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Measuring the fitness of symbiotic rhizobia

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Abstract The legume-rhizobia symbiosis is an important model system for research on the evolution of cooperation and conflict. A key strength of this system is that the fitness consequences of greater or lesser investment in cooperative behaviors can be measured for each partner. Most empirical studies have characterized the fitness of symbiotic rhizobia exclusively by their numbers within nodules, often estimated using nodule size as a proxy. Here we show that the relationship between nodule size and rhizobial numbers can differ drastically between strains of the same species. We further show that differences in accumulation of the storage polyester poly-3-hydroxybutyrate (PHB), which can support future reproduction, can be large enough that even direct measurements of rhizobial numbers alone can lead to qualitatively incorrect conclusions. Both results come from a comparison of strains differing in production of the ethylene-inhibitor rhizobitoxine (Rtx). A broader study (using three legume-rhizobia species pairs) showed that PHB/cell cannot be reliably estimated from its correlation with rhizobia/nodule or nodule size. Differences in PHB between strains or treatments will not always make major contributions to differences in fitness, but situation-specific data are needed before PHB can safely be neglected.

Keywords Cooperation · Symbiosis · Evolutionary stability · Cheating · Poly-β-hydroxybutyrate · Rhizobitoxine · Offspring quality

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1 Introduction

Rhizobia are soil bacteria that fix nitrogen inside legume root nodules. This symbiosis is an important source of nitrogen for both natural and agricultural ecosystems. It is also a premier model system for understanding the mechanisms responsible for the evolutionary persistence of cooperation, despite conflicts of interest among partners. Key to the value of this symbiosis as a model system is the fact both legume and rhizobial fitness are readily measured. Legume fitness is typically estimated by measuring seed biomass (Kiers et al. 2007), plant biomass (Ratcliff and Denison 2009), or both (Heath and Tiffin 2007). The effect of symbiosis on rhizobial fitness is typically estimated by counting or estimating rhizobial reproduction within legume nodules (Kiers et al. 2003; Simms et al. 2006; Heath and Tiffin 2007; Gubry-Rangin et al. 2010; Sachs et al. 2010). Nodule mass has often been used as a proxy for rhizobia/nodule, but Oono et al. (2009) warned that correlations between nodule mass and rhizobia per nodule might differ among strains.

Furthermore, changes in soil populations over years may depend on not only on the numbers of rhizobia released from nodules, but on resources rhizobial cells have acquired there which affect subsequent survival and reproduction (Denison and Kiers 2011). Nonetheless, estimates of rhizobial fitness typically ignore poly-3-hydroxybutyurate (PHB), a storage polyester which rhizobia can accumulate to more than 50% of cell dry weight (Bergersen et al. 1995; Tavernier et al. 1997). In the absence of external carbon, stored PHB can support the production up to three offspring per cell (Ratcliff et al. 2008). Two nodules containing the same number of rhizobia could thus differ up to three-fold in their contribution to soil populations.

If multiple traits contribute to fitness, then measuring just one of them can result in biased fitness measurements,

especially if there are negative correlations among traits. For example, among plants, finite resources result in a trade-off between seed size and seed number (Henery and Westoby 2001). Greater seedling establishment and survival of large-seeded plants can counterbalance their reduced fecundity (Moles and Westoby 2004), so resources per seed are key to fitness estimates. Similarly, the finite supply of plant-derived carbon available to symbiotic rhizobia may result in a trade-off between rhizobial reproduction and PHB synthesis, two processes which compete for the same resources. Strains of rhizobia that allocate more carbon to storing PHB for future reproduction and less to reproduction within nodules might thus falsely be deemed less fit than strains that pursue the opposite strategy, if the only fitness component measured was rhizobia per nodule. Genetic variation exists for PHB accumulation (Bergersen et al. 1995: Tavernier et al. 1997; Mercan 2002), but tradeoffs between PHB accumulation on symbiotic reproduction have not been quantified.

