Resource hoarding facilitates cheating in the legume-rhizobia symbiosis and bet-hedging in the soil

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Dedication

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Abstract

The carbon that rhizobia in root nodules receive from their host powers both reproduction and the synthesis of the storage polyester poly3-hydroxybutyrate (PHB), as well as N_2 fixation, which mainly benefits the host. Rhizobia escaping nodules can use stored PHB to survive starvation and reproduce up to 3-fold, but PHB synthesis is energetically expensive and trades-off with N_2 fixation. As a result, PHB synthesis is a central mechanism in the evolution of conflict between rhizobia and legumes, and should be included in estimates of rhizobial fitness. Some rhizobia have evolved sophisticated mechanisms to increase PHB accumulation, such as the production of rhizobitoxine, a chemical inhibitor of legume ethylene synthesis. Rhizobitoxine reduces host growth, decreasing rhizobia per nodule for all strains on a plant, but substantially increases PHB accumulation for rhizobitoxine-producing rhizobia. In addition to enhancing reproduction, PHB has a role in bet-hedging: when starved, free-living high-PHB rhizobia divide asymmetrically, forming dormant, high-PHB 'persisters' that survive long-term starvation and antibiotic treatment, and low-PHB 'growers' that are sensitive to these stresses. *Sinorhizobium meliloti* integrates bet hedging and phenotypic plasticity, forming fewer high-PHB persister cells when low competitor density predicts shorter-term starvation. Declining populations may select for delayed reproduction when there is a trade-off between reproduction and longevity, as there is with starving *S. meliloti*.

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INTRODUCTION

> "The most important unanswered question in evolutionary biology, and more generally in the social sciences, is how cooperative behaviour evolved and can be maintained in human or other animal groups and societies" –Robert May, 2005 in his last presidential address to the Royal Society.

The "problem of cooperation" is not limited to cognitive animals capable of forming societies. Even simple cooperative acts, such as the production of an extracellular enzyme by a soil bacterium, can face potential destabilization by cheaters that benefit from this action without paying for it. Similar conflict among different symbiont genotypes interacting with a single host organism can result in evolutionary breakdown in mutualism. Classic mutualism theory thus predicts that cooperation will evolve and be stable when symbionts are genetically uniform within a host, have repeated interactions with potential partners, are inherited with a partner, or have limited options outside mutualism (Herre et al. 1999). With the possible exception of repeated interactions, the rhizobia/legume symbiosis satisfies none of these conditions. Rhizobia are capable saprophytes, form nodules after a single interaction with the host, and are dispersed through the soil independently from their host legume's seeds (Denison 2000). Further, multiple strains of rhizobia inhabiting the same legume plant can vary widely in the benefits they provide the host, from cooperators that fix substantial amounts of nitrogen to cheaters that fix little or no nitrogen (Burdon et al. 1999). Worse yet, an inherent conflict of interest exists: the more legume-supplied energy rhizobia use to fix N_2, the less they have for their own reproduction and growth (Denison 2000). So why haven't cheating rhizobia displaced more cooperative strains?

Host sanctions, the selective punishment of cheaters by the legume, may be important in maintaining mutualism by lowering the fitness of cheaters below that of cooperators. Kiers et al. (2003) forced otherwise cooperative rhizobia to cheat by replacing atmospheric nitrogen gas with argon. The legume limited the size of nodules containing cheaters, apparently by reducing O_2 supply to the nodule interior. Those nodules deprived of N_2 contained approximately one third as many rhizobia as nodules receiving N_2. This raises a new question: in the face of such effective sanctions, why are poor mutualists globally persistent (Erdman 1950; Ezedinma 1964; Burdon et al. 1999; Heath and Tiffin 2007)? A comprehensive understanding of the evolution of cooperation requires that we explain the apparently stable coexistence variation in symbiont benefit. This thesis works towards this goal by improving our understanding of evolutionary conflict between rhizobia and legumes by focusing on a specific mechanism: rhizobial synthesis of the polyester poly-3-hydroxybutyrate (PHB).

PHB is an energy-dense polyester accumulated by many bacteria, including rhizobia, often in excess of 50% cell dry weight (Bergersen and Turner 1990a; Tavernier et al. 1997). PHB synthesis and nitrogen fixation directly compete for the same pool of reductant, and as a result, tradeoff with each other (Anderson and Dawes 1990). If storing energy and carbon as PHB increases rhizobial fitness, then the interests of the legume and rhizobia will collide over PHB synthesis.

How large is the conflict between legumes and rhizobia over PHB accumulation? The following paragraphs attempt to gauge the potential benefit of PHB to rhizobia and the potential cost of its synthesis to legumes, and are intended only as a rough estimate. While there are many studies that measure rhizobial PHB accumulation in culture and in symbiosis, none measure PHB in ecologically useful units, such as PHB/cell. Instead, only the % PHB by dry weight or g PHB/g protein is reported. With the dual caveats that the following data uses a standard curve optimized for *Sinorhizobium meliloti* and may not be valid for other species of rhizobia, and was developed over a range of 0-0.35 pg PHB/cell (but has been shown to be linear up to ~36 pg/cell in other microbes (Kacmar et al., 2005), I provide some unpublished data from my research. The following are maximum PHB measurements for nodular rhizobia: 1.9 pg/cell in *Bradyrhizobium elkanii*, 1.05 pg/cell in *Bradyrhizobium japonicum*, 0.64 pg/cell in *Rhizobium etli*, and 0.25 pg PHB/cell in *S. meliloti.* If *B. elkanii* and *S. meliloti* use a similar amount of PHB to reproduce during starvation, then *B. elkanii* may be capable of storing enough PHB to increase its numbers up to 10-fold after nodule senescence. The benefit to rhizobia from PHB accumulation may indeed be large.

The cost of PHB synthesis to legumes will be a combination of the metabolic cost of synthesizing PHB, and the effect of traits linked to PHB synthesis. Bergersen and Turner (1990b) have estimated that for soybean (*Glycine max*), PHB synthesis by *B. japonicum* can consume the equivalent energy and carbon of two days worth of nitrogen fixation, a significant but modest resource draw. A more direct approach to assessing the effect of

PHB synthesis on legume fitness is through the use of knockout mutants. In common bean (*Phaseolous vulgaris*), plants inoculated with a PHB (-) mutant of *R. etli* produced about a third more seeds with about a third more nitrogen per seed (Cevallos et al., 1996), suggesting that the total cost of PHB synthesis to legume fitness is more substantial than the metabolic cost of PHB synthesis would suggest. In their experiment, PHB(-) rhizobia fixed nitrogen for a longer period of time, demonstrating that PHB synthesis can be linked to other rhizobial behaviors that have an effect on legume fitness.

It is unlikely that conflict over PHB accumulation will result in complete breakdown of mutualism. The benefit of fixing less N_2 and thereby accumulating more PHB should diminish as PHB/rhizobia saturates, resulting in weak selection for completely ineffective rhizobia. Instead, the conflict over PHB is more likely to result in the persistence of suboptimal symbionts and a profusion of coevolutionary adaptations that increase PHB synthesis (by rhizobia) and reduce PHB synthesis (by legumes).

In Chapter 1, I determine whether hoarding PHB can provide a fitness benefit to rhizobia. Like fat in mammals, PHB is most likely to be beneficial when exogenous carbon and energy are limiting. I thus used two independent methods to generate populations of genetically identical rhizobia that varied in PHB content, then starved these rhizobia and measured reproduction and survival over 165 days. Stored PHB linearly increased both reproduction (up to 3 fold) and survival (up to 150 days). Stored PHB thus increases rhizobial fitness, and can provide a chemical route through which cheating can evolve.

Because of the tradeoff between N_2 fixation and PHB accumulation, genotypes that fix less nitrogen may gain more PHB as a side-effect. Such simple cheating strategies, however, may easily be countered by legume sanctions. Successful cheating may require a more sophisticated approach. In Chapter 2, I examine the fitness consequences of rhizobial biochemical manipulation of the legume. Rhizobia that produce rhizobitoxine block the legume's production of the hormone ethylene. Relative to an isogenic, rhizobitoxine (-) mutant coinfecting the host, these rhizobia gained 47% more PHB. Because rhizobitoxine production increases PHB accumulation relative to rhizobitoxine (-) competitors, but as a consequence reduces the fitness of both the competing rhizobia and host, rhizobitoxine production can be considered cheating.

The legume-rhizobia symbiosis has become an important model system for research on the evolution of cooperation partly because the fitness of each symbiont is easily measured. However, widely-used methods for measuring rhizobial fitness are incomplete, typically estimating the number of rhizobia within a nodule but ignoring stored resources such as PHB. Fitness estimates could safely ignore PHB only if, in a particular system, rhizobia never accumulate enough PHB to have major effects on survival or reproduction, or if the amount accumulated can be predicted from other variables that are measured. In Chapter 3 I examine the relationship between rhizobial reproduction in nodules, their PHB accumulation, and nodule size (the most widely-used proxy for rhizobial fitness) in three legume-rhizobia species pairs. Nodule size was an

excellent predictor of rhizobial reproduction, but it was generally uncorrelated with PHB accumulation. I conclude that an accurate interpretation of the evolutionary dynamics of this system requires that future work consider PHB in fitness estimates of symbiotic rhizobia.

In Chapter 4, I show that rhizobia do not simply use PHB to reproduce and survive when starved, but rather use PHB to bet-hedge resource limitation. When starved, *Sinorhizobium meliloti* allocates PHB to its offspring asymmetrically, producing a low-PHB 'grower' cell primed for further reproduction if food becomes available soon, and a high-PHB 'persister' cell capable of surviving both long-term starvation and antibiotic exposure. This behavior may allow rhizobia escaping from senescing nodules to survive until the following year, while also producing offspring that are competitive for late-season nodulation opportunities. This work has significance beyond legume-rhizobia coevolution, as it is the first to document maternally-imposed bet-hedging in a microbe.

Reproductive timing is a key life history trait for many organisms. In Chapter 5, I examine the effect that changes in overall population size exert on selection for reproductive timing. When there is a tradeoff between reproduction and longevity, population decline favors delayed reproduction, even if the number of offspring produced is the same, because future offspring increase allele frequencies more than current offspring. For most organisms, short-term declines are about as common as short-term increases. Alleles that accelerate reproduction when the overall population is growing

and delay reproduction during population declines can thus be favored by natural selection. Reliable cues of population increase, such as the constant availability of high-quality food and cues linked to these resources (such as food odors) should therefore favor expression of physiological states that promote early reproduction, even at the expense of longevity. On the other hand, predictors of population decline, such as food limitation, crowding, heat stress, and the ingestion of toxins commonly found in "famine foods", should favor delayed reproduction and greater longevity. In the context of this thesis, this work provides evolutionary context for the benefit of high-PHB 'persister' rhizobia that delay reproduction during starvation. More broadly, it provides a single hypothesis that explains the congruent effects that environmental factors such as dietary restriction, food odors, and phytotoxins exert on reproduction and longevity in many different species.

In Chapter 6, I examine the effect of rhizobial cell density during starvation, an environmental cue predicting the duration of starvation, on bet hedging by *S. meliloti*. High competitor density inhibits use of PHB for reproduction, resulting in the production of a greater ratio of high to low-PHB cells. Further, rhizobia starved at higher density delayed using PHB for reproduction and survived longer during starvation. *S. meliloti* thus integrates phenotypic plasticity and bet hedging adaptively, hedging their bets against long-term starvation less when cues predict a shorter duration of starvation.

CHAPTER 1: POLY-3-HYDROXYBUTYRATE (PHB) SUPPORTS SURVIVAL AND REPRODUCTION IN STARVING RHIZOBIA

Summary

Rhizobia in root nodules benefit their host by fixing atmospheric N_2. In return they receive carbon, which they use to reproduce inside nodules. They also store energy in the polyester poly-3-hydroxybutyrate (PHB), which may enhance survival in the soil during the long interval between hosts. There can be a conflict of interest between rhizobia and legumes over the rate of PHB accumulation, due to a metabolic tradeoff between N_2 fixation and PHB accumulation. To quantify the benefits of PHB to rhizobia under carbon limitation, populations of genetically uniform rhizobia with high versus low PHB (confirmed by flow cytometry) were generated by fractionating *Sinorhizobium meliloti* via density gradient centrifugation, and also by harvesting cells at early versus late stationary phase. These rhizobia were starved for 165 days. PHB use during starvation was highly predictive of both initial reproduction and long-term population maintenance. Cultured *S. meliloti* accumulated enough PHB to triple their initial population size when starved, and to persist for ~150 days before the population fell below its initial value. We also determined *in planta* PHB accumulation in *S. meliloti* over 21 days and showed that PHB can reach levels that would support significant increases in reproduction and survival.

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Introduction

Rhizobia (*Sinorhizobium, Rhizobium, Bradyrhizobium, Mesorhizobium,* and *Azorhizobium*) are α-proteobacteria capable of symbiotically infecting legume roots. Legumes supply rhizobia in nodules with reduced carbon (Minchin and Pate 1973), which rhizobia respire to power fixation of atmospheric N_2 into NH_3. Symbiotic rhizobia may sequester some of this carbon in bacterial storage polymers, such as the polyester poly-3-hydroxybutyrate (PHB). PHB accumulation often exceeds 50% cell dry weight (Bergersen and Turner 1990a; Tavernier et al. 1997).

Various ways in which rhizobia might use PHB to benefit their plant hosts have been proposed, including respiration to protect nitrogenase from O_2-inactivation "until the last stages of seed development" (Bergersen et al. 1991) and fueling the differentiation of some rhizobia into larger bacteroids (Lodwig et al. 2005), which thereby lose the ability to reproduce (Denison 2000).

Alternatively, rhizobia might use PHB in ways that enhance their own fitness. PHB could be used to provide the energy and carbon required for bacterial reproduction or stress tolerance, perhaps after rhizobia have escaped senescing nodules into the soil. PHB is synthesized by many species of bacteria, where it has been shown to improve survival during starvation (Tal and Okon 1985; James et al. 1999; Kadouri et al. 2002), as well as improve tolerance to high temperatures, H_2O_2 exposure (Kadouri et al. 2003; Ruiz et al. 2004), UV-irradiation, desiccation, and osmotic stress (Kadouri et al. 2003).

Biochemically, PHB synthesis directly competes with N-fixation for reductant (Anderson and Dawes 1990). Thus, in theory, nitrogen-fixing rhizobia bacteroids face a tradeoff between nitrogen fixation and PHB accumulation. Physiological and mutagenesis studies support this hypothesis. Romanov et al. (1977, 1980) found a negative correlation between the rate of nitrogen fixation and PHB accumulation. Cevallos *et al.* (1996) and Peralta *et al.* (2004) demonstrated that a PHB (-) mutant of *Rhizobium etli* fixed significantly more nitrogen than the isogenic PHB (+) wildtype. Non-fixing *nifH* mutants of *Rhizobium etli* (Cermola et al. 2000) and *Bradyrhizobium japonicum* (Hahn and Studer 1986) have been shown to accumulate more PHB than their isogenic nitrogen-fixing parental strains.

The quantitative tradeoff between N_2-fixation and PHB accumulation is most direct in species of rhizobia where the N_2-fixing bacteroids themselves accumulate PHB. In some legume species, nodules contain both highly differentiated N_2-fixing bacteroids, which have lost the ability to reproduce, and also undifferentiated reproductive rhizobia. Only the latter typically accumulate PHB (Denison 2000). In these nodules, increasing N_2 fixation by bacteroids would potentially compete with PHB hoarding by reproductive rhizobia in the same nodule, but the tradeoff is less direct than when individual bacteroids must allocate available carbon between N_2 fixation and PHB accumulation. If PHB increases rhizobial fitness, increasing survival or reproduction inside senescing nodules or later in the soil, then PHB accumulation during symbiosis would serve as a pathway

for rhizobia to divert host carbon from nitrogen fixation into their own reproductive success. This would set up a conflict of interest between host and symbiont that could influence the coevolution of legumes and rhizobia.

