were fixed binomial variables. Assumptions of normality and homogeneity of variance were assessed graphically (Zuur *et al.* 2009); seed number and nodule number were square root transformed and nodule weight was log trans-

formed to improve the fit to model assumptions. The significance of each random effect was determined with the likelihood ratio statistic using a chi-squared test with one degree of freedom, which is generally conservative for random effects (Pinheiro & Bates 2009). Significance of fixed effects was assessed with F-tests using type III sums of squares (lme4 package, Bates *et al.* 2013).

Plant benefits from symbiosis

To determine whether plants gained fitness benefits from symbiosis in harsh and benign soil contexts, we used ANOVA with type III sums of squares to compare fitness components for inoculated and uninoculated plants within each soil context (car package; Fox & Weisberg 2011). The full model included inoculation, soil type, their interaction, and block as fixed effects. Assumptions of normality and homogeneity of variance were assessed graphically (Zuur *et al.* 2009).

Experiment 2: *Validation of rhizobium fitness components*

We tested whether rhizobium fitness proxies, nodule size and nodule number, were positively correlated with number of culturable viable rhizobium progeny in our focal wild population. *Medicago* forms indeterminate nodules in which only a fraction of rhizobia are reproductively viable (Bronstein 1994; Thrall *et al.* 2007, 2008; Oono *et al.* 2011). Therefore, number of viable culturable progeny (colony forming units; CFU) per nodule provides a more accurate measure of rhizobium fitness than would number of rhizobium genome equivalents per nodule. For 57 *M. polymorpha* nodules, we quantified the relationship between CFU and nodule size with a two-way ANCOVA including nodule area as a continuous variable, soil type as a categorical variable, and their interaction. These nodules were randomly sampled from the six natural focal sites. Thus, both Experiment 1 and 2 estimate parameters for the same wild population (Method S1).

RESULTS

Genotypic selection

Selection did not significantly differ between the two soil environments (no soil type by trait interaction, Table S3 and Table S4); lineage means were subsequently averaged across soil types. Directional selection favoured cheating in rhizobium lineages but not in plant lineages (Table 1; Fig 1): rhizobium lineages that provided less host fitness benefit were more fit than those that provided more benefit (Table 1; Fig 1). In neither environment was fitness of a plant lineage related to its effect on rhizobium fitness (Table 1; Fig 1). Total and direct directional selection gradients were qualitatively similar. First-order multivariate models that simultaneously accounted for direct and indirect directional selection on both standardised symbiotic traits (host seed number and seed size) explained 65% of the variance in rhizobium genotype mean fitness measured as nodule weight ($F_{2,14} = 13.0$, $P = 0.0006$) and 58% of the variance in rhizobium genotype mean fitness measured as nodule number ($F_{2,14} = 9.63$, $P = 0.002$), based on the multiple R^2 of the models. Analogous models on plant

Table 1 Standardised directional selection gradients on symbiotic trait investment for plant (A) and rhizobium (B) genotypes with the effect of soil type removed statistically

	β	β′	β	β′
(A) Plant	Seed number		Mean seed weight	
Nodule number	0.05	0.09	−0.01	−0.01
Mean nodule weight	−0.01	0.06	0.01	0.00
(B) Rhizobium	Nodule number		Mean nodule weight	
Seed number	**−0.12****	−0.06	**−0.39*****	**−0.25****
Mean seed weight	**−0.13*****	**−0.10*****	**−0.32*****	**−0.23****

Total directional selection gradients (β) were estimated with a first-order model containing only the focal trait, while direct directional selection gradients (β′) were estimated from a first-order multivariate model that simultaneously accounts for selection on both symbiotic traits. Significant gradients are presented in boldface, **$P < 0.05$, ***$P < 0.01$.

genotypes explained a significant portion of variance in neither plant fitness component.