We first assessed the consistency, across strains of the same rhizobial species, of correlations between nodule mass and rhizobia per nodule. We used *Bradyrhizobium elkanii* strain USDA61, a natural isolate that produces the ethylene inhibitor rhizobitoxine (Rtx), and its isogenic Rtx(−) mutant, using Siratro (*Macroptilium atropurpureum* (DC) Urb.) as the host plant. We previously found that the Rtx strain reduces host growth relative to the mutant (Ratcliff and Denison 2009).

In assessing the importance of adding PHB measurements to the usual estimates of rhizobial numbers, we consider three possibilities. First, PHB/cell could safely be ignored if (in a given experiment) it were constant across strains and treatments, or not great enough in any strain or treatment to support significant rhizobial survival or reproduction. Second, if PHB/cell had some consistent positive correlation with rhizobia/nodule, then treatments or strains with more rhizobia/nodule could reliably be characterized as having greater fitness than those with fewer rhizobia/nodule. However, fitness differences would be greater than calculated from rhizobia/nodule alone, so we would still need enough paired measurements of PHB and rhizobial numbers to determine the slope of the correlation between them. Finally, if rhizobia/nodule and PHB/cell vary independently or are negatively correlated due to tradeoffs, and PHB levels are not negligible, then rhizobial fitness assays need to measure both parameters.

To determine if variation in PHB accumulation significantly affects fitness, we again used the Rtx(+) and isogenic Rtx(−) strains of *Bradyrhizobium elkanii* USDA61. We previously found that the Rtx strain accumulates nearly twice as much PHB/cell (Ratcliff and Denison 2009), but the biological significance of this difference has yet to be determined. We also assessed the second reason that measuring PHB/cell might not be necessary, namely, the hypothesis that PHB/cell can be estimated from correlations with rhizobia/nodule or nodule mass. These experiments used three legume species: siratro, alfalfa (*Medicago sativa* L.), and common bean (*Phaseolus vulgaris* L.), along with their associated rhizobia.

2 Materials and methods

Plants were grown from surface-sterilized seeds in hydroponic growth pouches in modified N-free Fahraeus nutrient solution as previously reported (Ratcliff et al. 2008). Rhizobia for plant inoculation were grown to stationary phase in 50 mL TY broth in 125 mL Erlenmeyer flasks incubated at 22°C and shaken at 100 RPM.

To see whether correlations between rhizobia per nodule and nodule mass are consistent across strains, and to assess the contribution of PHB to rhizobial fitness, Siratro (cv. Siratro) was inoculated with either rhizobitoxine-producing Rtx(+) *Bradyrhizobium elkanii* strain USDA61 or its isogenic Rtx(−) mutant or RX17E (Ruan and Peters 1992). After 6 weeks of growth, a total of 50 nodules were randomly harvested from five plants.

To see whether PHB/cell could be estimated from nodule size or rhizobia/nodule, we examined three legume-rhizobia species pairs. Alfalfa (cv. Rebound 5.0) was inoculated with *S. meliloti* 1021. Common bean (cv. Royal Burgundy) was inoculated with *Rhizobium etli* strain CE3. Siratro was inoculated with Rtx(−) *B. elkanii* strain RX17E. Five nodules were randomly harvested from each of three alfalfa plants at 7, 14, 21 and 28 days from nodule emergence (60 nodules total); each plant was sampled once. Sampling was the same for common bean, except that nodules were only removed 7 and 14 days from emergence (30 nodules total). Eight nodules were randomly harvested from six siratro plants after 84 days of growth. In all experiments, only nodules >1 mm in length were sampled.

