penalize rhizobia that fail to fix N₂ inside their root nodules. We prevented a normally mutualistic rhizobium strain from cooperating (fixing N₂) by replacing air with an N₂-free atmosphere (Ar:O₂). A series of experiments at three spatial scales (whole plants, half root systems and individual nodules) demonstrated that forcing non-cooperation (analogous to cheating) decreased the reproductive success of rhizobia by about 50%. Non-invasive monitoring implicated decreased O₂ supply as a possible mechanism for sanctions against cheating rhizobia. More generally, such sanctions by one or both partners may be important in stabilizing a wide range of mutualistic symbioses.

Mutually beneficial symbiotic relationships between species are ubiquitous, but their evolutionary persistence is puzzling in many cases. If each individual plant or animal host is infected by a single symbiont lineage, then the host and symbiont have a shared interest that may favour cooperation. This is especially so if the symbiota are transmitted vertically, from parent to offspring. However, many mutualisms involve multiple symbiont genotypes per individual host and horizontal transmission of symbionts among unrelated host individuals. In this case, each symbiont lineage is selected to increase its own growth and fitness selfishly, at the expense of its host and the other lineages. This is the classic Tragedy of the Commons problem, common to economic and social theory. The tragedy is that while the symbionts as a group could obtain more resources from their host with prudent cooperation, this is not evolutionarily stable because each symbiont lineage gains by selfishly pursuing its own short-term interests.

One possible solution is selection imposed by hosts rewarding

\[ \hat{\beta} = \frac{0.023, \hat{\gamma} = 0.005, \hat{\delta} = 0.001, \hat{\theta} = 0.001} \]

Host sanctions and the legume–rhizobium mutualism

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Explaining mututalistic cooperation between species remains one of the greatest problems for evolutionary biology. Why do symbionts provide costly services to a host, indirectly benefiting competitors sharing the same individual host? Host monitoring of symbiont performance and the imposition of sanctions on ‘cheats’ could stabilize mutualism. Here we show that soybeans

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**Figure 1** Rhizobia fixing N₂ grow to larger numbers in whole-plant and split-root experiments. Rhizobia allowed (N₂:O₂) or prevented (Ar:O₂) from fixing N₂ by experimental manipulation of atmosphere at the whole-plant (a, b) or split-root (c, d) level were counted (antibiotic media), from nodules (a, c), on the root surface (b, d) and in the surrounding sand (b) or water (d). Counts from water (d) were multiplied by ten for scaling. Significant differences by ANOVA or paired t-test: a, P < 0.005, N = 11 pairs; b, root fraction, P < 0.01, and sand fraction, P < 0.01, N = 11; c, P < 0.001, N = 12 plants; d, root fraction, P = 0.24, and water fraction, P < 0.01, N = 12.
Rhizobia fixing N₂ grew to larger numbers in the single-nodule experiment. Figure 2

fixation. Rhizobia vary greatly in the benefits they provide to cooperation or punishing less cooperative behaviour. Some mutualisms may be stabilized by the tendency of the root nodules of their host legume plants. N₂ fixation is clearly beneficial to the host plant, because it supplies nitrogen needed for growth and photosynthesis. But N₂ fixation (at rates that greatly exceed the nitrogen needs of rhizobia) is energetically costly to the bacteria, and hence reduces the resources that could be allocated to their own growth and reproduction. A single legume plant is typically infected by several different bacterial lineages, creating a potential tragedy of the commons. Consequently, if plants treat fixing and non-fixing nodules similarly (that is, no sanctions), natural selection will favour rhizobia that invest very little in N₂ fixation. Rhizobia vary greatly in the benefits they provide to legumes. Strains that fix little or no N₂ after they form root nodules on legumes are common in some soils. Given the cost of N₂ fixation, why haven’t these cheats completely displaced non-fixing rhizobia from infecting their roots?