Genetic variation in symbiotic traits

Plant lineages exhibited significant genetic variance in seed number, mean seed weight, nodule number and mean nodule weight (Fig 2a–d; Table 2). Soil environment influenced the effect of plant lineage on mean seed weight, nodule number, and mean nodule weight (plant lineage by destination soil interaction; Table 2). Rhizobium strains exhibited significant genetic variance in mean seed weight, nodule number and mean nodule weight, but not seed number (Fig 2e–h; Table 2). Both plant and rhizobium fitness components were greater in benign non-serpentine soil (Fig 3c–d; soil, Table 2). Spatial location in the greenhouse affected seed number, mean seed weight and nodule number (block; Table 2). Because plant lineage by rhizobium lineage (G × G) interactions were not detected, the genotypic effects of lineages and strains were considered independently in the preceding selection analyses (Table 2).

Plant benefits from symbiosis

Engaging in symbiosis benefited plants in both high and low quality environments. Symbiotic plants produced more, larger seeds than those grown without rhizobia (seed number: $F_{1,1290} = 204.1$, $P < 0.0001$, Fig 3a; mean seed weight: $F_{1,1290} = 191.4$, $P < 0.0001$, Fig 3b). Symbiosis improved plant fitness more on serpentine than non-serpentine soil (seed number: $F_{1,1290} = 14.1$, $P < 0.0001$, Fig 3a; mean seed weight: $F_{1,1290} = 33.2$, $P < 0.0001$, Fig 3b). On serpentine soil, symbiotic plants produced 1123% more seeds that were 976% heavier than non-symbiotic plants, whereas on non-serpentine soil, symbiotic plants produced 228% more seeds that were 98% heavier than non-symbiotic plants (Table S5).

Validation of rhizobium fitness components

Rhizobia gained greater fitness benefits (CFU per nodule) from larger nodules ($F_{1,53} = 10.12$, $P = 0.0025$, adjusted $R^2 = 0.2436$), with the following relationship: log (CFU per
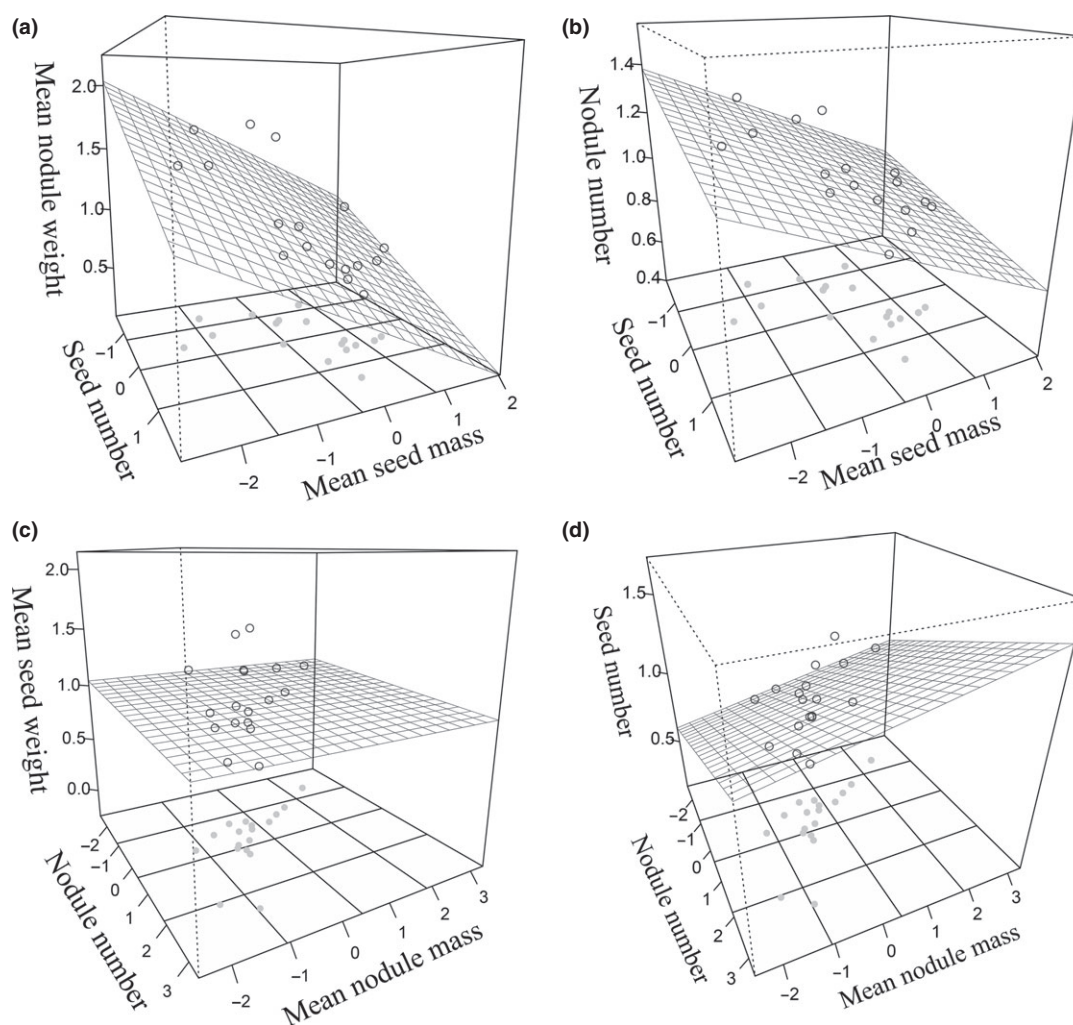
Figure 1 Selection favours cheating among rhizobium genotypes, but not among plant genotypes. Significant linear planes of selection on rhizobium genotypic values for two symbiotic investment traits (mean seed weight and seed number), estimated using two different rhizobium fitness components, mean nodule weight (a) and nodule number (b). Non-significant linear planes of selection on plant genotypic values for two symbiotic investment traits (mean nodule weight and nodule number) estimated using two different plant fitness components, mean seed weight (c) and seed number (d). The genotype mean for each rhizobium or plant genotype is depicted in the three-dimensional space (open circle) and projected onto the two-dimensional symbiotic investment trait plane (grey circle). As selection did not differ significantly between soil environments (Table S3), these surfaces were visualised from averages across soil types of fitness and trait values for each genotype. Open circles, genotype means along 3-D selection surface; filled circles, genotype means along 2-D trait plane.