Rhizobia with disrupted PHB synthesis genes have been shown to be less competitive for nodulation (Willis and Walker 1998; Aneja et al. 2005) and when free-living reproduce less under starvation (Cai et al. 2000; Povolo and Casella 2004) than wildtype cells. A complete absence of external carbon sources may not be required for PHB to provide a reproductive benefit. Cai *et al.* (2000) found that PHB synthase and depolymerase mutants of *S. meliloti* 1021 reproduced 7- and 5-fold when starved, respectively, while PHB (+) rhizobia reproduced to 9.7-fold their inoculum population size. The overall high reproduction in their experiments suggests that some exogenous carbon was present, but the increased reproduction of the wildtype rhizobia reflects the reproductive benefit of PHB accumulation.

While informative, these studies may confound the fitness consequences of energy and carbon storage in PHB with other effects of a functional PHB metabolism. Synthesis and degradation of PHB may improve rhizobial fitness by stabilizing cellular redox conditions and relieving TCA cycle inhibition under low oxygen conditions (Anderson and Dawes 1990; Dunn 1998; Poole and Allaway 2000). Furthermore, PHB synthase (*phbC)* knockout mutants are pleiotropic, exhibiting reduced growth on a variety of carbon sources (Cevallos et al. 1996; Lodwig et al. 2005; Wang et al. 2007a), and can

lack production of the extracellular polysaccharide succinoglycan (Aneja et al. 2004). These factors may reduce symbiotic (Leigh et al. 1985) and saprophytic fitness. As a result it is not known if PHB accumulation, per se, is responsible for the superior fitness of PHB(+) rhizobia. In the context of the legume-rhizobia symbiosis, it is the quantitative tradeoff between nitrogen fixation and PHB accumulation, not the mere ability to make PHB, that would result in evolutionary conflict. The existence of this conflict assumes that the relationship between PHB/cell and rhizobial fitness is positive, a hypothesis we test in this paper.

We generated phenotypic variation in PHB accumulation (measured by flow cytometry) among five genetically identical populations of *S. meliloti* using two independent methods. We then starved these cells and measured the effect of quantitative variation in cellular PHB accumulation on reproduction and long-term survival. To link these results to PHB levels in symbiotic rhizobia, we developed a flow-cytometric protocol for measuring PHB/cell in the reproductively viable (non-bacteroid) cell fraction from alfalfa nodules, and measured PHB accumulation by *S. meliloti* in symbiosis.

Materials and Methods

Starvation

Sinorhizobium meliloti 1021 populations with either high or low PHB accumulation were starved for 165 days to determine if PHB hoarding confers a fitness advantage when carbon is limiting. High- and low PHB-containing cells were generated by fractionating a

single population by buoyant density and by growing rhizobia to late (7 day incubation) and early (3 day) stationary phase (Patel and Gerson 1974) in M9 minimal media (Miller 1992) using 20g/L mannitol as the carbon source.

PHB accumulation increases bacterial buoyant density (Pedrós-Alió et al. 1985). Late stationary phase *S. meliloti* grown in M9 were suspended in 0.5 mL of 85% Percoll and 15% phosphate buffered saline (PBS) in sterile 6x50mm glass test tubes, sealed with Parafilm and centrifuged at 15,000 × *g* for 30 minutes in an Eppendorf 5415 D microcentrifuge. When centrifuged, Percoll forms a density gradient. Rhizobia separated on the density gradient were divided into three fractions of equal volume containing cells with high (1.138-1.119 g/mL), medium (1.119-1.109 g/mL) and low (≤1.109 g/mL) density. Marker beads (Amersham Biosciences, Sweden) were used to determine density.

Prior to starvation, all cells were double washed in carbon-free M9 media (containing no mannitol, 0.1 g/L thiamine, and HPLC-grade water). All glassware used for making C-free M9 was rendered C-free prior to use by acid washing in 0.6 M HCl for 1 hour, then pyrolizing at 550 °C overnight.

The initial population size (rhizobia/mL) was determined by dilution plating. Cells were diluted to $5 \cdot 10^5$ cells/mL in C-free M9; 1 mL aliquots were placed in sterile 2 mL microcentrifuge tubes and inverted daily to resuspend. Three replicate tubes were harvested at each time point and the number of viable rhizobia was determined by

plating. Rhizobia were fixed, resuspended in PBS + 20% glycerol, and stored at -80°C until the end of the experiment, when PHB/cell of all samples was determined by flow cytometry.

Plant growth

Alfalfa (*Medicago sativa* L. cv. Rebound 5.0, from C. Scheaffer, University of Minnesota), was grown in hydroponic growth pouches (Mega International. Minneapolis, MN). Plants were grown in a Conviron E7/2 growth chamber on a 16 hour photoperiod, light intensity of 500 μE m^{-2} s^{-1} photosynthetically active radiation, and a day/night temperature of 22/15 °C. Seedlings were inoculated with $5 \cdot 10^7$ cells/plant of *S. meliloti* strain 1021 (from M. Sadowsky, University of Minnesota). Five nodules were harvested from three plants at 7, 14, 21, and 28 days after emergence; each plant was only sampled once. All nodules were weighed and surface sterilized by a 10 second dip in 95% ethanol and five minute submersion in 3% bleach (.18% NaOCl). Nodules were washed five times in sterile DI water, and crushed in sodium ascorbate extraction buffer (Arrese-Igor et al. 1992). Nodule extracts were centrifuged at 100 × *g* for 10 minutes, and the supernatant was separated from nodule debris. Extracted rhizobia were pelleted at 5000 × *g* for five minutes, resuspended in starvation buffer (Wei and Bauer 1998), and stored at 4 °C.

Flow Cytometry

Generating PHB standards

High- and low-PHB containing *S. meliloti* were generated by culturing cells for 4, 8, 12, or 16 days with high or low gas exchange. $5 \bullet 10^7$ cells were inoculated into five replicate 125 mL Erlenmeyer flasks per treatment combination containing 50 mL yeast mannitol broth with 20 g mannitol/L (Somasegaran and Hoben 1994). Low gas exchange was imposed by covering the flask mouth with stretched sterile Parafilm and aluminum foil; Parafilm was omitted for high gas exchange. All flasks were harvested simultaneously. 40 mL of each sample was removed and centrifuged at 2,600 × *g* for 10 min. Pellets were double washed in DI water and dried for 2 days at 70 ºC in 13x100 mm screw-cap glass test tubes. Cellular PHB was determined by propanolysis and analysis following the protocol of Riis and Mai (1988) on an Agilent 6890N gas chromatograph with autosampler and HP-5 column (from J&W Scientific, 30 m, 0.32 mm ID, 0.25 μm film size). The remaining 10 mL of cells were fixed in 30% ethanol for 30 minutes and diluted to 10^7 cells/10 μL aliquot and stored at -80 °C in PBS +20% glycerol. Five samples were discarded due to contamination or PHB extraction errors.

Preparing cells for PHB analysis

Rhizobia were fixed and diluted to either $5 \bullet 10^5$ or $5 \bullet 10^6$ cells/mL (all cells in a run were diluted to the same concentration) in PBS and stained with Nile Red (NR), a fluorescent probe that binds PHB (Gorenflo et al. 1999). Diluted rhizobia were stained with 1% NR stock solution (100μg/mL in DMSO) for one hour, then pelleted at 5,000 × *g* for 5 minutes and resuspended in PBS to remove excess stain. Cells were analyzed on a

BentonDickson FACSCalibur, exciting with a 15mW 488nm argon laser. NR fluorescence was measured in channel FL2 (585+/-42 nm bandpass filter).

Determining PHB/cell through Nile Red staining

Background fluorescence from NR-stained bacteria is minimal (Kacmar et al. 2005). Thus, ten samples of *S. meliloti* cells of known PHB accumulation (by gas chromatography above) were used to generate a NR vs. PHB standard curve, by regressing geometric-mean NR fluorescence on mean PHB/cell (pg). These were stained and sampled under the same conditions as the cells run for PHB determination. PHB/cell was then determined by converting a sample's mean NR fluorescence to PHB/cell through the standard-curve regression function. A new standard curve was fit for each cytometry session that PHB was quantified.

Cell counts

Fixed rhizobia were diluted to between $5 \bullet 10^5$ to 10^7 cells/mL and data were acquired for 15 seconds on the flow cytometer. If the population of cells on the FSC x SSC (forward-by side-scattering) plot was overlapped by instrument noise, rhizobia were stained with 1% propodium iodide (PI) stock solution (1 mg/mL, in water) and incubated at least five minutes to label cells (specifically, DNA) before being rerun. Cell counts/mL (C) of the original samples was determined by:

$$C = F/(t \bullet R \bullet D)$$

where F is the number of cells acquired (after removing noise by gating using the FSC x SSC plot, or alternatively the number of cells fluorescing above background in channel FL3, if stained with PI), t is the time in seconds of data acquisition, R is the flow rate in mL/sec of the cytometer, and D is the dilution performed prior to staining. The flow rate (R) of the cytometer was determined by regression: four 12x75 mm FACS tubes were filled with ~1mL DI water and weighed with 0.1 mg precision. These were run for 1, 2, 3 and 4 minutes on the flow cytometer, and then weighed again to determine the volume removed. The slope of the linear regression for volume removed versus time in seconds was used for R. The flow rate was recalculated each session to correct for any variation.

To determine if the flow cytometer accurately counted cells, we diluted 60 samples of cultured *S. meliloti* to between $5 \cdot 10^5$ and $3.5 \cdot 10^7$ cells/mL, then determined rhizobial density in each culture by both drop plating on TY agar (Somasegaran and Hoben 1994) and flow cytometry. For flow analysis all cultures were diluted to $5 \cdot 10^5$ to $1 \cdot 10^7$ cells/mL.

Statistics

JMP 7.0 was used for all statistical analyses. Single-factor ANOVA and t-tests were used to determine if means varied significantly. Linear regression was used to describe the relationship between PHB accumulation and reproduction/survival. Assumptions of these parametric tests were checked and met for all data with the exception of PHB accumulation in alfalfa nodules, where the variance in PHB/cell increased with time.

Before analysis, these data were power transformed with $\lambda = -1$ to homogenize the variance. Tukey's HSD was used to determine if rhizobial populations fractionated by density contained significantly different amounts of PHB. In calculating the standard error of PHB used during starvation, we conservatively assume there was no covariance between initial PHB accumulation and PHB content after starvation.

Results

Generating rhizobia with high versus low PHB accumulation

S. meliloti cultured to late stationary phase accumulated an average of 0.50 pg PHB/cell, significantly more than the 0.30 pg PHB/cell accumulated by early stationary phase cells ($t=10.43$, d.f.=4, $p=.0004$. 2-sample t-test of NR fluorescence). When split by buoyant density, cells from the dense, medium and buoyant fractions accumulated 0.40, 0.30, and 0.18 pg PHB/cell, respectively ($F_{2,8}= 120.9$, $n=9$, $p<.0001$, single-factor ANOVA. All means significantly different at $\alpha=.01$).

Measuring PHB with the flow cytometer

PHB/cell by gas chromatography was highly correlated with Nile Red fluorescence ($p<.0001$, $r^2=.95$, Figure 1-1). The intercept for this regression was not significantly different from 0 ($t=.04$, d.f.=35, $p=.96$. 2-sided t-test), indicating that *S. meliloti* cells without PHB possess no detectable background fluorescence when stained with NR.

The effect of PHB hoarding on starving rhizobial fitness

We examined the extent to which PHB use during starvation correlated with the ability of *S. meliloti* to reproduce and persist during long-term starvation. When starved, cells from all treatments initially reproduced, with population sizes peaking after 29-36 days of starvation (Figure 1-2). Reproduction was measured as the difference between the maximum population size attained during starvation and the initial population size. Rhizobia using more PHB during starvation (a direct consequence of starting with more PHB) reproduced significantly more ($p=.003$, linear regression, Figure 1-3A).

S. meliloti consumed the majority of stored PHB in the first 29-36 days of starvation; after which all *S. meliloti* populations steadily decreased in size (Figure 1-2). Linear regressions of relative population size (viable cells on day n, divided by cells inoculated) versus days of starvation, during this population decline (days 29-36 through 165, $p<.0001$ for all treatments) were used to calculate the time elapsed before each treatment returned to its initial inoculum population size. Populations of *S. meliloti* with initially higher PHB persisted significantly longer than those with less PHB ($p=.0005$, linear regression, Figure 1-3B), with a threefold range in survival time over the 2.8-fold PHB range tested.

Identifying undifferentiated rhizobia in indeterminate nodules

Alfalfa nodules typically contained two *S. meliloti* populations that could be distinguished by flow cytometry because they varied >10 fold in forward scatter, indicating a large size difference (Shapiro 2003). A bimodal distribution in cell size was

confirmed by microscopy (data not shown). Only the small-celled population accumulated PHB, as measured by NR fluorescence (R_2 in Figure 1-4). PHB accumulation and small size are both consistent with these being the undifferentiated, reproductive rhizobia (Denison 2000; Lodwig et al. 2003). The correlation of viable cells/nodule with PHB-containing cells further supports this conclusion. Linear regression of flow-cytometric counts onto plate counts had a slope near one and an intercept near zero for both cultured *S. meliloti* and PHB-containing *S. meliloti* from alfalfa nodules (Table 1-1). However, cytometric counts of total rhizobia (including those without PHB, assumed to be bacteroids) significantly overestimated viable rhizobia numbers (Table 1-1).

PHB accumulation by undifferentiated rhizobia

Symbiotic *S. meliloti* accumulated a within-nodule mean of 0.04 to 0.25 pg PHB/cell during the first three weeks of growth (Figure 1-5). Mean PHB/cell within a nodule increased over this period ($p=.0011$, $n=43$, linear regression).

Discussion

PHB serves as a reproductive currency for starving *S. meliloti,* increasing short-term fitness by providing the carbon and energy required for reproduction. PHB linearly increased long-term population maintenance of starving *S. meliloti,* mainly because the population increased more before declining. Early and late stationary phase cells doubtless vary in traits other than PHB accumulation (Kolter et al. 1993), which may also

be the case for cells varying in buoyant density. Nevertheless, variation in the amount of PHB used during starvation explained the great majority of variation in reproduction and population persistence (96 and 99%, respectively), with all five populations falling on the same lines. This suggests that the relationship between PHB catabolism and reproduction and survival during starvation is robust to variation in other phenotypic traits in *S. meliloti*.

S. meliloti can store energy in glycogen and extracellular polysaccharides (EPS) (Zevenhuizen 1981; Leigh et al. 1985) as well as PHB. The tight correlation between PHB used during starvation and reproduction/persistence suggests that energy storage in other polymers, at least in cultured cells, is either exceptionally well-correlated with PHB accumulation or is of negligible importance under our conditions.

To quantify PHB accumulation by symbiotic *S. meliloti*, we developed a flow cytometric technique for measuring PHB accumulation in the viable undifferentiated cells that constitute the reproductive population of rhizobia within an indeterminate nodule. *S. meliloti* 1021 within alfalfa nodules accumulated a maximum of 0.25 pg PHB/cell in the first three weeks of symbiosis; about half of the maximum PHB accumulation found in cultured cells. Most nodules contained *S. meliloti* with little PHB, but these rhizobia may accumulate more PHB as the nodule ages. Rhizobia associated with legumes that form determinate-type nodules may accumulate PHB faster. Wong and Evans (1971) found

that *Bradyrhizobium japonicum* accumulated 40% PHB by cell dry weight over a ten-day period.