Rhizobia were separated from each crushed nodule by centrifugation, and the number of rhizobia/nodule was determined by flow cytometry as described in Ratcliff et al. (2008). For nodules occupied by rhizobia with nonswollen bacteriods (*Bradyrhizobium* and *Rhizobium*), all rhizobia were counted. For nodules occupied by rhizobia with terminally differentiated bacteriods (*Sinorhizobium*), only nonswollen rhizobia (previously shown to be reproductively viable) were counted. Flow cytometric counts of nonswollen rhizobia have been shown to accurately measure colony forming units (CFU; Ratcliff et al. 2008). PHB content was determined by flow cytometry of Nile Red stained cells as described in Ratcliff et al. (2008).

To estimate the fitness contribution of PHB to *B. elkanii*, we added the estimated reproductive contribution of stored PHB to direct counts of rhizobia/nodule. The relationship between PHB/cell and PHB-supported reproduction has

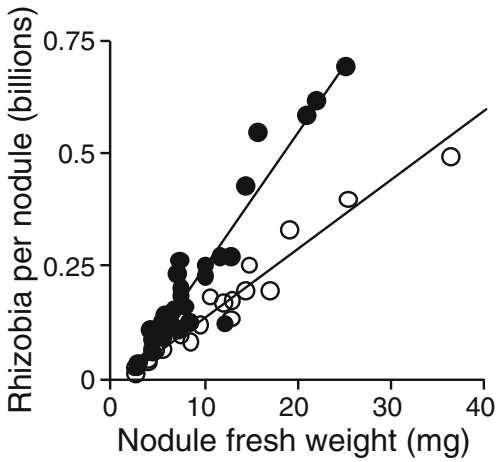

Fig. 1 Nodule weight is correlated with the number of rhizobia within, but this correlation is strain-dependent. For a single strain of rhizobia, nodule weight was highly correlated with the number of rhizobia inside. However, Rtx(−) rhizobia formed nodules that contained twice as many rhizobia per mg of nodule than did the Rtx(+) strain

been quantified in *S. meliloti* but not *B. elkanii*; in *S. meliloti* one picogram of PHB can support the production of 5.3 offspring (Ratcliff et al. 2008). For *B. elkanii* we assumed that stored PHB could be used to generate comparable cellular biomass during reproduction. But we would only expect the number of offspring produced per picogram of PHB to be similar if these two species have similar cell size. We therefore compared the dry weight of low-PHB cells of the two species by culturing cells in rich media (TY) for 3 days. Because the biomass per cell of *S. meliloti* and *B. elkanii* was not significantly different (t_{11}=0.68, p=0.53), we assumed that the reproductive contribution of PHB in *B. elkanii* was comparable to that of *S. meliloti*.

All statistics were calculated in JMP 9.0. ANCOVA, with the rhizobial strain (Rtx(+) or (−)) used as the cofactor, was used to test relationships between nodule weight and rhizobia/nodule and between rhizobia/nodule and PHB/rhizobia. Fitness of Rtx(+) and (−) strains and PHB/rhizobia was compared with two-sided t-tests that did not assume equal variance between treatment groups. The relationship between nodule mass, rhizobia/nodule and PHB/rhizobia under single strain inoculation was determined by simple linear regression. Assumptions of these parametric tests were checked and satisfied.

3 Results

Nodule weight was well-correlated with rhizobia/nodule for either Rtx(+) or Rtx(−) rhizobia, but this relationship was inconsistent between the two strains (Fig. 1, $F_{1,45}$=74.7, p< 0.0001, effect of interaction between strain and nodule weight on rhizobia/nodule, ANCOVA). The slope of rhizobia/nodule (billions) on nodule fresh weight (mg) for Rtx(−) rhizobia was 0.3, while the same slope for Rtx(+) rhizobia was only 0.15. In other words, a nodule containing Rtx(−) rhizobia has about twice as many rhizobia as a nodule of similar weight containing Rtx(+) rhizobia.