nodule) = 3.91 + 0.42(nodule area) + error. The CFU per nodule, and the linear dependence of CFU per nodule on nodule area, did not differ between soil types (Table S6; Fig 4).

DISCUSSION

To our knowledge, this is the first study to simultaneously measure selection for cheating on both host and symbiont mutualist genotypes from a natural population. As predicted by antagonistic coevolutionary models of mutualism, rhizobia experienced selection for cheating (Axelrod & Hamilton 1981; Bull & Rice 1991; Ferriere *et al.* 2002; West *et al.* 2002; Sachs *et al.* 2004; Foster & Kokko 2006). Although all rhizobia were highly beneficial, genotypes that conferred less host benefit (i.e., smaller and fewer seeds) were more fit (i.e., obtained larger and more numerous nodules) than more-cooperative

genotypes. In striking asymmetry, we detected no selection on cheating by plant lineages. Challenging models of asymmetric mutualisms, which predict that selection will favour cheating by hosts interacting with captive symbionts (Johnstone & Bshary 2002), the relative fitness of plant genotypes was unrelated to the fitness they conferred to symbiotic rhizobia.

Mutualism theory: implications of genotypic selection for symbiont cheating

Evidence that selection favours cheating in rhizobia supports a fundamental assumption of mutualism theory: that pleiotropic costs of cooperation can disadvantage more-cooperative genotypes, which, if unchecked, would facilitate an evolutionary increase in cheating (Sachs *et al.* 2004). Pleiotropic costs might arise if allocating limiting resources to partner benefit (e.g.,

Table 2 Mixed model ANOVAs for symbiotic fitness components for 18 *M. polymorpha* lineages and 17 *E. medicae* strains grown in all possible factorial combinations in two contrasting soil environments in the greenhouse