Many genes have been shown to affect PHB accumulation in rhizobia, indicating that PHB accumulation is a polygenic trait. Mutants show that genetic variation exists for PHB accumulation (Cai et al. 2000; Cermola et al. 2000; Encarnacion et al. 2002; Aneja et al. 2005; Wang et al. 2007a; Wang et al. 2007b), but the extent to which indigenous rhizobia vary in these genes (and their phenotypic consequences) remains to be determined.

Estimates of symbiotic rhizobial fitness typically consider only the number of viable rhizobia per nodule (Kiers et al. 2003) or nodule size, which is correlated with viable rhizobia per nodule (Simms et al. 2006; Heath and Tiffin 2007). We found that rhizobia can accumulate enough PHB in symbiosis to double their numbers subsequently (perhaps after escaping back into the soil), so rhizobial fitness estimates would be improved by including information on cellular PHB.

The results of this study suggest that, all else being equal, rhizobia should be under strong directional selection for increased PHB accumulation, which would tend to decrease nitrogen fixation. Yet field studies examining this mutualism have found that rhizobial populations are highly polymorphic for host benefit (Erdman 1950; Burdon et al. 1999; Heath and Tiffin 2007). Host sanctions of cheating rhizobia (Denison 2000) may counter

the selective advantage rhizobia would obtain by maximizing PHB accumulation. Kiers *et al*. (Kiers et al. 2003) showed that sanctions reduced the number of viable rhizobia inside soybean nodules by up to a factor of three, but PHB wasn't measured. Given that the reproductive advantage high-PHB cells obtained over low-PHB cells in this study is also on the order of threefold, a comparison of PHB accumulation between sanctioned and non-sanctioned rhizobia would improve our understanding of the efficacy of sanctions in selecting against cheating rhizobia.

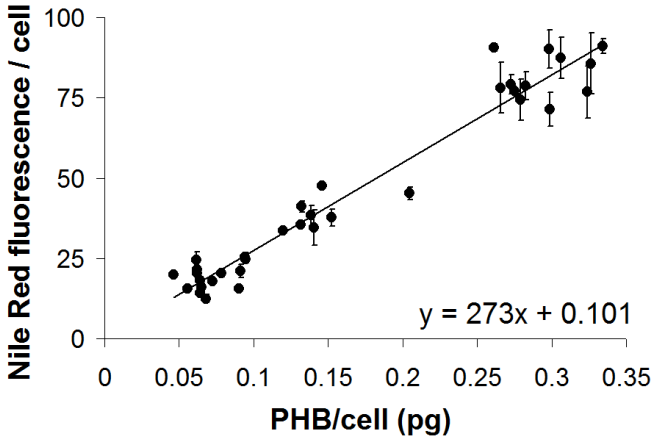

Figure 1-1. Standard curve relating Nile Red fluorescence to gas-chromatographically-determined PHB/cell (in picograms) in *Sinorhizobium meliloti*. The line shown is a least squares linear regression. Shown are means ± SEM of 3 replicates.

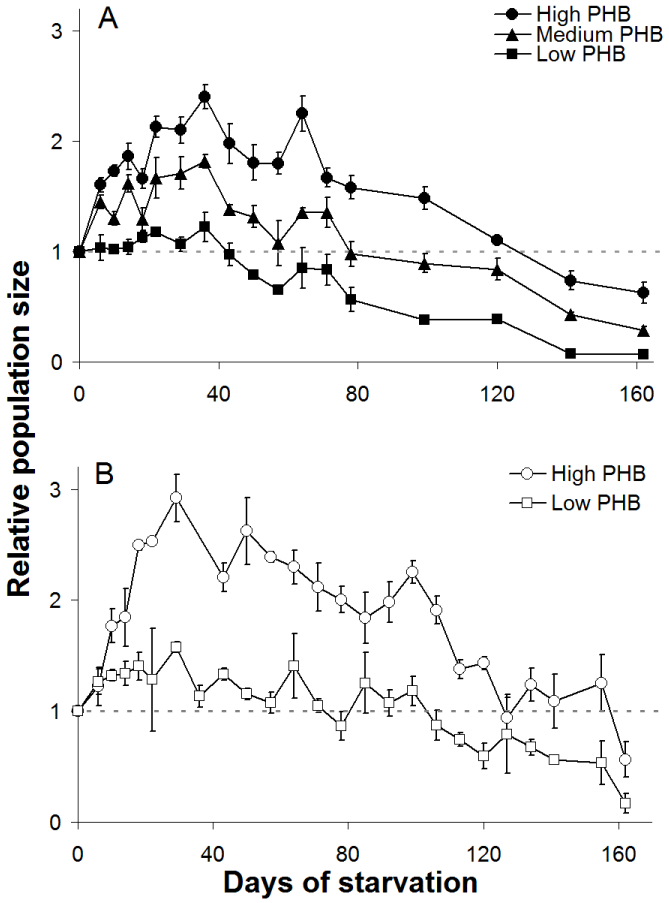

Figure 1-2. Starving *Sinorhizobium meliloti*. Rhizobia with high (0.4 pg/cell), medium (0.3 pg/cell) and low (0.18 pg/cell) mean PHB accumulation were obtained by separating stationary phase cells into high, medium and low buoyant density fractions, respectively (A). In B, high (0.5 pg/cell) and low (0.3/cell pg) mean PHB accumulation were generated by harvesting cells at late or early stationary phase culture, respectively. Relative population size, the number of viable cells divided by the initial population size, was determined by plating. The dashed gray line in A depicts the time-zero population size. Shown are means ± SEM of 3 replicates.

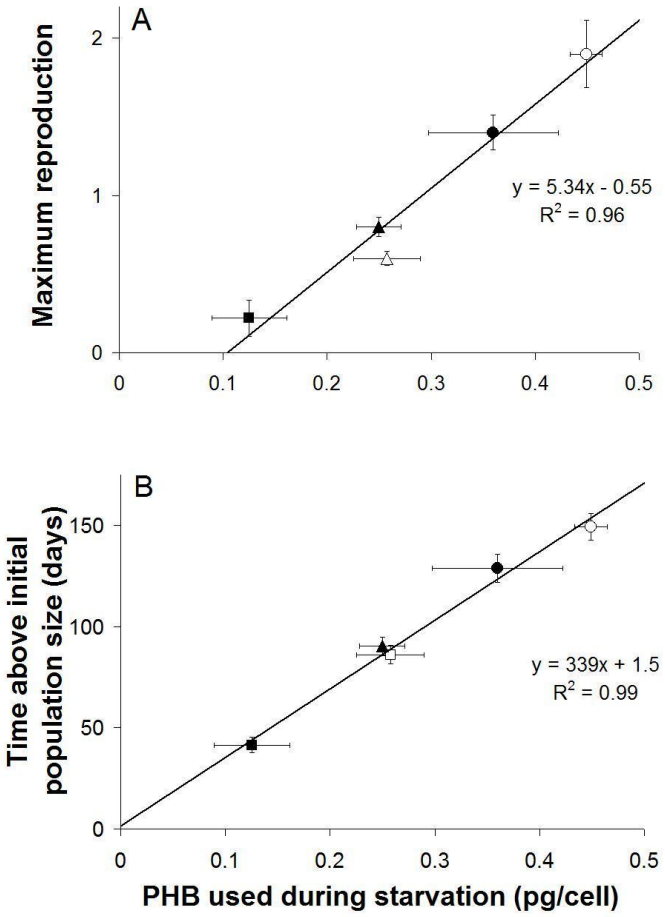

Figure 1-3. PHB linearly increases rhizobial fitness under starvation. PHB was used as a source of energy and carbon for reproduction (A); and increased the ability of cells entering starvation with more PHB to maintain larger population sizes (B). PHB used during starvation was determined by subtracting mean PHB/cell at the population size peak (at this point PHB/cell was constant for the rest of the experiment) from initial PHB/cell for each treatment. PHB/cell was determined by flow cytometry. Maximum reproduction (A) was measured as the difference between the maximum number of viable cells measured and the number of inoculated cells. Population maintenance over long-term starvation (B) was determined by (i) linearly regressing viable population size on

time for all points after the population size peak, then (ii) using the regression function to estimate the time taken for the population to return to its initial size. Treatment symbols used are the same as in Figure 1-2. Shown are means ± SEM.

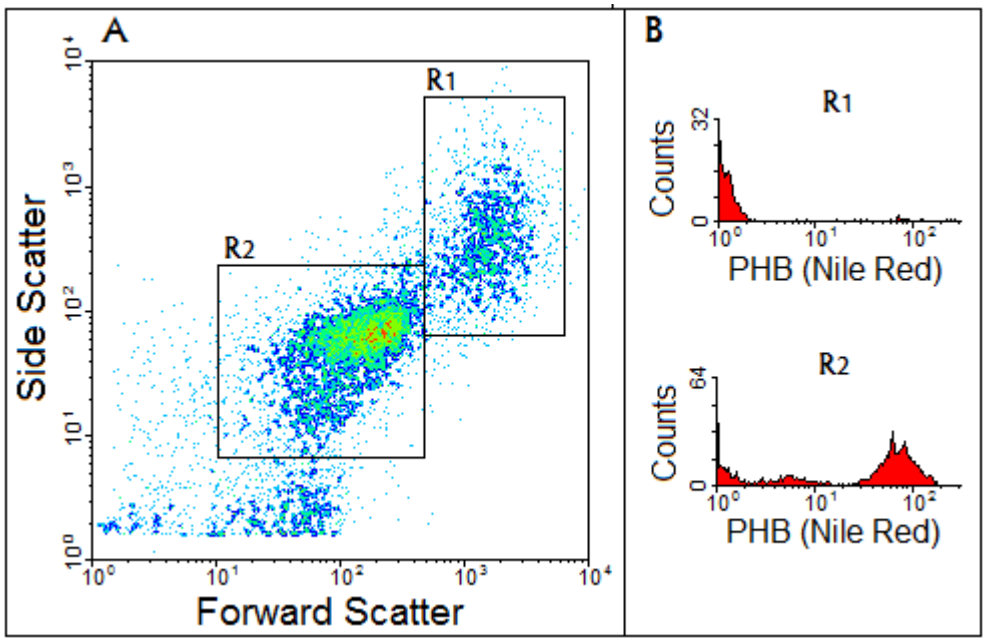

Figure 1-4. Flow cytometry of *Sinorhizobium meliloti* from an alfalfa nodule. Bacteroids (R_1) possess a higher forward scatter (because they are larger) than undifferentiated cells in R_2 (A), and did not contain detectable levels of PHB, unlike undifferentiated cells (B).

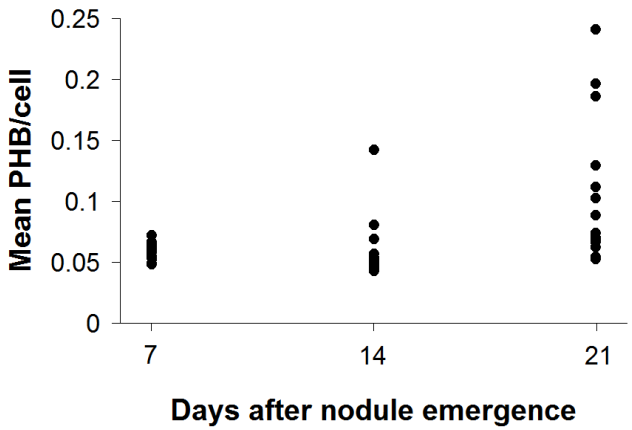

Figure 1-5. Mean PHB accumulation of undifferentiated *S. meliloti* in symbiosis with alfalfa. Each point represents a single nodule. Five nodules were harvested from three plants at each time point. Mean PHB/cell (picograms) determined by flow cytometry. Nodules harvested after 28 days of growth were beginning to senesce and were excluded.

Table 1-1 Linear regression of flow cytometric cell counts on the viable nodule population size (determined by plate counts) in *S. meliloti*

Source	Slope	Intercept as a % of plate count range	r^2
Culture	1.06	5.7%***	.84
Nodule (all rhizobia)	0.30****	.92%	.83
Nodule (cells w/ PHB)	1.17	2.3%	.71

*Two-way t-tests that the slope ≠1 and the intercept ≠ 0 significant at the ***=p<.001 and ****=p<.0001 level. n > 40 for all treatments. All regressions were highly significant (p<.0001).*

CHAPTER 2. RHIZOBITOXINE-PRODUCERS GAIN MORE POLY-3-HYDROXYBUTYRATE IN SYMBIOSIS THAN COMPETING RHIZOBIA, BUT REDUCE PLANT GROWTH

Summary

Legume sanctions against rhizobia that fix less N should exert strong selection for highly cooperative genotypes of rhizobia, but strains providing little host benefit are common. One reason may be that some rhizobia chemically manipulate the host, undermining the efficacy of sanctions. Here we show that the ethylene inhibitor rhizobitoxine is an example of such a manipulation. Rhizobitoxine-producing rhizobia decreased legume growth, but benefited relative to an isogenic, nonproducing strain on the same plant by accumulating 47% more of the storage polyester poly-3-hydroxybutyrate (PHB).

Citation: Modified from Ratcliff WC and Denison, RF (2009). Rhizobitoxine producers gain more poly-3-hydroxybutyrate in symbiosis than do competing rhizobia, but reduce plant growth. The ISME Journal. 3: 870-872

Introduction

Rhizobia are soil bacteria well known for fixing N_2 in legume root nodules. Strains that divert resources from N_2 fixation to their own immediate reproduction, or to synthesis of poly-3-hydroxybutyrate (PHB) to support later reproduction (Ratcliff et al. 2008), could outcompete more-mutualistic strains in other nodules on the same plant (Denison 2000; West et al. 2002a). This conflict between rhizobia and legumes is limited by host sanctions against nodules containing rhizobia that fix little N_2, reducing reproduction of cheating rhizobia to half that of more-beneficial strains (Kiers et al. 2003). Given host sanctions, why are less-beneficial rhizobia still common in some soils? Two possibilities (Kiers and Denison 2008) are that they escape sanctions by sharing nodules with more-mutualistic strains, or manipulate their hosts biochemically, avoiding sanctions despite poor performance.

Some rhizobia make the ethylene-inhibitor rhizobitoxine (Rtx), which increases nodules per plant and competitiveness for nodule formation (Duodu et al. 1999; Yuhashi et al. 2000; Okazaki et al. 2003). Increasing nodules per plant does not provide a fitness advantage for Rtx+ rhizobia relative to nearby Rtx(-), unlike increased nodulation competitiveness. Rhizobia continue producing rhizobitoxine inside nodules (Ruan and Peters 1992), consequently increasing nodule size (and, presumably, rhizobia per nodule) in *Vigna radiata* (Duodu et al. 1999). No effects on PHB acquisition have been reported. Rhizobia could benefit by using rhizobitoxine to manipulate the host into forming larger

nodules (allowing more rhizobial reproduction inside), or by increasing their synthesis of PHB to support later rhizobial reproduction (Ratcliff et al. 2008), but the carbon cost of either excessive nodule growth or PHB synthesis could decrease the fitness of the legume host. Here we examine the effect of rhizobitoxine on Siratro growth, rhizobial reproduction, and PHB accumulation inside nodules.