The legume–rhizobium system offers exceptional opportunities to test the sanctions hypothesis. We can force rhizobia to cheat by replacing air (N₂:O₂, 80:20 v/v) with a gas mixture (Ar:O₂, 80:20 v/v) containing only traces of N₂ (about 0.03% v/v). We estimate that this treatment reduces N₂ fixation to about 1% of normal, based on a Kₘ (half-saturation N₂ concentration) of about 3% [17]. This method allows precise control of when and where rhizobia fix N₂ without possible confounding effects associated with non-fixing strains. We used this method with soybean (Glycine max) and its symbiont Bradyrhizobium japonicum. These rhizobia are often mutualistic, but ‘ineffective strains’, which take plant resources but fix little or no N₂, are widespread. We forced rhizobial cheating in: (1) whole plants; (2) one-half of the root system; or (3) individual nodules. In each case, we imposed cheating by exposing target nodules to a nearly N₂-free atmosphere and exposed control nodules to air. In the absence of sanctions, we would expect rhizobia fixing little N₂ to direct more resources to their own growth and reproduction. In contrast, if host plants detect the near-cessation of N₂ fixation and apply effective sanctions, then we would predict greater growth and reproduction in the rhizobia allowed to fix N₂ normally.

As predicted by the sanctions hypothesis, forcing rhizobia to cheat by preventing N₂ fixation led to a significant decrease in their fitness. N₂-fixing rhizobia consistently grew to larger numbers than non-fixing rhizobia in nodules, whether cheating was forced at the plant (Fig. 1a), half-root (Fig. 1c), or nodule level (Fig. 2). In addition, there was a twofold difference (after one plant generation) in release of rhizobia into surrounding sand (Fig. 1b) or nutrient solution (Fig. 1d). Furthermore, rhizobia that had fixed N₂ in nodules had greater survival in sand over five months than rhizobia from the non-fixing treatment (paired t-test, P < 0.01, N = 12).

The decrease in fitness of the non-fixing rhizobia was associated with a decrease in resource allocation to non-fixing nodules by host plants, as indicated by nodule mass. In experiments where rhizobia were forced to cheat at the half-root or individual-nodule level, each host plant had both fixing and non-fixing nodules, allowing selective partitioning of resources by the host plant. Consistent with the sanctions hypothesis, final nodule fresh weight was higher

<table>
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<tr>
<th>O₂ permeability (% initial)</th>
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<tr>
<td>Ar:O₂</td>
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<tr>
<td>N₂:O₂</td>
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Figure 3 O₂ relations in single nodules where rhizobia were allowed to fix (N₂:O₂) or prevented from fixing (Ar:O₂) N₂. Within 48 h, non-fixing nodules had significantly lower nodule interior O₂ concentration under 20% O₂, as calculated from leghaemoglobin oxygenation (paired t-test, P < 0.001, N = 6), and significantly lower O₂ permeability (paired t-test, P < 0.05, N = 6), relative to controls. Data are presented as % of initial concentration to standardize for any initial differences. A correction for increasing nodule size in controls would have further increased permeability differences between the treatments.
in N₂-fixing nodules, both in the split-root experiment (paired t-test, \( P < 0.001, N = 12 \)) and in the single-nodule experiment (paired t-test, \( P < 0.05, N = 6 \)). In addition, root dry weights were higher on the N₂-fixing side in the split-root experiment (paired t-test, \( P < 0.05, N = 12 \)). These results demonstrate how differences in resource allocation \(^{15,16} \) at the nodule level are linked to differences in rhizobial fitness.

What is the mechanism by which these sanctions are carried out? Host plants could impose sanctions on non-fixing nodules by attacking rhizobia directly or by decreasing the supply of any resource required for growth \(^{8,9} \). It appears that a decrease in O₂ supply may be the primary mechanism. Nodule interior O₂ concentration and nodule O₂ permeability were both lower in non-fixing nodules within 48 h of the initiation of the experiment (Fig. 3). This decrease in nodule interior O₂ concentration, previously seen in whole-plant experiments \(^{20} \), is the opposite of what would have happened if photosynthate supply had decreased enough to limit respiration in the nodule interior. A lack of significant differences between treatments in O₂-saturated respiration rate (paired t-test, \( P = 0.47, N = 6 \)) also indicated that photosynthate supply did not limit respiration more in non-fixing nodules. Nodule O₂ permeability responds to various conditions that affect nitrogen supply and demand \(^{15,16} \), but responses to soil nitrogen are in the opposite direction (that is, greater O₂ permeability when less nitrogen is available) \(^{21} \) from the response we found to differences in N₂ fixation. Our results therefore appear to be a specific response to rhizobial defection.