	Sqrt seed number	Weight of a seed	Sqrt nodule number	Log weight of a nodule
Random effects				
Lineage × strain × soil	0.00	0.00	0.00	0.00
Lineage × strain	0.32	1.19	0.00	0.32
Lineage × soil	1.02	**37.97******	**11.46*****	**4.58***
Strain × soil	0.00	0.00	0.00	0.47
Lineage	**218.82******	**641.12******	**305.77******	**208.75******
Strain	0.00	**5.04***	**86.62******	**606.12******
Fixed effects				
Soil	**667.48******	**68.04******	**24.44*****	**11.95***
Block	**40.74******	**10.35***	**16.81******	0.55

Shown are chi-squared values from a log likelihood ratio test for random effects and F values from a mixed model ANOVA for fixed effects. Significant values are presented in bold-face. *$P < 0.05$, **$P < 0.025$, ***$P < 0.001$, ****$P < 0.0001$.
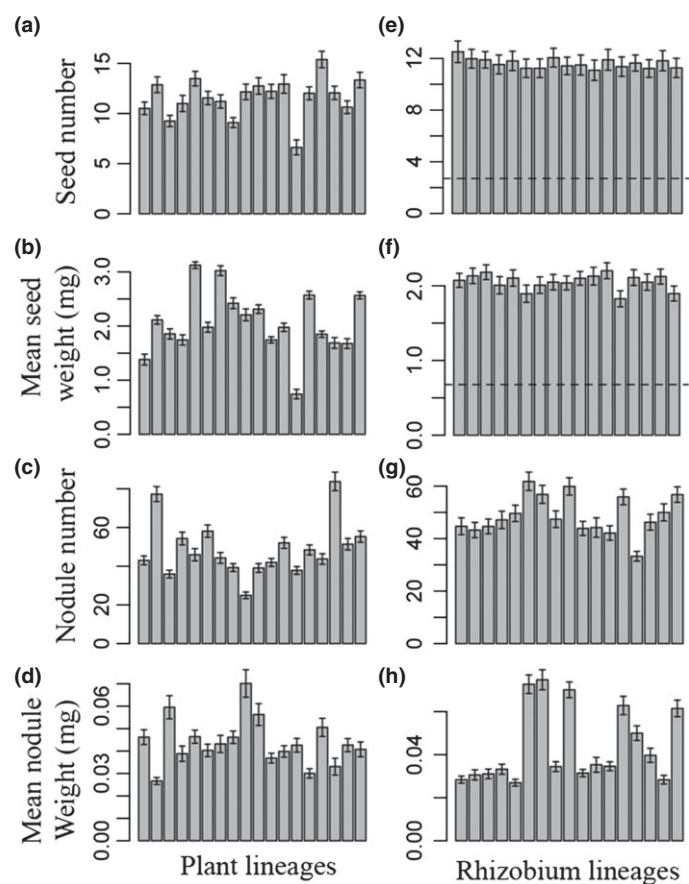

Figure 2 Lineage means (± SE) for plant (a–d) and rhizobium lineages (e–h) for each of the four measured traits. Lineages are displayed in increasing numeric order for both plants (P1–P18) and rhizobia (R1–R17). In (e and f) the mean values for plants not inoculated with rhizobia are indicated with a dashed line.

fixing nitrogen) trades off with allocating to individual fitness benefit (e.g., microbial energy storing compounds) (Kiers & Denison 2008).
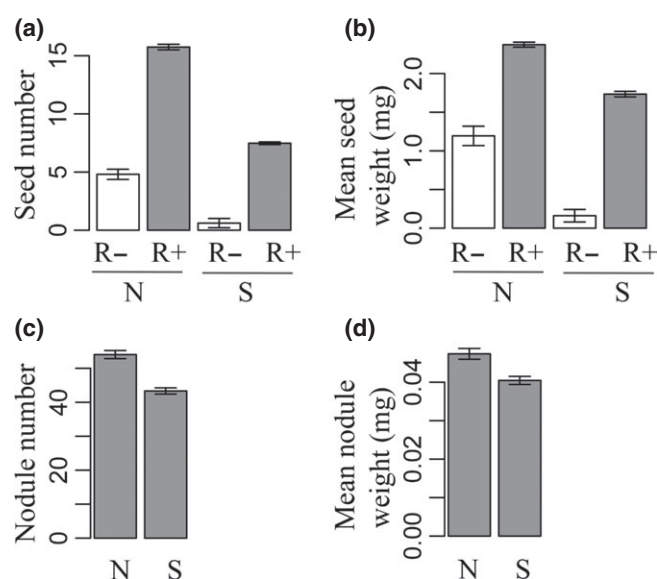