Materials and Methods

We used USDA61, a wild-type Rtx-producing *Bradyrhizobium elkanii,* and the isogenic Rtx(-) Tn5 insertion mutant RX18E (Ruan and Peters 1992). Six Siratro plants (*Macroptilium atropurpureum* cv. Siratro), grown in pouches were inoculated with either 10^5 Rtx(+) or Rtx(-) rhizobia, or with $5 \cdot 10^4$ rhizobia of each strain. Plants were grown as described by Ratcliff et al. (2008) for 12 weeks before harvesting. At harvest, nodules were randomly sampled; rhizobia per nodule and PHB per cell were determined flow-cytometrically as previously described (Ratcliff et al. 2008), but using forward scattering (FSC), calibrated against Nile Red fluorescence. Calibration data came from two preliminary experiments where plant growth, nodule harvest and analysis of rhizobia were as described, except plants were harvested after 4 or 6 weeks of growth. Plants were oven dried and weighed for biomass. Strain identity in nodules from co-inoculated plants was determined by plating 10 single-cell isolates from each nodule on TY agar (per liter: 5 g tryptone, 3 g yeast extract, 0.66 g $CaCl_2 \cdot 2H_2O$ plus 150µg/mL Kanamycin) selective for RX18E. Mixed nodules were rare and were excluded from analysis. Statistics were computed with JMP 7.0.

Results/Discussion

Forward scatter (FSC) was linearly correlated with Nile Red fluorescence, and thus PHB (Ratcliff et al. 2008) ($p < 0.0001$, $r^2 = 0.87$, Figure 2-1). The intercept was not significantly different from 0 ($t = 0.08$, d.f. = 88, $p = 0.94$ two sided t-test).

Rtx(+) rhizobia reduced Siratro shoot growth 37% ($t=2.29$, d.f.=12, $P=0.045$, 2-sample t-test, Figure 2-2a inset) under single-strain infection. They reproduced less: $2.01 \cdot 10^8$ per nodule, versus $1.25 \cdot 10^9$ Rtx(-) rhizobia/nodule ($t = 11.81$, d.f. = 62, $p < 0.0001$ two sided t-test, Figure 2-2a.), perhaps because worse plant health limited nodule growth, but accumulated 96% more PHB than the Rtx(-) genotype ($t = 9.82$, d.f. = 62, $p < 0.0001$ two sided t-test, Figure 2-2a).

Single-strain inoculation is useful to measure Rtx effects on plants. It is inappropriate for measuring Rtx effects on rhizobial fitness, however, because benefits to rhizobia from healthier plants are shared only by one strain, whereas, in the field, plant-level benefits are shared with several competing strains infecting each plant (Hagen and Hamrick 1996; Silva et al. 1999). Therefore, we tested Rtx effects on rhizobial fitness by co-inoculating siratro with both Rtx (+) and (-) rhizobia, then assaying rhizobia/nodule and PHB accumulation. Rtx(+) rhizobia gained an average of 47% more PHB than the Rtx(-) mutant ($t = 4.49$, d.f. = 47, $p < 0.0001$, two sided t-test, Figure 2-2b). In contrast to single-strain inoculation, Rtx(+) rhizobia suffered no penalties to reproduction, with

$6.11 \cdot 10^8$ rhizobia/nodule versus $6.55 \cdot 10^8$ rhizobia in Rtx(-) nodules ($t = 0.3$, d.f. = 47, $p = 0.77$ two sided t-test, Figure 2-2b). Compared to single-strain inoculation, co-inoculation significantly decreased the number of Rtx(-) rhizobia/nodule and increased the number of Rtx(+) rhizobia/nodule ($F_{3,108} = 30.62$, $p<0.0001$, single-factor ANOVA, differences assessed at $\alpha = 0.05$ with Tukey's HSD).

Siratro infected with the Rtx(+) strain alone or coinoculated with both strains formed an average of 16.2 and 15.16 nodules/plant, respectively, significantly more than the average of 4.5 nodules per plant formed by the Rtx(-) strain alone ($F_{2,16} = 9.77$, $p = 0.0019$, single-factor ANOVA, differences assessed at $\alpha = 0.05$ with Tukey's HSD.) In order for an increase in nodules per plant to provide the Rtx(+) strain a fitness benefit, Rtx(+) rhizobia must found more nodules than an Rtx(-) strain during competition. In contrast to other work (Yuhashi et al. 2000; Okazaki et al. 2003), we found that Rtx(+) rhizobia were no more competitive than Rtx(-), founding only 42% of nodules during coinoculation, but this difference was not significant ($t = 0.84$, $p = 0.42$, d.f. = 11, two sided t-test).

Where otherwise-similar Rtx(-) and Rtx(+) strains often share individual host plants, their similar reproduction inside nodules and the greater PHB accumulation of the Rtx(+) rhizobia (Figure 2-2b) will favor the spread of the latter. Rhizobitoxine reduces Siratro growth (Figure 2-2a insert), but this only reduces reproduction of Rtx(+) rhizobia relative to an Rtx(-) strain during single-strain infection (Figure 2-2). There is a metabolic tradeoff between PHB synthesis and N_2 fixation (Anderson and Dawes 1990), confirmed

by tests using mutants (Hahn and Studer 1986; Cevallos et al. 1996), but it is not clear how much of the negative effect of the Rtx(+) strain on plant growth was due to increased PHB accumulation. We suggest that production of plant-hormone mimics by rhizosphere bacteria is often manipulation rather than mutualism (Kiers and Denison 2008). Rhizobitoxine may not be the only way in which rhizobia inside nodules manipulate their hosts biochemically.

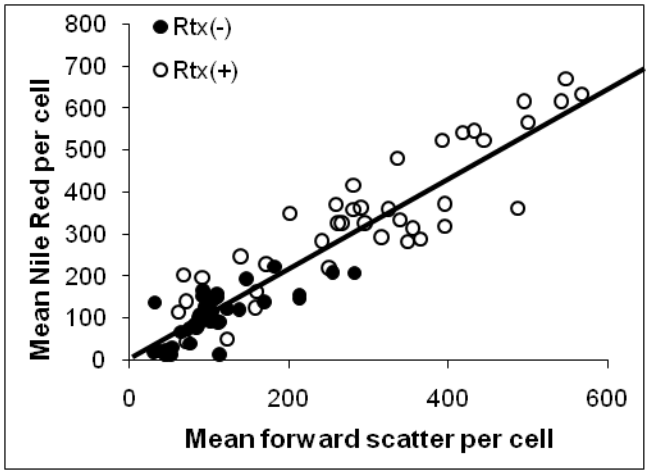

Figure 2-1. Forward scatter is highly correlated with Nile Red, a PHB stain. Each point represents one nodule (means of thousands of cells). Filled symbols are Rtx(-), open symbols Rtx(+).

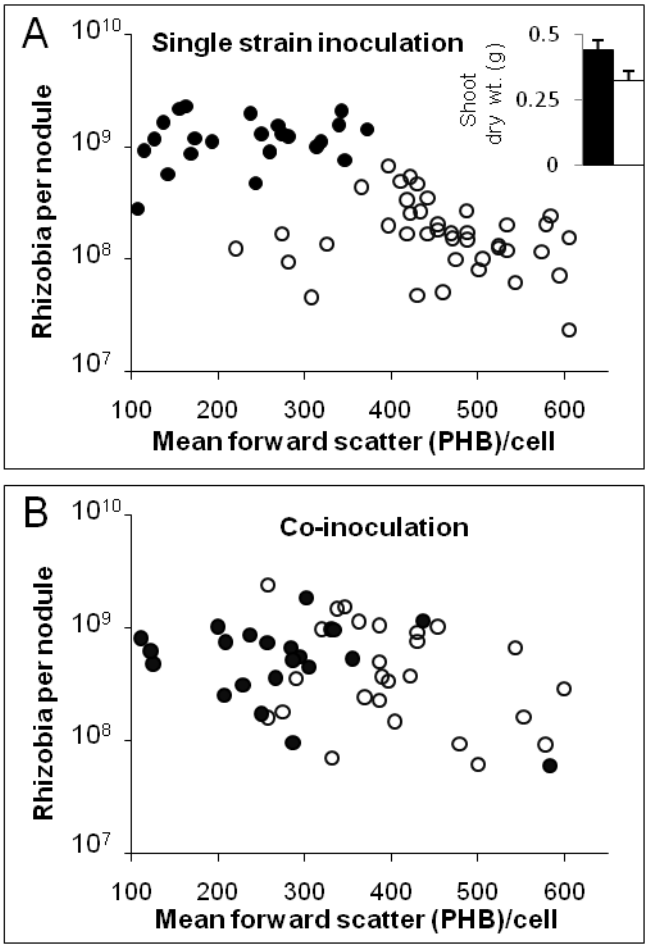

Figure 2-2. Rhizobitoxine production by *Bradyrhizobium elkanii* reduces plant growth (A, inset) but benefits the producing strain. (A) Under single-strain inoculation, which is not representative of field conditions, the benefit of more PHB was outweighed by less reproduction. (B) With mixed inoculation, Rtx(+) rhizobia still accumulate more PHB than their Rtx(-) competitors, but negative effects of rhizobitoxine on plant growth (fewer rhizobia per nodule) are shared. Each point represents the average of a nodule. Error bars = SEM. Each point represents one nodule (means of thousands of cells). Filled symbols are Rtx(-), open symbols Rtx(+).

CHAPTER 3: INCLUDING POLY-3-HYDROXYBUTYRATE (PHB) IN ESTIMATES OF SYMBIOTIC RHIZOBIAL FITNESS

Summary

In the last decade the legume-rhizobia symbiosis has become an important model system for understanding the evolution and stability of cooperation. Central to this work is the ability to measure the fitness consequences to both partners of cooperative and cheating behaviors. So far nearly all empirical studies characterize the fitness of symbiotic rhizobia by their numbers within nodules (sometimes estimated using nodule size as a proxy), ignoring the large potential contribution of the storage polyester poly-3-hydroxybutyrate (PHB) to future reproduction. We found that, in three legume-rhizobia species pairs, nodule size was an excellent predictor of the number of rhizobia within, although the number of reproductive bacteria per gram of nodule varied nearly 100-fold among host species. In all cases, the rhizobia also accumulated enough PHB to contribute significantly to future reproduction or survival during starvation conditions. For the siratro-*Bradyrhizobium* and alfalfa-*Sinorhizobium* pairings, there was no correlation ($r^2<0.04$) between nodule size and rhizobial PHB content, and even within the common bean-*Rhizobium* symbiosis the correlation was weak ($r^2=0.24$). Accurate estimates of rhizobial fitness should therefore include actual measurements of PHB, which can now be quantified rapidly with high-throughput flow cytometry, which also gives direct counts of rhizobia per nodule.

Citation: in review at the Journal of Evolutionary Biology

Introduction

Rhizobia (α-proteobacteria of the genera *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*) 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 (Kiers and Denison 2008). Key to the value of this symbiosis as a model system is the fact that 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); whereas the effects of symbiosis on rhizobial fitness is typically estimated by counting or estimating their reproduction within legume nodules (Kiers et al. 2003; Kiers et al. 2006; Simms et al. 2006; Heath and Tiffin 2007; Heath and Tiffin 2009; Heath 2010).

This approach to measuring rhizobial fitness has at last two potential problems. First, rhizobial numbers are often approximated from nodule size. Is this valid? Second, estimates of rhizobial fitness typically ignore poly-3-hydroxybutyurate (PHB), a storage polyester that rhizobia can accumulate to more than 50% of cell dry weight (Bergersen and Turner 1990b; Ratcliff et al. 2008). In *Sinorhizobium meliloti*, stored PHB can support the production up to three offspring in the absence of an external carbon source (Ratcliff et al. 2008), so two nodules containing the same number of rhizobia could potentially differ three-fold in the number of rhizobia released into the soil. It might be

safe to ignore this potential problem, however, if there were a consistent relationship between PHB/rhizobia and either nodule size or rhizobia/nodule. Third, it has not been shown that the number of rhizobia eventually released into the soil is directly proportional to the number of rhizobia inside a nodule prior to senescence. This paper will address the first two of these three problems.

During the last 150 years there has been a proliferation of fitness measures, all aimed at the same goal: to allow one to calculate allele frequencies in the next generation from genotype frequencies in the current generation (de Jong 1994). For most organisms it is difficult to measure genotype and allele frequencies over generations, and as a result fitness is often estimated by measuring life-history components, such as fecundity. But number of offspring is not a perfect predictor of representation in future generations. For example, seed size is positively correlated with fitness for many plants, particularly under stressful conditions (Moles and Westoby 2004).

Recent measures of rhizobial fitness have focused exclusively on fecundity within nodules (West et al. 2002b; Kiers et al. 2003; Simms et al. 2006; Heath and Tiffin 2007), while excluding traits linked to future reproduction and survival. However, there can be important negative correlations among life history traits that contribute to fitness. For example, among plants, there is a strong negative correlation between seed size and seed number (Henery and Westoby 2001). The finite supply of plant-derived carbon available to rhizobia in nodules may result in a similar trade-off between symbiotic rhizobial

reproduction and PHB synthesis. Strains of rhizobia that store PHB for future reproduction but therefore reproduce less within nodules might thus falsely be deemed less fit than strains that pursue the opposite strategy, if reproduction alone is measured. This problem could be avoided by measuring PHB per rhizobial cell as well as rhizobia per nodule. This might not be necessary, however, if there were consistent correlations between these two variables, of if both rhizobial numbers and PHB could be predicted from nodule weight. Here we test these hypotheses in three legume-rhizobia species pairings.

Materials and Methods

For all three species pairings, we grew plants hydroponically in a growth chamber until nodules could be harvested, and then assessed symbiotic rhizobial population size and PHB content via flow cytometry. Before planting, seeds were surface sterilized by a 10-s dip in 95% ethanol, followed by immersion in 3% bleach (0.18% NaOCl) for three minutes, and then 3% H_2O_2 for three minutes (Somasegaran and Hoben 1994). Seeds were rinsed five times in deionized (DI) water, germinated in sterile Petri plates, and then transferred to hydroponic growth pouches (Mega International, Minneapolis, MN. 55416). Plants were watered with a modified N-free Fahraeus nutrient solution (Fåhraeus 1957) with FeNaEDTA substituted for Fe-citrate to hold [Fe] constant. Plants were grown in a Conviron E7/2 growth chamber using a 16 hour photoperiod with 500 µE m^{-2} s^{-1} photosynthetically active radiation, and day/night temperatures of 22/15 °C.

Rhizobia for plant inoculation were grown to stationary phase in 50 mL TY broth in 125 mL Erlenmeyer flasks incubated at 22 ºC and shaken at 100 RPM. Alfalfa (*Medicago sativa* L. cv. Rebound 5.0, from C. Scheaffer, University of Minnesota), a legume that forms indeterminate nodules (Timmers et al. 2000), was inoculated with *S. meliloti* strain 1021 (from M. Sadowsky, University of Minnesota) at $5 \cdot 10^7$ viable rhizobia per plant. Common bean (*Phaseolus vulgaris* L. cv. Royal Burgundy, from Henry Fields Seed Co. Aurora, IN. 47001), was inoculated with *Rhizobium etli* strain CE3 (from M.A. Cevallos, Universidad Nacional Autónoma de México) at $5 \cdot 10^7$ viable rhizobia per plant. Siratro (*Macroptilium atropurpureum* (DC) Urb. cv. Siratro, from the late P. Graham, University of Minnesota), was inoculated with *Bradyrhizobium elkanii* strain RX18E or RX17E (Ruan and Peters 1992) (from M. A. Parker, State University of New York, Binghamton) at 10^5 viable rhizobia/plant. Both common bean (Sprent 1980) and siratro (Price et al. 1984) form determinate nodules. Five nodules > 1 mm in length were randomly harvested from each of three alfalfa plants 7, 14, 21 and 28 days from nodule emergence (for a total of 60 nodules); each plant was only sampled once. Sampling was the same for common bean, except that nodules were only removed 7 and 14 days from emergence (a total of 30 nodules). Eight nodules were randomly harvested from each of 12 siratro plants after 84 days of growth. Plants with ≤ 8 nodules had all nodules harvested; a total of 75 nodules were obtained. All nodules were weighed, and then surface sterilized by a 10-s dip in 95% ethanol and immersion in 3% bleach for three minutes. Nodules were rinsed five times in sterile DI water (Somasegaran and Hoben 1994), and then crushed into sodium ascorbate extraction buffer (Arrese-Igor et al. 1992). Crushed nodules were

centrifuged at 100 × *g* for 10 minutes, and the supernatant was decanted from nodule debris. Rhizobia in the supernatant were centrifuged at 5000 × *g* for five minutes and resuspended in starvation buffer (Wei and Bauer 1998), then kept at 4 °C. The number of rhizobia per nodule, the average amount of PHB per cell, and whether each *S. meliloti* cell from an alfalfa nodule was undifferentiated was determined flow cytometrically, as previously described in (Ratcliff et al. 2008).