The average size of Rtx(+) nodules was larger, however, so the two strains did not differ in rhizobia/nodule. Based on numbers alone, therefore, Rtx(+) and (−) strains appear equally fit (Fig. 2a; t_{49}=0.75, p=0.23, t-test). Rtx(+) rhizobia, however, accumulated 3.1-fold more PHB within nodules (Fig. 2b; t_{49}=7.7, p<0.0001, t-test). When we include the estimated reproductive contribution of PHB in our fitness estimate, we find that the higher-PHB Rtx(+) strain has an estimated 2-fold fitness advantage (Fig 2a; t=2.3, n=49, p=0.03, t-test). If we measured only rhizobial numbers within nodules, we would have incorrectly concluded that rhizobitoxine has no effect on rhizobial fitness.

Despite the possibility that PHB can make major contributions to rhizobial fitness, direct measurement of PHB per

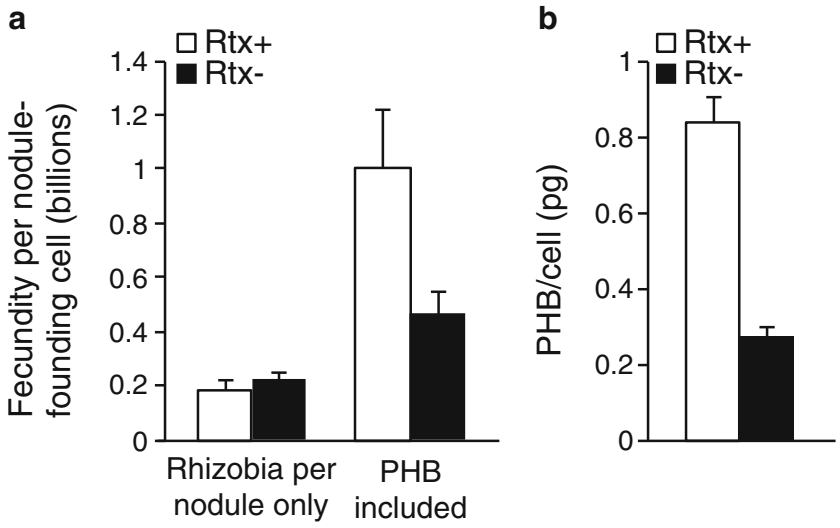

Fig. 2 Accurate estimates of rhizobial fitness require inclusion of the storage resource poly-3-hydroxybutyrate (PHB). **a** Both Rtx(+) and (−) rhizobia founding nodules left similar numbers of descendents, but **b** Rtx(+) rhizobia accumulated more than 3-times as much PHB. While symbiotic reproduction was similar, we estimate that the Rtx(+) strain has a 2-fold fitness advantage when the predicted reproductive benefit of PHB is added to direct counts of cells per nodule. Shown are means ± SEM