Figure 3 *Medicago polymorpha* individual fitness as the total number of seeds produced (a) and the mean weight of individual seeds (b), for plants inoculated with either water (R−, open bars) or rhizobia in water (R+, grey bars). *Ensifer medicae* fitness as the total number of nodules produced (c) and the mean weight of individual nodules (d) per plant. By non-serpentine (N) or a serpentine (S) soil environment. Bars are ± SE.
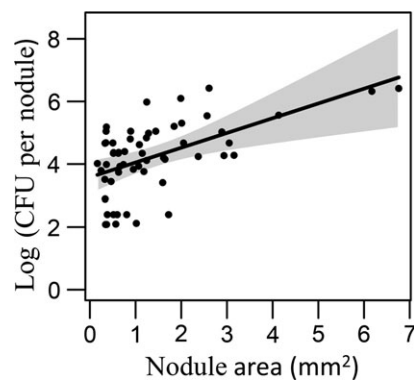

Figure 4 Number of viable rhizobium progeny (colony forming units; CFU) in a nodule as a function of nodule area. Linear regression (black line), individual nodules measured (black circles), 95% confidence interval of the regression based on the conditional plot of the linear model (grey band).

Stable mutualism therefore requires mechanisms that direct benefits to more-cooperative partners and purge less-cooperative genotypes (Foster & Kokko 2006). Hosts that encounter multiple symbionts can express relative counter-selection traits (e.g., partner choice, relative sanctions or partner fidelity feedback), which compensate more-beneficial symbionts for pleiotropic costs of cooperation. Legume-rhizobium symbioses feature such mechanisms (Heath & Tiffin 2009; Oono *et al.* 2009; Gubry-Rangin *et al.* 2010), which can provide more-cooperative strains with higher fitness than less-cooperative strains when plants host multiple symbionts (Heath & Tiffin 2009), even if less-cooperative strains are favoured when inoculated singly onto a plant (Sachs *et al.* 2010b). As expected if rhizobia experience such counter-selection in

nature, we found no completely uncooperative rhizobium genotypes in this natural population, which comports with recent reviews (Friesen 2012; Frederickson 2013).

An under-recognised problem in mutualism theory is that counter-selecting traits could eliminate their own selective advantage by purging less-cooperative symbionts (Frederickson 2013). Our finding that selection favoured less-cooperative rhizobium genotypes suggests an antagonistic coevolutionary explanation for the maintenance of such counter-selecting traits. If 'slight cheats' (Ferriere *et al.* 2002), such as the less-cooperative rhizobium genotypes in our sample, incur lower pleiotropic costs of cooperation, they could prosper on host genotypes or in other contexts where counter-selection mechanisms are weak (Simonsen & Stinchcombe 2014) and thus continue to selectively favour hosts with stronger counter-selection.

Pleiotropic costs of cooperation could also select for conditional cooperation (Akçay & Simms 2011), wherein symbionts adjust benefits in response to host actions or the performance of other strains on the host (Ghoul *et al.* 2013). There is no evidence for conditional rhizobium cooperators or cheats but such plasticity is plausible (Akçay & Simms 2011).

Legumes and rhizobia: fitness alignment or conflict?