Results/Discussion

Nodule weight significantly ($p < 0.0001$, Figure 3-1) predicted the total number of rhizobia per nodule in common bean ($r^2=0.75$), siratro ($r^2=0.52$) and alfalfa ($r^2=0.93$). In nodules of some legume species, including bean and siratro, all rhizobia are thought to retain the ability to reproduce, whereas in other host species, including alfalfa, rhizobial bacteroids (the differentiated, nitrogen-fixing form) are swollen and apparently nonreproductive (Oono et al. 2009). In alfalfa and some other hosts with indeterminate nodule growth, reproductive rhizobia are mostly found in the meristematic region at the nodule tip (Vasse et al. 1990). Because indeterminate nodules typically grow as a cylinder by tip extension (Sutton 1983), the volume of the nodule meristem (and hence the number of reproductive rhizobia) might not be expected to increase at the same rate as total nodule mass during growth. Nonetheless, we found that, even in alfalfa, nodule mass was an excellent linear predictor of the number of undifferentiated (putatively reproductive) rhizobia per nodule, obviously with different coefficients than for total rhizobia ($r^2=0.82$) (Figure 3-1). This consistent relationship may be due to small simultaneous increases in nodule diameter with increasing length, or to the colonization

of senescing, basal regions of alfalfa nodules by undifferentiated rhizobia (Timmers et al. 2000). Rhizobial density within nodules was highly variable among species pairs. Siratro nodules contained 5-fold more viable rhizobia g^{-1} than bean nodules, and 94-fold more viable rhizobia g^{-1} than alfalfa nodules ($F_{2,171}$=15.2, $p<0.0001$, effect of interaction between plant species and nodule mass on viable rhizobia per nodule, ANCOVA).

Although reproductive rhizobia of each species accumulated considerable amounts of PHB, nodule weight did not predict PHB per cell in either siratro or alfalfa, and it was a poor predictor of PHB per cell in common bean (Table 3-1). Average PHB content per cell should therefore be measured for each nodule. Widely used direct chemical measures of cellular PHB (e.g. Law and Slepecky 1961; Riis and Mai 1988) are impractical, requiring more rhizobia than any nodule in this study contained. Fortunately, flow-cytometric methods are far more rapid and sensitive than direct methods of PHB analysis and well-suited to high-throughput analysis of both rhizobial PHB and rhizobia/nodule (Ratcliff et al. 2008; Ratcliff and Denison 2009).

Given the importance of PHB to rhizobial fitness (Ratcliff et al. 2008) and the failure of proxies like nodule size or rhizobia per nodule to provide reliable predictions of this important variable, conclusions about rhizobial fitness from previous studies that failed to measure PHB (Kiers et al. 2003; Kiers et al. 2006; Simms et al. 2006; Heath and Tiffin 2007; Heath and Tiffin 2009; Heath 2010) might need at least quantitative revision. In the only study that examined the effect of differences among strains in both PHB and

symbiotic reproduction, rhizobia of a less-beneficial strain had significantly greater PHB content, but not greater population size (Ratcliff and Denison 2009). Failure to measure PHB would therefore have missed an important fitness difference in that study. Clearly, a more complete understanding of the evolutionary significance of cooperation and cheating requires that investigators include PHB accumulation in estimates of symbiotic rhizobial fitness.

The relationship between nodule weight and rhizobial population size was linear over the nodule size range of the three species studied (Figure 3-1). Because we used only a single genotype of each rhizobial species and didn't test contrasting experimental treatments, we cannot say whether the same linear relationships would hold up for comparisons among rhizobial strains or among experimental treatments (Oono et al. 2009). This would be an important question if rhizobia per nodule were a sufficient measure of rhizobial fitness, but that is only true for cases where PHB accumulation is negligible. Because measuring PHB/cell by flow cytometry also gives direct counts of rhizobial cells/nodule, estimates of rhizobial numbers from nodule weight will often be superfluous.

Because PHB can directly facilitate reproduction in rhizobia, the simplest approach to integrating rhizobial population size and PHB accumulation into a single fitness estimate is to add the reproductive potential of stored PHB to the number of viable rhizobia within the nodule. For *S. meliloti*, the relationship between PHB/cell and PHB-supported

reproduction has been quantified; a picogram of PHB can support the production of nearly 5 offspring (Ratcliff et al. 2008). Thus an alfalfa nodule that contains $5 \cdot 10^6$ *S. meliloti* with a mean PHB content of 0.2 pg $cell^{-1}$ would have a potential population size (without additional external carbon) of $7.6 \cdot 10^6$ cells. The actual fitness consequences of PHB accumulation will depend on environmental conditions, which may be difficult to measure empirically. When carbon does not limit the reproduction of free-living rhizobia, stored PHB may provide little benefit. However, carbon in the bulk soil often limits bacterial growth (Veen and Lindenmayer 1977). Even in the comparatively carbon-rich rhizosphere (Cheng et al. 1996), fluctuation in available resources and competition with other microbes may favor individuals with substantial PHB reserves. If 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 per nodule may be less important than differences in PHB per cell.

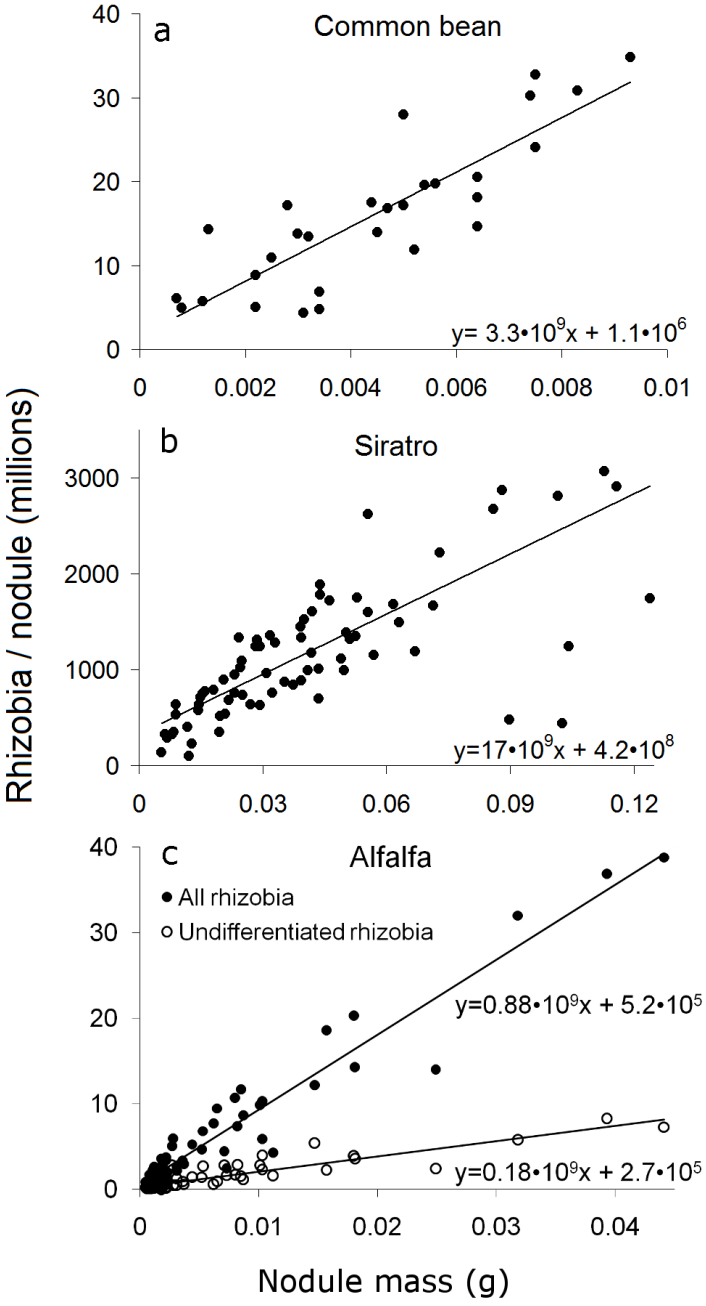

Figure 3-1. Nodule mass is an excellent predictor of symbiotic rhizobial population size in three legume-rhizobia species pairs.

Table 3-1. Nodule mass is a poor predictor of rhizobial PHB per cell

Species pair	Fraction analyzed	Mean PHB cell^{-1} (pg)	Range (pg)	Regression of PHB cell^{-1} (pg) on nodule weight (g)	r^2
Common bean *R. etli*	All rhizobia	0.38	0.45	y= 29x + 0.26**	0.24
Siratro *B. elkanii*	All rhizobia	0.34	0.54	y= x + .30	0.04
Alfalfa *S. meliloti*	Undifferentiated rhizobia	0.071	0.198	y= -0.46x + 0.07	0.01

** $p < 0.01$

CHAPTER 4: MATERNALLY IMPOSED BET HEDGING IN THE BACTERIUM *SINORHIZOBIUM MELILOTI*

Summary

Bet hedging through increased phenotypic variation can enhance geometric-mean fitness across unpredictable environments (Seger and Brockmann 1987). Metazoan bet-hedging usually involves maternally imposed phenotypic diversification among offspring (Hopper 1999; Lips 2001; Laaksonen 2004; Crean and Marshall 2009; Simons 2009), such as differences in seed dormancy. Virtually all known microbial bet-hedging strategies, in contrast, rely on low-probability stochastic switching of a heritable phenotype, by individual cells in a clonal group (van der Woude and Baumler 2004; Moxon et al. 2006; Veening et al. 2008; Gordon et al. 2009). This is ineffective at generating diversity when group size is small and there is little generational turnover between episodes of risk. Here we describe a novel microbial bet-hedging behavior that resembles maternal bet-hedging in metazoa. *Sinorhizobium meliloti* stores carbon and energy in the polyester poly-3-hydroxybutyrate (PHB) as a contingency against carbon scarcity (Ratcliff et al. 2008). We show that, when starved, dividing *S. meliloti* bet hedge by forming two daughter cells with different phenotypes. These have high and low-PHB levels, suited to long- and short-term starvation, respectively. The high-PHB phenotype was significantly more tolerant of antibiotic exposure as well as starvation, but it took longer to resume growth when given resources. The low-PHB cells may increase competiveness for current nodulation opportunities, while the high-PHB cells can survive for over a year without food, perhaps until the legume host is next available.

Citation: in review at Current Biology. There is no Introduction section in a CB report.

Results and Discussion

Genetically uniform *S. meliloti* accumulated large amounts of poly-3-hydroxybutyrate (PHB, Figure 4-1a) when grown in M9 minimal media with 10 g L^{-1} mannitol. We showed previously that, when starved, these rhizobia used stored PHB to reproduce, increasing about threefold within 29 days (Ratcliff et al. 2008). By then, the population of *S. meliloti* had differentiated into discrete high- and low-PHB phenotypes (Figure 4-1b). Some PHB was used in the process, as PHB levels in the high-PHB subpopulation were lower than initial (Figure 4-1a,b). Reproduction mainly produced new low-PHB cells, which increased to 195% of the original cell count. The number of high-PHB cells, which contained 16 times as much PHB as the low-PHB cells (Figure 4-1b; $t=34.7$, $p<0.0001$, $n=6$, t-test), remained constant during this period, at 96% of the original number of starved rhizobia.

Additional experiments using flow cytometry and fluorescence microscopy confirmed the bimodal distribution of PHB levels in the population and showed that both populations consisted of intact cells, based on their ability to exclude PI stain (Figure 4-2 a-f). This population of rhizobia remained differentiated into high and low-PHB phenotypes after more than 500 days of starvation, demonstrating that this differentiation is bistable.

The observed bimodal distribution of PHB per cell could be generated either by individual high-PHB rhizobia dividing asymmetrically to produce both high and low-PHB offspring (maternally imposed diversification), or by some rhizobia consuming all PHB for reproduction while some of their clonemates refrain from reproducing (stochastic diversification). To differentiate between these two hypotheses, we followed the fate of immobilized high-PHB rhizobia reproducing in starvation media over a 24 hour period, by microscopy. PHB was preferentially retained in the old-pole cells (Figure 4-1c-e), with the median PHB content of new-pole cells only 40% that of their old-pole parental cells ($t=7.09$, $p<0.0001$, $n=33$, matched-pairs t-test).

To determine if this asymmetric allocation of PHB among daughter cells can serve as a mechanism for bet hedging (defined and discussed below), we examined the survival of each phenotype during long vs. short-term starvation. Rhizobia were starved for either 14 or 528 days; then viability was assessed microscopically with YO-PRO-1, a fluorescent-green live/dead stain. After 14 days, there was no detectable difference in the viability of high- and low-PHB cells (Figure 4-3a; $t=0.96$, $p=0.37$, $n=10$, t-test). However, after 528 days, high-PHB cells had a five-fold survival advantage (Figure 4-3a; $t=9.5$, $p<0.0001$, $n=10$, t-test).

In addition to starvation, antibiotics produced by other microbes are potential sources of mortality in the rhizosphere (Samac et al. 2003; Riesenfeld et al. 2004). To determine if either phenotype was more antibiotic tolerant, we used two independent methods to

generate genetically-uniform populations of starved rhizobia that varied in the percentage of rhizobia with the high-PHB phenotype, then exposed these populations to ampicillin. Populations with mostly high-PHB rhizobia survived antibiotic exposure significantly better than those with mostly low-PHB rhizobia (Figure 4-3b; $p<0.0001$, $n=41$, $r^2=0.46$, linear regression). S. m. 1021 is normally killed by ampicillin, but the high-PHB phenotype is apparently tolerant. This suggests that the high-PHB bacteria may be dormant, as antibiotic tolerance is typical of dormant or 'persistent' bacteria (Keren et al. 2004). Most populations of bacteria contain a small proportion of persisters even in the absence of stress (Lewis 2007), but it has recently been shown that persistence can be stress induced (Dorr et al. 2010).

This apparent dormancy might be expected to have some cost, such as a delay in growth when starvation ends. To test this hypothesis, we subcultured populations of starved rhizobia that varied in the percentage of cells with the high-PHB phenotype (closed circles from Figure 4-3b) into rich media (TY) and measured the duration of lag phase. Populations containing a larger fraction of high-PHB cells had a significantly longer lag phase. An additional lag of 0.96 minutes per % high-PHB cells in the population, ($p=0.044$, $n=27$, linear regression), extrapolated to the two extremes, suggests a 96-minute difference between the two phenotypes. It appears that in *S. meliloti*, dormancy is induced by starvation, but only in the high-PHB old pole cell.

To be considered bet hedging, disparate phenotypes of a common genotype must be capable of rediversifying. If each phenotype is completely heritable, then diversification is the result of an evolutionary radiation and is not bet hedging (Seger and Brockmann 1987). We thus determined whether high- and low-PHB phenotypes are capable of rediversifying when put through an additional round of growth and starvation. Using density-gradient centrifugation, we generated fractions that were either enriched (48.4%) or depleted (11.2%) in high-PHB cells, from a single starved, dimorphic population of *S. meliloti*. These fractions were plated on rich media (TY) and 48 single-cell isolates were randomly selected from each fraction. All isolates accumulated PHB when grown in M9 mannitol media and then diversified into high- and low-PHB cells. Further, the phenotype of the founding cell did not affect this diversification: isolates drawn from the enriched and depleted fractions formed populations in which, after 29 days of starvation, the high-PHB phenotype constituted an average of 88.3% and 83.3% of the original cell count, respectively ($t=0.68$, $p=0.50$, $n=95$, t-test), and the low-PHB phenotype constituted 146% and 154.3% of the original cell count, respectively ($t=0.44$, $p=0.66$, $n=95$, t-test; Figure 4-4). The high- and low-PHB phenotypes do not show even epigenetic inheritance.