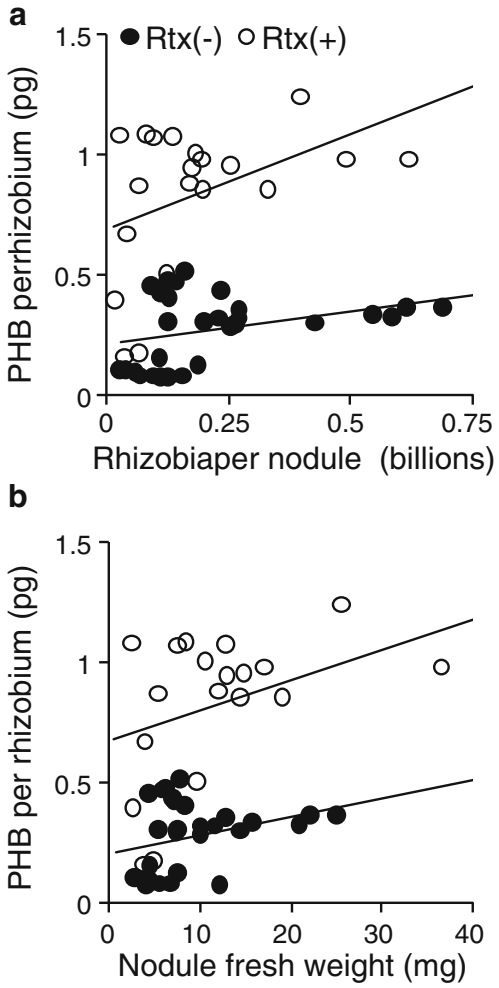

Fig. 3 PHB/cell is not predicted by rhizobia per nodule or nodule weight. **a** PHB content was poorly correlated with rhizobia/nodule or **b** nodule fresh weight. The rhizobitoxine-producing (Rtx+) strain of *Bradyrhizobium elkanii* reproduced less within nodules, per gram of nodule weight, than the otherwise isogenic Rtx (−) strain. Rtx(+) rhizobia, however, accumulated more PHB during symbiosis (**a**, **b**)

rhizobial cell will only be necessary if rhizobial PHB content cannot be accurately predicted from other nodule traits, such as rhizobia/nodule or nodule weight. Among nodules formed by the same rhizobial strain, we see a slight positive correlation between rhizobia/nodule and PHB/cell (Fig. 3a; $F_{1,45}$= 9.5, p=0.006 main effect of rhizobia/nodule on PHB/cell, ANCOVA). But rhizobia/nodule and nodule weight were poor predictors of PHB accumulation, explaining only 6% and 4.3% of the total variation in PHB/cell, respectively (Fig. 3a and b). These low correlations were found in all three legume-rhizobia species pairs. Reproductive (undifferentiated) rhizobia within common bean, siratro and alfalfa nodules accumulated PHB, in amounts that were poorly correlated with nodule weight and uncorrelated (all p>0.2; Table 1) with direct counts of rhizobia/nodule. Because of these poor correlations between PHB/cell and frequently measured nodule traits, it will often be necessary to measure both rhizobia/nodule and PHB/cell directly when estimating rhizobial fitness.

4 Discussion

Rhizobitoxine-producing and non-producing rhizobia provide a striking example of the importance of measuring both rhizobia/nodule and rhizobial offspring quality (particularly PHB/cell) for correct evolutionary inference as neither can be reliably estimated from nodule mass. Rtx(+) rhizobia support one-third less host growth, but display similar nodulation competitiveness (Ratcliff and Denison 2009), relative to an otherwise isogenic Rtx(−) mutant. There were twice as many Rtx(−) rhizobia as Rtx(+) rhizobia per gram of nodule. Therefore, using nodule mass as a proxy for rhizobial numbers would be inappropriate for comparisons between strains, despite the good within-strain correlation.

Even with an accurate estimate of the number of rhizobia within nodules, failure to measure PHB/cell would cause us to underestimate the fitness advantage that the Rtx(+) strain gains in nodules. This would lead to the incorrect conclusion that production of rhizobitoxine is not beneficial to the rhizobia (Fig. 2a). If that were so, then the reduced host benefit from this strain would not be considered 'cheating' (West et al. 2007). When PHB is included in our fitness

Table 1 Within-strain relationship between nodule mass, rhizobial population size and PHB/rhizobia

Species pair	Cell fraction analyzed	Mean PHB cell^{-1} (pg)	Range (pg)	Regression of rhizobia per nodule on nodule mass (g)	Regression of PHB cell^{-1} (pg) on nodule mass (g)	Regression of PHB cell^{-1} (pg) on rhizobia per nodule
Common bean	All	0.38	0.19–0.63	$y=3.3\cdot10^{9}x+1.1\cdot10^{6}$*	$y=29x+0.26$§	$y=3.6\cdot10^{-9}x+0.33$†
R. etli				$r^2=0.75$	$r^2=0.24$	$r^2=0.06$
Siratro	All	0.29	0.04–0.53	$y=2.7\cdot10^{10}x+1.2\cdot10^{8}$*	$y=-0.9x+0.3$	$y=-4\cdot10^{-11}x+0.32$
B. elkanii				$r^2=0.74$	$r^2=0.01$	$r^2=0.03$
Alfalfa	Un-differentiated	0.071	0.04–0.2	$y=1.8\cdot10^{8}x+2.7\cdot10^{5}$*	$y=-0.46x+0.07$	$y=-6\cdot10^{-10}x+0.07$
S. meliloti				$r^2=0.82$	$r^2=0.01$	$r^2=0.02$