Whether legume and rhizobium fitnesses are aligned or conflicted in mutualism is controversial (Friesen 2012; Kiers *et al.* 2012). In a recent meta-analysis, Friesen (2012) found that artificial and natural mutations beneficial for rhizobia pleiotropically benefit their hosts, which suggests widespread alignment of legume and rhizobium fitness. Specifically, nodule number and nodule biomass positively correlated with plant aerial biomass in single strain inoculation, and nodulation competitiveness positively correlated with symbiont effectiveness in multi-strain inoculation (Friesen 2012). Additionally, sequence conservation in rhizobium host infection genes, relative to those of a pathogen, suggests resolution of antagonism between rhizobia and hosts (Kimbrel *et al.* 2013). In contrast, Heath & Tiffin (2009) found fitness conflict, detected as a negative correlation between legume and rhizobium fitness across pairs of host and symbiont genotypes (G × G interactions) in single-strain inoculation. The robust natural selection for rhizobium cheating we documented across a large sample of natural partner genotypes (genotype main effects) in single-strain inoculation, with rigorous validation of fitness components, supports fitness conflict, congruent with fitness trade-offs found in other studies (Laguerre *et al.* 2007; Sachs *et al.* 2010b).

Why might studies differ in whether they detect mutualist fitness alignment or conflict? As explained above, expression of host choice could determine the correlation between host and symbiont fitness. Alignment often occurs when experimental designs allow hosts to counter selection favouring cheats by preferentially choosing or allocating to more-beneficial partners (Nyholm & McFall-Ngai 2004; Kiers & Denison 2008; Jandér *et al.* 2012; Heath & Tiffin 2009; Sachs *et al.* 2010b), whereas restricting a host to one strain can reveal fitness conflict (Heath 2010; Sachs *et al.* 2010a), as in the present study. However, in a host with weak relative counter-selection mechanisms, multi-strain inoculation could reveal conflict not visible in the single-strain scenario: less-cooperative rhizo-bia might 'free-ride' by disproportionately benefitting from the greater overall host vigour provided by more cooperative strains (Denison *et al.* 2003). Our observation of host-symbiont fitness conflict in single-strain inoculation suggests that rhizobial fitness might depend more on relative counter selection mechanisms than on overall host vigour.

Another explanation could be that early fitness alignment shifts to conflict later in the symbiosis. Theory predicts selection for cheating will increase toward late stages of a mutualistic interaction as the probability of continued interaction decreases (Trivers 1971; Axelrod & Hamilton 1981; Bull & Rice 1991). Furthermore, the ontogeny of many symbioses facilitates a shift from fitness alignment to conflict. Symbionts might initially benefit from increased host performance, but this fitness alignment could break down once hosts divert resources to reproduction (Frederickson 2009). Also, early host-symbiont fitness alignment via kin selection among the few symbionts infecting a young host might decline as increasing numbers of symbionts reduce symbiont relatedness (West *et al.* 2002). Thus, studies that measure host fitness as juvenile biomass may detect fitness alignment (e.g., Friesen 2012), whereas studies that measure reproductive output (e.g., present study; Heath 2010) may detect fitness conflict.

Finally, the coevolutionary history of genotypes may affect observed patterns of fitness alignment. In studies that partner non-coevolved mutualists, general vigour variation among naïve partner combinations might drive strong positive partner fitness covariance, obscuring underlying fitness trade-offs (Fry 1993).

Genetic selection gradients

The magnitudes of genetic selection gradients (Rausher 1992) favouring rhizobium cheating in the present study were moderate to strong, falling within or above the range of median values observed across traits in macro-organisms (Kingsolver & Diamond 2011). Selection favoured rhizobium cheating when we measured rhizobium fitness on a per-pot basis; the magnitude of this selection would be even greater if fitness were measured on a per-nodule basis (Oono *et al.* 2009) because our least-beneficial genotypes generated more numerous, larger nodules. If selection for cheating is monotonic, a soft-selection scenario, in which each host plant associates with a single rhizobium genotype and rhizobium fitness is globally regulated, would project declining rhizobium cooperation with each generation, which could eventually break down the mutualism.