Starving high-PHB *Sinorhizobium meliloti* face a trade-off between reproduction and survival. Metabolically active bacteria have a higher potential growth rate, but they are more susceptible to antibiotics (Lewis 2007) and to exhausting their energy reserves. Our data show that, when starved, *S. meliloti* can divide into one metabolically active cell,

apparently primed for further reproduction or nodulation as resources allow, and one less-active old-pole persister, which can survive for a year or more without food. The lower metabolic activity of the persister would enhance survival under starvation even if initial resource allocation were equal, but survival is further enhanced by greater allocation of PHB to the persister. Interestingly, some mathematical models suggest that equal allocation of resources among offspring enhances maternal inclusive fitness, even if there is some environmental variability (Smith and Fretwell 1974; McGinley et al. 1987; Sadras 2007). These models do not include the possibility of qualitatively different strategies among offspring.

The term "bet hedging" is sometimes used loosely, but a rigorous definition includes lower expected arithmetic-mean fitness as well as greater expected geometric-mean fitness (Seger and Brockmann 1987; Simons 2009). If the duration of starvation is unpredictable, asymmetric division into both high- and low-PHB phenotypes could serve as a bet hedging mechanism by reducing variance in maternal inclusive fitness across starvation events. Without a record of the long-term frequency and severity of starvation and antibiotic exposure faced by *S. meliloti* in the field, however, we cannot determine conclusively whether this behavior evolved due to selection for diversification bet hedging. This limitation is nearly ubiquitous in studies of bet hedging: to our knowledge only two studies (Beaumont et al. 2009; Simons 2009) have shown that a putative bet hedging trait decreases a genotype's arithmetic mean fitness while increasing geometric mean fitness in the environment in which the trait evolved. Nonetheless, the asymmetric

division in starving *S. meliloti* and optimization of low and high-PHB phenotypes for growth and stress resistance, respectively, strongly suggest that this behavior evolved for the purpose of risk-spreading.

This is the first example of a microbial bet-hedging mechanism that allows a single maternal cell to effectively express a diversification strategy (via division into daughter cells with contrasting phenotypes) when exposed to stress. Most putative microbial bet-hedging strategies result from phase variation (van der Woude and Baumler 2004), contingency loci (Moxon et al. 2006) and epigenetically inherited phenotypic bistability (Veening et al. 2008; Gordon et al. 2009). Diversification is driven by low-probability (typically 10^{-1}-10^{-5} per individual per generation) stochastic switching that generates phenotypic diversity among a group of genetically identical cells, rather than phenotypic plasticity that responds adaptively to current conditions. Except at the higher frequency end of this range (10^{-1}), a small group founded by a single phenotype is unlikely to contain even a single variant, making the entire clonal group subject to extinction.

In contrast, the diversification strategies commonly found in metazoans (Hopper 1999; Lips 2001; Laaksonen 2004; Crean and Marshall 2009; Simons 2009) generate diversity among progeny every generation, and thus are effective even when group size is small and stress frequent. For example, a plant producing as few as two seeds might still have significant variation in dormancy among its offspring. For rhizobia, dispersal in the rhizosphere and subsequent competition for different nodulation opportunities would

favor similar bet-hedging, especially if saprophytic reproduction (and hence the number of generations in a patch of the rhizosphere) is limited. Successful nodulation requires that rhizobia encounter the root of a compatible legume during a period of active nodulation, and outcompete other rhizobia for root entry (Gage 2004). Individual rhizobial cells released from nodules early in a growing season may, by forming both high- and low-PHB phenotypes when starved, take advantage of late-season nodulation opportunities the same year, while also increasing the chances of surviving until new hosts are next available. In agricultural environments, viable rhizobial populations can persist in soil for years without a compatible host (Kucey and Hynes 1989). To some extent, this may be due to prolonged survival of individual cells.

Experimental Procedures

Rhizobial strains and culture

All work was done with *Sinorhizobium meliloti* strain 1021 and the GFP-labeled derivative pDG71 (Gage 2002). Before starvation, rhizobia were cultured in 50 mL of M9 minimal media (Miller 1992) + 10 g L^{-1} mannitol in 125 mL Erlenmeyer flasks shaking at 100 rpm at 22° C for 6 days. pDG71 was cultured with the presence of 5 µg mL^{-1} tetracycline.

Starvation

S.m. 1021 were harvested by centrifugation from growth flasks and double washed in carbon-free M9 media (containing no mannitol, 0.1 g L^{-1} thiamine and HPLC-grade water). Caution was taken to ensure that starvation media remained carbon free. All

glassware used was acid-washed (0.6M HCl for one hour), rinsed in DI water and pyrolyzed at 550°C overnight. All starvation experiments were initiated at a density of 5 × 10^5 - 5 × 10^6 cells mL^{-1} unless otherwise noted, with cell density determined by either plate counting or FACS analysis as previously described (Ratcliff et al. 2008).

Flow cytometry

All flow cytometry was carried out on a Benton Dickson FACSCalibur. PHB per cell was quantified by staining with the red fluorescent lypophyllic dye Nile Red as previously described (Ratcliff et al. 2008). Live/dead analysis was conducted by using the red fluorescent DNA stain propidium iodide (PI) (Ratcliff et al. 2008). 1% of the PI stock solution (1 mg PI mL^{-1} in H_20) was added to cells at 5 × 10^5 - 5 × 10^6 cells mL^{-1}, incubated for 5-10 minutes and then assayed flow cytometrically, collecting data on the FL3 detector (>670nm filter). Live and fixed (30% EtOH for 30 minutes) controls were run to determine the fluorescence range of viable and killed cells, and viability for all samples was determined by gating.

Following the fate of PHB in pairs of dividing cells

To determine if high- and low-PHB phenotypes were generated by maternal or stochastic processes, we developed a method to follow individual cells during culture in liquid media. We employed 8-well Nunc Lab-Tek II chamber slides, which allow culture of rhizobia on a coverslip under 0.5 mL liquid media. The inner surface of each slide was first marked with ~0.5 mm diameter dot from a permanent marker (Sharpie), allowing us

to repeatedly find the same field of view, then treated with poly-D-lysine (0.1 mg mL^{-1} for 5 minutes, removed via pipette and double washed in sterile DI water). Slides were air-dried in a laminar flow hood, and 10 μL of high-PHB rhizobia at 10^8 cells mL^{-1} were placed over the marked region of the slide. Rhizobia were allowed to adhere to the poly-D-lysine treated coverslip for 30 minutes, then the medium was removed by pipette and the marked region was rinsed twice with 50 μL of C-free M9 to remove free cells. Rhizobia were starved in 300 μL C-free M9. Rhizobia were imaged on an Olympus IX70 inverted epifluorescence microscope equipped with a 4MP SPOT digital camera at time zero and after 24 hours. We quantified PHB/cell by staining with Nile Red; first fixing with 125 μL 95% EtOH for 30 minutes then adding NR 1% stock solution (1mg mL^{-1} in DMSO), incubating for 60 minutes, and reimaging. PHB/cell in the old and new cell pairs was quantified by measuring the median NR fluorescence intensity of each cell in ImageJ after thresholding to remove background fluorescence.

Assaying viability during long/short starvation

S.m. 1021 cells starved in M9-C were assayed for PHB content and viability simultaneously via microscopy, using the red fluorescent PHB stain Nile Red and green-fluorescent live/dead stain YO-PRO-1 (shown to be comparable in action to propidium iodide, Figure 4-5). 1% of the staining solution (1 mg mL^{-1} Nile Red and 0.1 mM YO-PRO-1 in DMSO) was added to rhizobia at ~5 x 10^6 cells mL^{-1}, incubated for 60 minutes at room temperature, and then excess stain was removed by centrifugation. For each sample at least 30 fields of view were randomly imaged on an Olympus IX70 microscope

for both NR and YO-PRO-1 fluorescence. RGB stacked images were decomposed into their 3 single color elements and only the red and green components were kept for NR and YO-PRO-1 fluorescence emissions, respectively. These two images were combined into a single stack and registered in ImageJ. Both NR and YO-PRO-1 emit a small amount of fluorescence in the green and red spectra, respectively; we removed this potentially confounding effect with the Spectral Unmixing plug-in (written by J. Walter) in ImageJ. Median NR and YO-PRO-1 fluorescence intensity were measured for each cell, and as with flow cytometry, viability and high/low PHB content determined by gating.

Assaying antibiotic survival

To determine if the high-PHB phenotype was more antibiotic-tolerant, we generated groups of starved S.m. 1021 with varying percentages of high-PHB phenotype cells, and then exposed these populations to ampicillin at 500 μg mL^{-1} for 90 minutes. We used two independent methods to generate variation in the % high-PHB cells. Because high-PHB rhizobia are more dense than low-PHB cells (Ratcliff et al. 2008), we used density gradient centrifugation to fractionate five populations of starved cells with bimodal PHB content into high (1.138-1.119 g mL^{-1}), medium (1.119-1.109 g mL^{-1}) and low (≤1.109 g mL^{-1}) density as previously described in (Ratcliff et al. 2008). We also generated variation in the % high-PHB cells by starving S.m. 1021 at high ($5 \cdot 10^6$), medium ($5 \cdot 10^5$) and low (10^5) densities for three days, cells starved at higher densities reproduced less and thus formed population with a larger fraction of high-PHB cells. Viability was

assayed flow cytometrically for both experiments; however we used PI in the centrifugation experiment, and YO-PRO-1 in the density-of-starvation experiment. In both experiments rhizobia were left at 4° C overnight before viability assays were performed to give susceptible cells time to die.

Measuring the lag phase of low and high-PHB phenotypes

Populations of S.m. 1021 with varying percentages of high-PHB cells, generated by starving rhizobia at varying densities (above), were subcultured into rich media (TY) and incubated at 30° C for 30 hours. Each population was sampled at 0, 4, 12, 18, 24, and 30 hours of starvation and population size determined via flow cytometry. Fold increases in population size for 12-30 h growth was compared to the average of 0 and 4 h, in which no growth was detected. Gompertz growth curves were fit to the number of cell doublings over time using the iterative parameter fitting algorithm in JMP 7.0 (SAS institute). The parameter of the Gompertz growth curve that describes the duration of lag phase was then used as the response measurement for the experiment.

Statistical analyses

All statistics were computed in JMP 7.0. All t-tests were two-sided (unless otherwise noted).

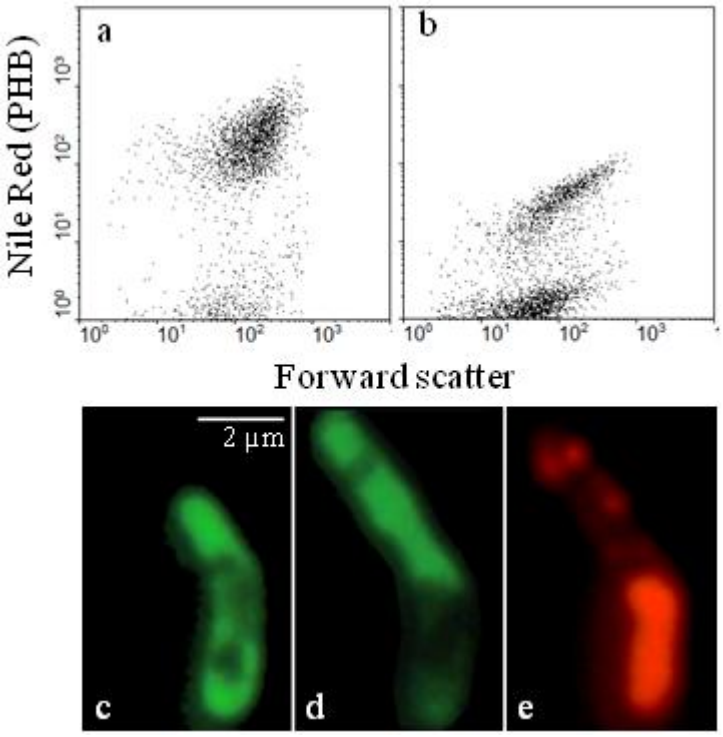

Figure 4-1- *Sinorhizobium meliloti* differentiates into high- and low-PHB phenotypes when starved. PHB was measured flow cytometrically using the fluorescent probe Nile Red (NR). **a**, All cells initially contained large amounts of PHB. **b**, After 29 days of starvation, these cells were differentiated into distinct high- or low-PHB phenotypes. **c**, GFP-labeled *S. meliloti* from a uniformly high-PHB population were immobilized, starved, and imaged after zero (c) and 24 hours (d,e). **e**, NR staining indicates that PHB allocation is asymmetric; the new-pole cell contains little PHB while the old-pole cell retains the majority of the remaining maternal PHB.

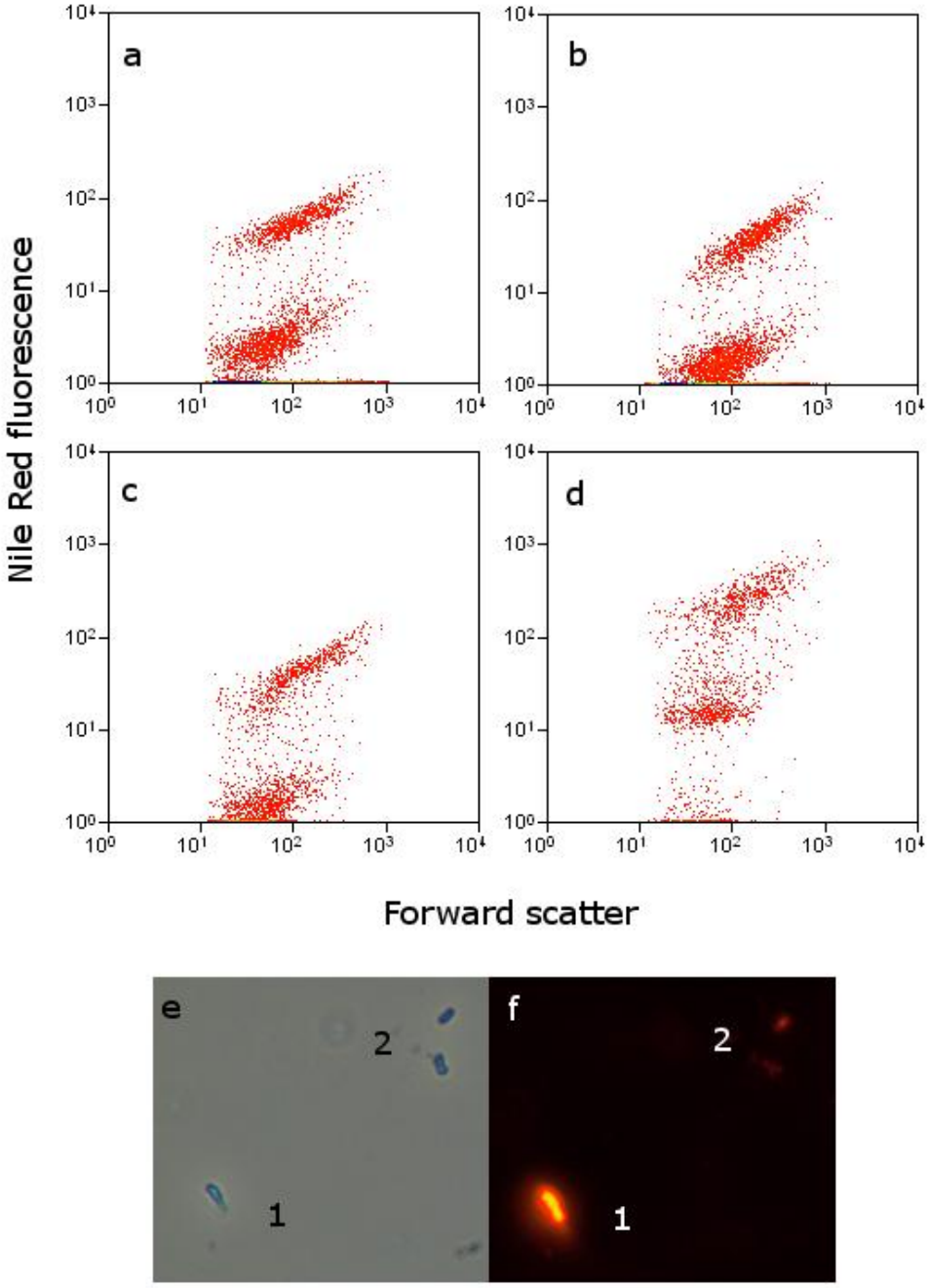

Figure 4-2 - Confirmation of the bimodality of PHB/cell and rhizobial viability. **a**, Unfixed rhizobia were stained with NR, confirming bimodality of PHB. **b**, Unfixed

rhizobia were stained with both NR and DNA stain propidium iodide (PI). Failure of PI to increase fluorescence of unfixed rhizobia shows cells did not uptake PI, so were intact and presumed viable. **c,** As a control, fixed cells were stained with NR and **d**, fixed cells were stained with both PI and NR. Staining DNA with PI increases red fluorescence for fixed cells (c,d), confirming that unfixed cells are intact (a,b). **e**, *Sinorhizobium meliloti* str. 1021 from panel c (fixed and stained with NR) imaged on an Olympus IX70 microscope under bright field microscopy and **f**, fluorescence microscopy. A high PHB cell is annotated as 1, and a pair of low PHB rhizobia as 2.