*p<0.0001, §p<0.01

† The regression is significant (p=0.033), but removal of a single outlier results in a loss of significance (y=x+0.3, r^2 =0.035, p=0.11)

estimate, however, we find that Rtx-producing rhizobia have a 2-fold fitness advantage (Fig. 2a), and are thus cheating, benefiting from an action that is costly to the host and other rhizobia that share the host.

Rhizobitoxine inhibits 1-aminocyclopropane-1-carboxylic acid synthase, a rate-limiting step in legume ethylene biosynthesis (Yasuta et al. 1999). Ethylene is a negative regulator of nodulation in many legumes (Sugawara et al. 2006), and Rtx-producing rhizobia have been shown to increase the total number of nodules that legumes form (Duodu et al. 1999; Okazaki et al. 2003; Yuhashi et al. 2000). However, causing plants to form additional nodules will increase the relative fitness of the Rtx(+) strain only if Rtx(+) rhizobia preferentially occupy these additional nodules. If the additional nodules are equally available to Rtx(−) competitors, then an increase in total nodulation will not lead to any evolutionary increase in the relative frequency of Rtx(+) rhizobia. Rhizobitoxine somehow increases the amount of PHB synthesized by symbiotic rhizobia, a benefit that is specific to individual nodules and increases rhizobial fitness even when multiple strains infect a single host (Ratcliff and Denison 2009). While the mechanistic basis of Rtx-mediated PHB accumulation is currently unknown, the simplest hypothesis is that Rtx increases legume allocation of resources to the nodule. Alternatively, Rtx may interfere with host sanctioning (Kiers et al. 2003), allowing strains that fix less nitrogen to avoid punishment. Further experiments will be required to test these hypotheses.

The lack of a consistent correlation among nodule weight, rhizobia/nodule, and PHB/cell (Fig. 3a and b; Table 1) means that PHB must be directly quantified, not estimated. The extent to which natural selection among rhizobia is acting on within-nodule reproduction vs. PHB accumulation is unknown. However, the existence of complex traits like rhizobitoxine that increase PHB accumulation rather than rhizobial reproduction suggests that the balance of selection may, in some cases, favor increased PHB accumulation.

Chemical quantification of cellular PHB (e.g. Law and Slepecky 1961; Riis and Mai 1988) is relatively insensitive, requiring more rhizobia than any nodule in this study contained. Fortunately, flow-cytometric methods are far more rapid and sensitive than chemical methods of PHB analysis and are well-suited to high-throughput analysis of both rhizobial PHB and rhizobia/nodule (Ratcliff et al. 2008; Ratcliff and Denison 2009). This approach has the further advantage that, with most flow cytometers, both PHB/cell and rhizobia/nodule can be measured simultaneously.

By increasing survival and reproduction between hosts, PHB is of general importance to rhizobial fitness. There are, however, two conditions under which PHB accumulation would not contribute to fitness. PHB can be safely ignored if symbiotic rhizobia do not accumulate enough PHB to support significant reproduction or survival, or if carbon in senescing nodules, the bulk soil or the rhizosphere does not limit reproduction. While much remains to be learned about rhizobial ecology, sustained saprophytic reproduction is probably rare. Work by van Veen et al. (1997) has shown that carbon in the bulk soil often limits bacterial growth, but even in the comparatively carbon-rich rhizosphere, fluctuation in available resources and competition with other microbes may favor rhizobia with substantial PHB reserves. If there are situations where most rhizobia with PHB reserves below some threshold are unable to survive until the next nodulation opportunity, then differences among rhizobial strains in the number of rhizobia/nodule may sometimes be less important than differences in PHB/cell.

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