A key assumption in these experiments is that nitrogen supply is unlikely to limit the growth or reproduction of rhizobia in non-fixing nodules directly. Much of the nitrogen needed for nodule growth is imported from the phloem, even in nodules that are exporting much larger quantities of nitrogen to the xylem \(^{22} \). Even under the conservative assumption that plants force complete nitrogen autonomy on rhizobia in non-fixing nodules, we still estimate that even 1% of the N₂ fixation rate in air would provide enough nitrogen to prevent any direct limitation on rhizobial growth. Specifically, if we assume an N₂ fixation rate in air of 2.6 mg N per g of dry weight of nodule per h (0.276 mmol H₂ and 3 mol H₂ per mol N₂) \(^{22,23} \) and 2.5 mg bacteroid N per dry weight of nodule (15 mg bacteroid protein and 6 mg protein per mg N) \(^{22} \), the bacteroids (the differentiated, N₂-fixing form of rhizobia) in nodules exposed to air would fix enough nitrogen to double in less than an hour. Even at only 1% of the N₂-fixation rate in air, bacteroids in the Ar:O₂ treatment would fix enough nitrogen to have quadrupled their numbers during the 240 h duration of our single-nodule experiments.

The nodules in our experiment contained only one strain of rhizobia. Mixed nodules can occur, but there is little information about their frequency under field conditions. The potential tragedy of the commons that results from multiple strains per host \(^{12} \) could also apply to mixed individual nodules. Mixed nodules might reduce the evolutionary effects of nodule-level sanctions if cheats sharing a nodule with mutualists are somewhat protected from nodule-level sanctions. The sanctions reported here are less severe than the flower abortion seen in some yuccas \(^{11} \). If rhizobial cheats accumulate more resources than mutualists in the same nodule, as seen by electron microscopy \(^{16} \), this could perhaps explain the persistence of cheats, despite the fitness cost of cheating in single-strain nodules.

Sanctions directed at specific bacteroids within nodules could be effective in mixed nodules, but only in species in which bacteroids retain the ability to reproduce. Ironically, the most recent evidence for sanctions against bacteroids comes from pea nodules \(^{26} \). In contrast to soybean nodules, bacteroids in pea nodules leave no descendants \(^{6,27,28} \), so denying them resources would have no direct effect on the evolutionary maintenance of cooperation. Only undifferentiated rhizobia, which never fixed N₂, escape into the soil after pea nodules senesce (Fig. 4). Whole-nodule sanctions, such as cutting off O₂ supply, could affect the survival and reproduction of all rhizobia in the nodule interior. This would impose selection on whichever form is reproductive, and therefore central to the evolution of a given species.

Our results support the hypothesis that legumes select for more cooperative rhizobia by imposing sanctions on the basis of the amount of N₂ that rhizobia fix once established inside nodules. The hypothesis that host sanctions could lead to the evolutionary stabilization of the legume–rhizobium mutualism has been shown previously to be theoretically robust \(^{8,9} \). More generally, sanctions are one way in which the host can control the resource environment of their symbiont, and hence impose a selective environment that favours cooperative behaviour. Mechanisms that can do this, such as sanctions and other more indirect methods \(^{4,5} \), could be important in stabilizing a wide range of mutualistic symbioses. This is because they can favour cooperation when cooperation is otherwise hardest to explain: when there are many symbiont strains per host and there is horizontal symbiont transmission among unrelated host individuals \(^{4,5} \).

**Methods**

We used an Ar:O₂ atmosphere with only traces of N₂ (about 0.03%, by mass spectrometry) to mimic rhizobial cheats that suddenly stop fixing N₂. In future experiments, we could alter the timing and composition of gas treatments to simulate rhizobia with different fixation patterns (for example, fixing N₂ at 25% of potential).