We detected significant selection on a rhizobium genotype's contribution to both seed size and seed number, but only seed size exhibited significant genetic variation. Thus, continued selection on contribution to seed size, but not seed number, could cause evolution of rhizobium population composition. Significant selection on seed number suggests that very small differences in symbiotic investment in host seed number produce strong differences in rhizobium fitness. Low symbiont genetic variation for investment in seed number could reflect a 'ghost of selection's past' (Frederickson 2013), whereby purifying selection against cheating has reduced standing genetic variation for the trait.

No selection for legume cheating

We found no evidence of selection on cheating by the host plant, despite abundant variation among plant genotypes for both plant and rhizobium fitness components. Thus, host plants do not maximise their fitness by contributing less to symbionts, but neither do they benefit from contributing more to their symbionts. This finding contradicts models of asymmetric mutualisms (Johnstone & Bshary 2002; Frean & Abraham 2004). While the absence of vertical co-transmission would favour less-cooperative strategies in both host and symbiont (Bull & Rice 1991; Denison et al. 2003; Sachs et al. 2004), other factors might asymmetrically disfavour cooperation in symbionts, but not hosts. First, if each symbiont associates with a single host, whereas a host associates with multiple symbionts, symbionts evolving under natural conditions could experience a more dramatic tragedy of the commons, whereby resources provided to a host by cooperative symbionts could indirectly aid less-cooperative, competing, 'free-riding' symbionts (Denison et al. 2003; Foster & Kokko 2006). Second, symbiosis may be less costly to hosts than to symbionts (Rutter & Rausher 2004). Thus, a host's allocation to symbiont fitness might not trade-off with its own fitness. For example, plant photosynthates allocated to symbionts may not be costly if photosynthesis is sink-limited (Douglas 2008). Third, temporal differences in symbiotic benefit might make cheating more costly for hosts (Douglas 2008). While rhizobia produce many progeny early in the symbiosis during nodule formation, host plants do not set seed until later. Thus, rhizobia that provide low benefits to a host could still benefit from the interaction if the host dies before reproducing, whereas a host that does not support its symbionts may fail to reproduce.

Finally, experimental design might have obscured selection for cheating in hosts. For example, hosts may not cheat until the end of the relationship, when they might kill rhizobia and recover resources invested in them (West et al. 2002). Future experiments could detect such cheating by measuring density of rhizobium released to the soil. Plant cheating might also be revealed by measuring other rhizobium traits, for example storage compounds such as PHB and rhizopines, which enhance future free-living rhizobial survival (Kiers & Denison 2008).

Physical environment and selection on cooperation

Despite dramatic shifts in soil nitrogen availability and toxicity across soil environments, which strongly affected mean fitness of both plants and rhizobia, patterns of selection on cheating by hosts and symbionts were remarkably similar between environments, which suggests broad relevance across the ecological range of the mutualism. This finding supports a recent assertion that environmental context may only weakly affect patterns of host-symbiont fitness alignment in costly, horizontally acquired mutualisms (Chamberlain & Holland 2009), despite the potential for conditionality (Bronstein 1994).

Consonant with mutualism theory (Hochberg et al. 2000; Neuhauser & Fargione 2004), plants gained an order of magnitude greater fitness benefit from rhizobia in the harsher serpentine soil environment. Neither harsh nor benign soil contexts shifted the interaction away from one of mutual benefit, as observed previously (Porter et al. 2011).

CONCLUSIONS

By analysing cheating as a continuous trait, we reveal an important role for antagonistic selection in mutualism evolution. Empirical evidence that pleiotropic costs of cooperation select for symbiont cheating supports a fundamental assumption in mutualism theory. Further, standing variation along the cooperating-cheating continuum within a population of cooperative symbionts suggests that host mechanisms that oppose cheating are effective yet imperfect (Heath & Stinchcombe 2013). Determining whether selection favours cheating in other mutualisms would improve understanding of the role of natural selection in maintaining cooperation in mutualisms (Frederickson 2013).

STATEMENT OF AUTHORSHIP

SSP designed the study and performed the research. SSP and ELS analysed the data and SSP wrote the first draft of the manuscript and ELS contributed substantially to revisions.

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