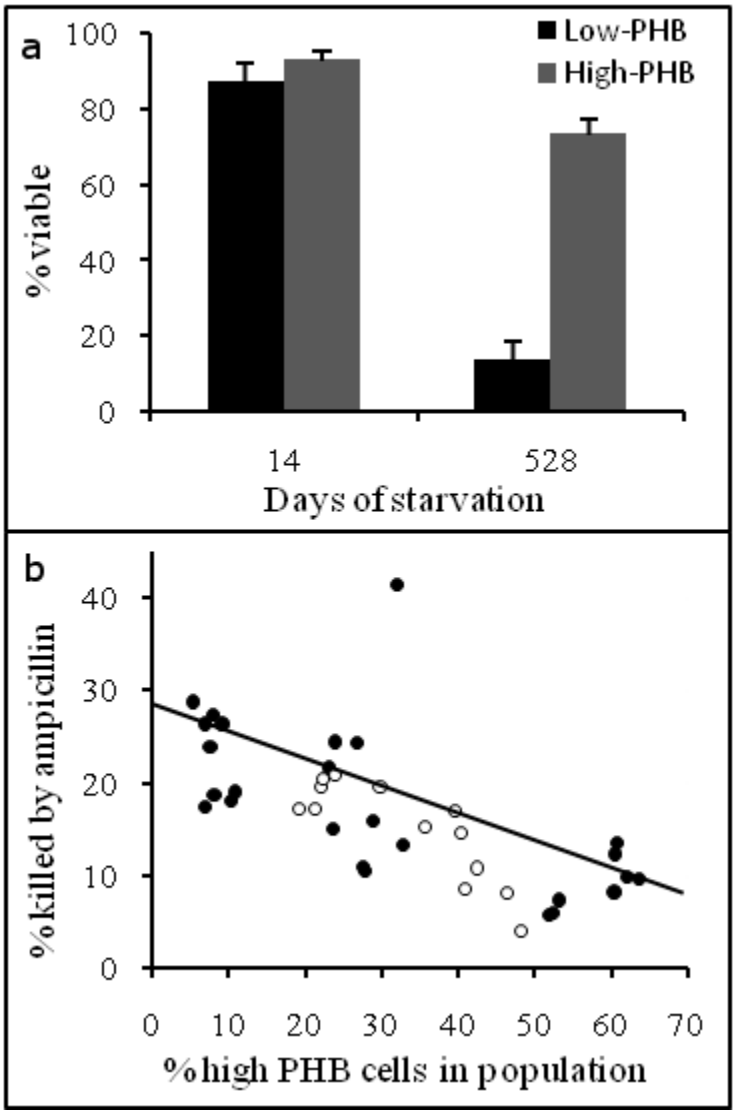

Figure 4-3- The high-PHB phenotype is starvation- and antibiotic-tolerant. **a,** Shortly after phenotypic differentiation (14 days of starvation), both high and low-PHB rhizobia were still equally viable, as shown by live/dead staining. However, after 528 days of starvation, high-PHB cells were significantly more viable than low-PHB cells. Plotted are means ± SEM. **b,** The high-PHB phenotype was also more tolerant of antibiotic exposure. Populations of *S. meliloti* that varied in the percentage of high vs. low-PHB

cells were generated via density gradient centrifugation (open circles) or by starving rhizobia at varying densities (closed circles). These rhizobia were then challenged with ampicillin and assayed for mortality (PI or YO-PRO-1 uptake) by flow cytometry. Populations containing a greater fraction of high-PHB cells were less susceptible to ampicillin. There was no significant difference in linear regression slopes ($t=0.8$, $p=0.43$, $n=41$, ANCOVA) or intercepts ($t=1$, $p=0.32$, $n=41$, ANCOVA) for the two methods of manipulating the frequency of high-PHB cells.

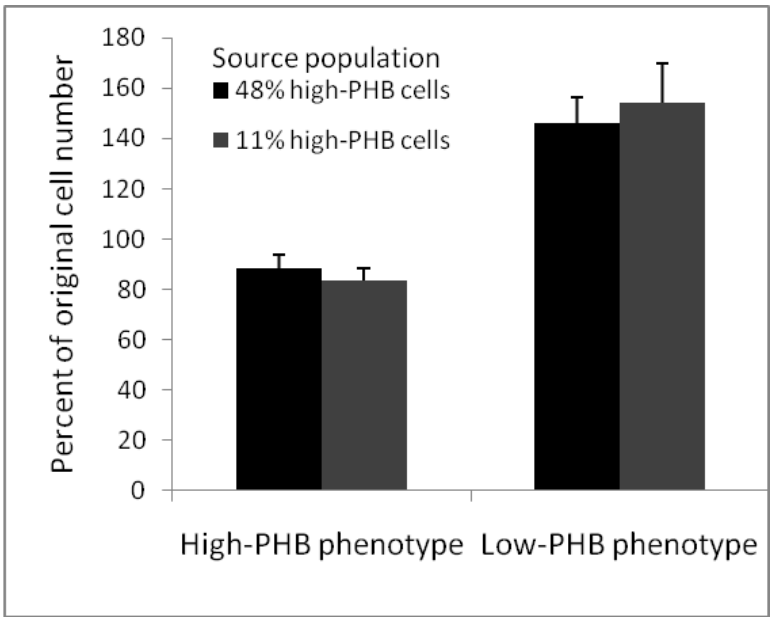

Figure 4-4- High and low-PHB phenotypes are not heritable. Two populations of starved *S. meliloti* were generated using density gradient centrifugation in which high-PHB cells were either depleted (11%) or enriched (48%). 48 isolates drawn from each fraction were grown in M9 media + 10 g L^{-1} mannitol, and then starved. Shown are the mean number of high and low-PHB cells formed after 29 days of starvation for the 48 isolates taken from each fraction. Error bars are the SEM. The phenotype of the ancestral cell had no discernable effect on the bet-hedging behavior.

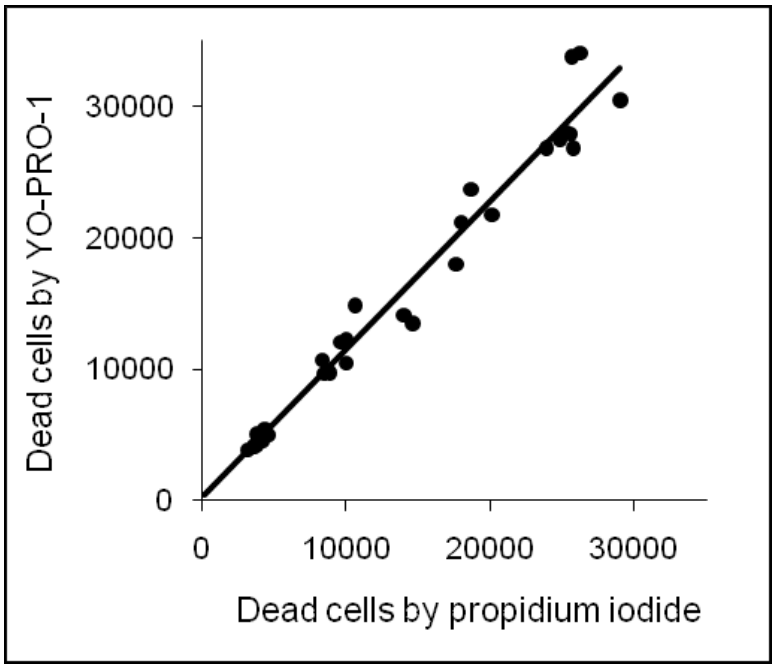

Figure 4-5- Comparison of the fluorescent live-dead stains propidium iodide (PI) and YO-PRO-1. S*inorhizobium meliloti* str. 1021, grown in M9 + 10 g L^{-1} manntiol, was exposed to 0-50% ethanol (in 5% increments) for two minutes. These rhizobia were stained with YO-PRO-1 or PI and analyzed flow cytometrically. For both stains live and fixed (30% EtOH for 30 minutes) controls were run to establish the fluorescence range of live and dead cells; and then viability was determined by gating. Staining with PI: as described in the methods section. Staining with YO-PRO-1: rhizobia were stained with 1% of the YO-PRO-1 stock solution (0.1 mM in DMSO) for 30-90 minutes, and then assayed flow cytometrically, collecting data on the FL1 detector (530±15nm filter). YO-PRO-1 was highly correlated with PI ($p<0.0001$, $n=33$, $r^2=0.97$, linear regression) but was slightly more sensitive, staining 12.5% more cells than PI ($t=32.84$, $p<.0001$, two-sided t-test on regression slope).

CHAPTER 5: WHEN STRESS PREDICTS A SHRINKING GENE POOL, TRADING EARLY REPRODUCTION FOR LONGEVITY CAN INCREASE FITNESS, EVEN WITH LOWER FECUNDITY

Background: Stresses like dietary restriction or various toxins increase lifespan in taxa as diverse as yeast, *Caenorhabditis elegans*, *Drosophila* and rats, by triggering physiological responses that also tend to delay reproduction. Food odors can reverse the effects of dietary restriction, showing that key mechanisms respond to information, not just resources. Such environmental cues can predict population trends, not just individual prospects for survival and reproduction. When population size is increasing, each offspring produced earlier makes a larger proportional contribution to the gene pool, but the reverse is true when population size is declining.

Principal findings: We show mathematically that natural selection can favor facultative delay in reproduction when environmental cues predict a decrease in total population size, even if lifetime fecundity decreases with delay. We also show that increased reproduction from waiting for better conditions does not increase fitness (proportional representation) when the whole population benefits similarly.

Conclusions: We conclude that the beneficial effects of stress on longevity (hormesis) in diverse taxa are a side-effect of delaying reproduction in response to environmental cues that population size is likely to decrease. The reversal by food odors of the effects of dietary restriction can be explained as a response to information that population size is less likely to decrease.

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Introduction

Food odors can reverse the life-extending effects of dietary restriction (Libert et al. 2007). Intermittent fasting increases longevity in vertebrates and *Caenorhabditis elegans,* even when there is little to no reduction in overall calorie consumption (Anson et al. 2005; Honjoh et al. 2009). A crowding pheromone delays reproduction and extends lifespan in *C. elegans* (Golden and Riddle 1982; Fielenbach and Antebi 2008). Water that contained starving pond snails induces delay in pond snail egg development, doubling embryo maturation time (Voronezhskaya et al. 2004). Repeated mild heat stress extends lifespan in *Drosophila* at the expense of fecundity (Hercus et al. 2003). Cold stress induces diapause in *Drosophila*, halting reproduction and extending lifespan (Tatar et al. 2001). Low doses of many plant-defense toxins, including some that are not antioxidants, can extend lifespan (Mattson and Cheng 2006). We show that phenomena like these can be explained as responses of reproductive timing to information that predicts, not an individual's own particular prospects, but rather changes in overall population size.

The evolution of reproductive delay has previously been explained by three main hypotheses. First, reproductive delay can increase fitness when older individuals are more reproductively successful than they would have been earlier (Perrin and Rubin

1990; Komdeur 1992; Koons et al. 2008), even if fecundity with delay is less than a younger individual might have achieved under better conditions (Stearns and Koella 1986). Second, reproductive delay can act as a bet-hedging mechanism, increasing fitness in unpredictable environments (Wilbur and Rudolf 2006; Koons et al. 2008). The third hypothesis, by far the most widely-cited in the aging literature, is that reproductive delay during periods of adversity promotes survival until conditions improve, thereby increasing individual fecundity and fitness (Tatar et al. 2001; Barbieri et al. 2003; Alcedo and Kenyon 2004; Wood et al. 2004; Anson et al. 2005; Walker et al. 2005; Libert and Pletcher 2007; Fielenbach and Antebi 2008; Monaghan et al. 2008). We show that delaying reproduction can be adaptive even when none of the above hypotheses are true. Our alternative hypothesis considers the fitness consequences of plasticity in reproductive timing in response to environmental cues predicting changes in overall population size.

Changes in population size play a fundamental role in determining the evolutionary consequences of the timing of reproduction (Fisher 1930; Hamilton 1966). Stable population size favors early reproduction, because of the risk of dying before the next opportunity to reproduce. In growing populations, early reproduction is favored even more strongly, because each offspring added to a smaller current gene pool is a larger proportional contribution than one added to a larger future gene pool (Williams 1957; Hamilton 1966). Conversely, natural selection favors delayed reproduction when overall population size is decreasing (Hamilton 1966; Pianka 1999). Evolution of reproductive timing in shrinking populations has often been considered "somewhat academic"

(Charlesworth 1980) because "a population with a negative growth rate would soon go extinct" (Hughes and Reynolds 2005). However, short-term fluctuations in population size are common. Here, we show that facultative delay in reproduction during periodic population declines enhances fitness. A key point is that, if organisms can use environmental cues to predict population decline and consequentially delay reproduction, fitness can be enhanced even without increases in lifetime fecundity.

Results

There are two kinds of cues relevant to delaying reproduction: those specific to individuals and those that apply to the population as a whole. As an example, dietary restriction may directly affect individuals, and may also provide information about future survival and reproduction of an individual or the population as a whole. We will start with some simplifying assumptions before considering more general cases.

Consider a semelparous species with haploid genetics and no parental care. Each individual reproduces only once, at either one or two years of age, then dies. Assume that all reproduction occurs in summer and juveniles (or adults delaying reproduction) die only in winter.