**Whole-plant experiment**

Seeds of a dwarf cultivar of soybean (Glycine max; cv. T243, Strain PI 548224, USDA Soybean Germplasm Collection) were sterilized, germinated and planted into autoclaved 700 ml chambers made from stacked Magenta GA-7 culture boxes filled with quartz sand. An air-driven pump recirculated sterile N-free nutrient solution in each chamber. Bradyrhizobium japonicum strain USDA 110 ARS was injected into the sand at the base of each seeding, 7 d after planting. Plants were grown with photosynthetically active radiation of 600 μE m⁻² s⁻¹ and 14 h photoperiod. Replicate plants were grouped into four blocks based on acetylene reduction estimates of initial nitrogenase activity and
randomly assigned to N2-fixing and non-fixing treatments. Either N2O2 or ArO2 was delivered through perforated plastic tubing 1 cm above the base, at 100 ml min−1. Three months after planting, nodules were removed from roots. Roots were cut, vortexed, and sonicated in a FS20 ‘watch-bath’ type sonicator in 0.01% Tween 20. The extractant was diluted 10-fold and spread on MAG antibiotic-containing plates. intact nodules were removed from roots, counted, weighed and crushed in a tissue homogenizer, diluted and plated. Sand fraction, which was homogenized for 30 min in a sterile flask containing sterile 0.01% Tween 20, on a flack rotator. A liquid subsample was removed from the sand mixture 3 cm below the water line, diluted by 10 and plated. Colonies grew for 10 d at 32°C and colony-forming units (c.f.u., mean of eight plates) were recorded.

**Split-root experiment**

Seeds of G. max semiwild variety ‘S0066’ were sterilized, germinated, and inoculated with approximately 107 cells per seedling. Twelve plants, each with two similar root halves (resulting from regrowth after root-tip removal), were transplanted to hydroponic chambers, with similar nodule numbers on each half of a chamber divided by a silicone gel seal. Chamber halves were randomized into two treatments, either N2O2 or ArO2 (80:20, v/v) at 130 ml min−1. 5-d after transplanting, H2 production was measured to confirm disruption of N2 fixation by ArO2. Five weeks after transplanting, roots, nodules and rhizobia in nutrient solution were processed as described above for the 12 replicates, each a paired comparison. For survival assays, nodule homogenate was diluted and added at an estimated 105 rhizobia per g sterile sand. Twenty weeks later, rhizobial populations were determined by plate counts.

**Single-nodule experiment**

Six independent replicate experiments used G. max ‘S0066’ grown in plastic growth pouches and inoculated as above. Fifteen days later, two nodules of equal size were selected per plant. Fixing and non-fixing treatments were randomized. Chambers of 2 cm diameter were positioned around intact nodules, with 250 ml min−1 of humidified N2O2 or ArO2 flowing through each chamber. Fractional oxygenation of leghaemoglobin under air, N2O2 permeability, and O2-saturated respiration rate were measured daily as previously described11,14. Briefly, nodules were exposed successively to 20, 0, 70 and 0% O2 while fractional oxygenation of the nodule protein leghaemoglobin was measured by non-invasive spectrophotometry. O2 permeability was calculated from the rate of increase in oxygenation after switching to 70% O2, after correcting for respiration, which was calculated from the rate of oxygen decrease as interior O2 fell from O2-saturated to O2-limited concentrations after switching to 0% O2. After 10 d, nodules were weighed, crushed, and assayed for c.f.u. per nodule and g of nodule. Analyses of variance and Tukey’s studentized range test for whole-root, and paired crushed, and assayed for c.f.u. per nodule and g of nodule. Analyses of variance and paired comparison. For survival assays, nodule homogenate was diluted and added at an estimated 105 rhizobia per g sterile sand. Twenty weeks later, rhizobial populations were determined by plate counts.

12. Burdon, J. J., Gibson, A. H., Searle, S. D., Woods, M. J. & Brockwell, J. Analysis of social interactions in the mouse, we performed a recessive ENU mutagenesis screen that determine the consequence of mutations in living organisms. Large-scale production of mouse mutations with the point mutation N-ethyl-N-nitosourea (ENU) is a key strategy for analysing the human genome because mouse mutants will reveal functions unique to mammals, and many may model human diseases. To examine genes considered conserved between human and mouse, we performed a recessive ENU mutagenesis screen that uses a balancer chromosome, inversion chromosome 11 (refs 4, 5). Initially identified in the fruitfly, balancer chromosomes are valuable genetic tools that allow the easy isolation of mutations on selected chromosomes. Here we show the isolation of 230 new recessive mouse mutations, 88 of which are on chromosome 11. This genetic strategy efficiently generates and maps mutations on a single chromosome, even as mutations throughout the genome are discovered. The mutations reveal new defects in haematopoiesis, craniofacial and cardiovascular development, and fertility.