An individual increases its lifetime fecundity by delaying reproduction only if

$$S' F_2' > F_1' \qquad [1]$$

where S' is the focal individual's chance of surviving to reproduce in year 2 if it delays reproduction, and F_1' and F_2' are its expected fecundity as a one- or two-year-old. Given the trade-off between current and future reproduction -- semelparity is an extreme example -- delaying reproduction might increase fitness if 1) fecundity increases with age or experience, or 2) fecundity will increase due to improvement in conditions (e.g., weather or food). To emphasize our main point, we initially assume that neither is true. This would be the case if there is no benefit to age and if favorable and unfavorable periods last long enough, relative to the generation time, that an individual cannot wait until conditions change to reproduce. We therefore assume no difference between years 1 and 2, so $F_2' = F_1'$. Because S' cannot exceed one, delaying reproduction cannot increase expected lifetime fecundity.

While sometimes used synonymously, lifetime fecundity is not the same as fitness. Natural selection depends, not on absolute numbers, but on proportional representation in the population (Hamilton 1966; Charlesworth 1980). If we calculate changes in proportional representation immediately after year-2 reproduction, then a rare allele for facultative delay in reproduction will increase in frequency, within a population of first-year reproducers, if and only if

$$S' F_2' > F_1 J_1 F_2 \qquad [2]$$

where F_1 and F_2 are year-1 and year-2 fecundity for the overall population and J_1 is the fraction of those juveniles born in year 1 that survive to reproduce in year 2. In terms of the information available to the focal individual in year 1 that is relevant to delaying

reproduction, we assume that carry-over effects of year-1 individual condition (fat reserves, etc.) to its year-2 fecundity F_2' are negligible, relative to shared-environment effects on year-2 fecundity of the whole population. Therefore $F_2' = F_2$. Delaying reproduction is then favored if

$$S' > F_1 J_1 \qquad [3]$$

The left side of Eqn. 3 is the focal-individual-specific chance of adult survival to year 2, while the right side is overall population change, the ratio of total population in year 2 to that in year 1. If S' takes its maximum possible value of 1.0, then delaying reproduction increases the focal individual's fitness (proportional representation in the population) if and only if population decreases from year 1 to year 2. Even if adult survival is uncertain, a more drastic population decrease can still favor delaying reproduction. For example, natural selection will favor facultative delay in reproduction if there is reliable information that the population will decrease by 50% (F_1J_1=0.5) and the individual-specific chance of adult survival S' is > 50%. Note that Eqn. 3 does not include J_2, so the benefits of delay also do not depend on whether juvenile survival is better in year 2 than in year 1.

The above analysis assumed that the duration of favorable or unfavorable conditions greatly exceeds individual lifespan and that older individuals receive no benefit from growth or experience, so that reproductive delay never increases individual fecundity. In real populations, however, such benefits may be common. Relaxing both assumptions,

we show that a genotype with facultative delay in reproduction in response to cues predicting population decline can invade a population of first-year reproducers and that facultative delay is an evolutionarily stable strategy (ESS). Further, we show that the increase in reproductive success that individuals gain by delaying reproduction until conditions improve does not necessarily increase their relative fitness.

Consider a semelparous population composed of a genotype (A) that reproduces at age one year (like an annual plant) and another genotype (FD) that facultatively delays reproduction for one year when conditions are bad (i.e., if population is likely to decrease), then reproduces the next year regardless of year quality (like some facultative biennials). Because no individual lives more than two years, we can enumerate all possible fitness effects of variation among years by considering four possible two-year combinations of good and bad year quality: BB, GG, GB, and BG. An initially rare FD can invade a population of A when the two-year growth rate (f , the ratio of individuals in spring of year 3 to those in spring of year 1) of FD is greater than the overall population growth rate, i.e., that of A. Let F be average adult fecundity, J be the average probability that a juvenile will survive to reproduce as an adult in the next year, δ (constrained so that $\delta > FJ-1$) be the difference in FJ between an average year and a good or bad year, S be the probability that an adult delaying reproduction during a bad year survives to reproduce the next year, and α be the reproductive advantage of second year adults. All other conditions are the same as in the first model.

During two successive good years (GG) both genotypes reproduce each year. Growth rates are then:

$$f_A = ((1+\delta)FJ)^2$$

$$f_{FD} = ((1+\delta)FJ)^2 \qquad [4]$$

In an expanding population $f_{FD} = f_A$ and so FD cannot invade a population of A.

Growth rates during two bad years (BB):

$$f_A = (1-\delta)^2 FJ^2$$

$$f_{FD} = S(1-\delta)FJ\alpha \qquad [5]$$

Here $f_{FD} > f_A$ if $(1-\delta)FJ < \alpha S$, meaning that FD can invade if overall population decline in a bad year is worse than the consequences of delay: increased reproduction as a two-year old (if $\alpha > 1$) but a decreased probability of surviving to reproduce.

Growth rates when a good year follows a bad year (BG):

$$f_A = (1+\delta)(1-\delta)FJ^2$$

$$f_{FD} = S(1+\delta)FJ\alpha \qquad [6]$$

Again, we find that $f_{FD} > f_A$ if $(1-\delta)FJ < \alpha S$.

Finally, when a bad year follows a good year (GB), both genotypes reproduce the first year, and the FD delays reproduction during the second. As before, we compare expected genotype growth rates across two years, but because the fitness consequence of FD's delay during the second year depends on year 3 quality, we calculated the expected value for 3rd year reproduction assuming G and B occur with equal probability:

$$f_A = (1+\delta)(1-\delta)FJ^2[\frac{1}{2}(1+\delta)FJ + \frac{1}{2}(1-\delta)FJ]$$

$$f_{FD} = S(1+\delta)FJ[\frac{1}{2}(1+\delta)FJ\alpha + \frac{1}{2}(1-\delta)FJ\alpha] \qquad [7]$$

As with BG and BB, we find that $f_{FD} > f_A$ if $(1-\delta)FJ < \alpha S$.

In short, neither genotype gains an advantage during a population expansion (Eq.4), but a rare FD can invade a population of A in any series of years involving population decline (Eq. 5-7).

Can FD maintain dominance once common? FD is an ESS if A cannot invade when rare. We find that f_A is not greater than f_{FD} during population expansion (Eq. 4), and so cannot invade, and that $f_A < f_{FD}$ during a series of years that include population decline when $(1-\delta)FJ < \alpha S$. Thus, FD is an ESS under the same conditions that it can invade a population of genotype A.

Because the success of the facultative-delay strategist FD depends on parameter values, we found the critical values under which FD dominates A, specifically focusing on the probability (*S*) that a 1-year old delaying reproduction survives to reproduce in year 2. Holding α, *FJ*, and δ constant, the minimum value for *S* required for FD to obtain a relative fitness advantage is:

$$S^* = \frac{(1-\delta)FJ}{\alpha} \qquad [8]$$

Qualitatively, Eqn. 8 shows that facultative delay in reproduction increases fitness when survival from year 1 to 2 (S) is large or when either survival from birth to year 1 (J) or fecundity (F) is small, so that population decreases. Large variation among years (δ) makes 1- δ small, favoring FD, as does any increase in fecundity with age (α). Equations 1-3 show that delay can be favored even if it does not increase fecundity, but there are also cases where delay will increase fecundity, as modelled in Equations 5-8. In such cases, can we partition the benefits of delay into those that depend on increased fecundity, versus those that result solely from increased representation in a future population?

The facultative-delay genotype (FD) postpones reproduction until its second summer if environmental cues predict a population decrease before then. If the second year is also unfavorable, then this delay has no effect on FD's individual fecundity, because its reproductive success in either bad year is equivalent. However, if the next year is favorable, delay results in an increase in FD's reproductive success. Therefore, to determine how much of the increase in FD's fitness is due to increases in its individual reproductive success, we subtract the relative fitness of FD f_{FD}/f_A during BG years (when delay increases FD's fecundity) from its relative fitness during BB years (when delay does not increase FD's fecundity):

$$\left[\frac{f_{FD}}{f_A}\right]_{BG} - \left[\frac{f_{FD}}{f_A}\right]_{BB} = \frac{S\alpha}{(1-\delta)FJ} - \frac{S\alpha}{(1-\delta)FJ} = 0 \qquad [9]$$

Surprisingly, perhaps, increased fecundity from delay makes no contribution to relative fitness. The benefit of increased individual reproductive success by FD is exactly balanced by increases in the reproductive success of the next generation of genotype A, which never delays reproduction. Thus, under our assumptions, the fitness benefit of reproductive delay is entirely due to increased proportional representation of the alleles causing delay, not an increase in reproductive success from waiting for better conditions.

The fitness consequences of variation in key life-history parameters are shown in Figure 5-1. Although age-linked increased reproduction by second year FDs (α) favors delay (Eqn. 8 and Fig. 1), $\alpha > 1$ is not required for selection to favor FD. Even if $\alpha < 1$ so that aging reduces reproductive success, reproductive delay can still be favored, so long as $(1-\delta)FJ/S < \alpha$. If stress is correlated with population decline (but not a perfect predictor), some bet-hedging in reproductive delay (Wilbur and Rudolf 2006; Koons et al. 2008) may be evolutionarily favored.

Discussion

Although some of our detailed predictions might depend on our specific assumptions, such as semelparity, tradeoffs between current and future reproduction are probably universal even in iteroparous species like humans. As with Williams' antagonistic pleiotropy hypothesis (Williams 1957), we accept multiple mechanisms for tradeoffs between reproduction and survival. Reproduction can increase immediate or subsequent

mortality due to harmful male-female interactions during mating (Tatar and Promislow 1997; Findlay et al. 2008), fights over mates or breeding territory, sexually-transmitted disease, or an increased risk of predation, in addition to the direct metabolic costs of reproduction and care of young. Body size, metabolic rate, blood pressure, and hormone levels that are optimal for reproduction are often not optimal in terms of longevity. For example, increased fecundity at the expense of longevity has been ascribed to high levels of insulin and insulin-like growth factor in taxa as diverse as yeast, *Drosophila*, *C. elegans*, and mammals (Tatar and Promislow 1997; Findlay et al. 2008). Therefore, physiological or behavorial responses that delay reproduction will often increase longevity as a side-effect.

Our evolutionary model makes several predictions that could be tested in various species (Rauser et al. 2009). First, cues that predict overall population decline may trigger different behaviors or physiological states than cues specific to an individual's own likely survival and reproduction. For example, low current food intake but high fat reserves might predict, respectively, an overall population decrease (FJ <1) but a greater individual chance (S') of surviving to reproduce in a subsequent year. In this case, two seemingly conflicting indicators both favor delaying reproduction, which will often increase longevity. The observation that food odors can partially reverse the effects of dietary restriction on longevity (Alcedo and Kenyon 2004; Libert et al. 2007) is consistent with this hypothesis, if food odors predict the availability of resources linked to overall population growth.

Second, cues unrelated to food supply that reliably predicted population decline over the evolutionary history of a species should also tilt the balance towards later reproduction, often increasing longevity. Facultative delayed reproduction in response to other cues of impending population decline, such as population density, weather, predation or territorial conflict may be common. These responses could be linked to undiscovered physiological mechanisms with possible medical applications. For example, the nematode *C. elegans* delays reproduction and extends lifespan by forming the relatively inactive dauer stage. Recovery from this state is stimulated by food but repressed by a pheromone that indicates high population density (Golden and Riddle 1982). The interaction of these signals acts in the direction predicted by our hypothesis, favoring earlier reproduction when overall population is likely to increase.

Third, directly harmful effects of environmental factors may sometimes be outweighed by indirect health benefits linked to the reduced fecundity they trigger. For example, moderate consumption of foods containing plant defensive toxins (e.g., glucosinolates, catechins, curcumin, resveratrol) can induce similar changes in gene regulation as dietary restriction (Mattson and Cheng 2006), delaying reproduction and increasing longevity (Wood et al. 2004). The xenohormesis hypothesis explains this as a form of interspecific eavesdropping: organisms have evolved to respond to stress-linked phytochemicals as an early warning of environmental degradation (Howitz and Sinclair 2008). Indeed, many of these plant defensive compounds are synthesized in response to stresses that slow plant

growth, and their ingestion may thus predict a reduction in food availability, starvation, and a decline in overall population size. Alternatively, ingestion of plants with high constituitive levels of defensive toxins may result from a lack of less-toxic preferred foods. Under this "famine food" hypothesis, ingestion of these toxins, as well as spoilage indicators such as fermentation by-products, predicts starvation and short-term population decline, favoring physiological changes that delay reproduction but improve short-term health.

Those focused on human health are naturally more interested in proximate mechanisms of aging than in ultimate evolutionary explanations. With respect to the former, we agree that it may be necessary to "generalize with caution" (Walker et al. 2005). However, our evolutionary argument is sufficiently general that it should apply to all species and to a wide variety of environmental cues.

Methods

Figure 5-1 was generated using Mathematica 7.0.

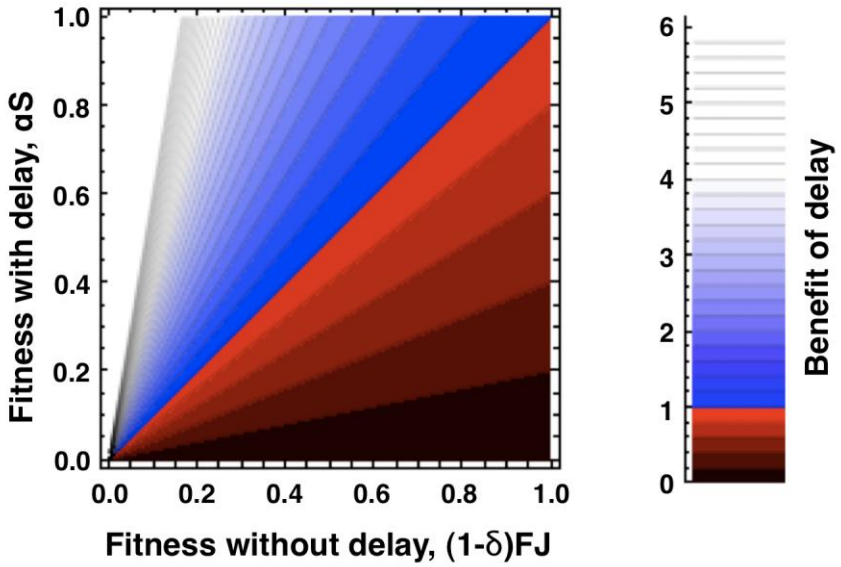

Figure 5-1. Fitness consequences of reproductive delay during population decline. An initially rare genotype (FD) that facultatively delays reproduction during a bad year is evolutionarily favored (blue shading) when αS, the increased fecundity of older individuals, reduced by the chance of dying before second-year reproduction, is greater than the overall change (decrease) in population size. This change, $(1-\delta)FJ$, is also the reproductive success of the initially predominant annual genotype (A). This fitness landscape does not change regardless of whether conditions are better next year or not, demonstrating that the fitness benefit of delay is due, not to an increase in fecundity with improved conditions, but rather to an increase in the proportional representation of FD in a shrinking population.

CHAPTER 6: INTEGRATION OF PHENOTYPIC PLASTICITY AND BET HEDGING IN FREE-LIVING *SINORHIZOBIUM MELILOTI*

Summary

Natural environments are variable. Two phenomena have repeatedly in response to this uncertainty: bet hedging and phenotypic plasticity. Despite a vast literature, little empirical work has examined the interaction of these strategies. The bacterium *Sinorhizobium meliloti* hedges its bets when starved, using energy and carbon stored in the polyester poly-3-hydroxybutyrate (PHB) to form two discrete phenotypes: high-PHB cells having long-term survival and low-PHB capable of rapid growth. Here we demonstrate that *S. meliloti* integrates bet hedging and phenotypic plasticity by using competitor density as a cue predicting the duration of starvation. When starved at high density, *S. meliloti* delays reproduction and forms fewer low-PHB offspring. In long-term experiments, this increased the number of viable *S. meliloti* after 42 days of starvation, but reduced early-starvation reproduction and thus early-starvation fitness.

Introduction

Temporal change in environmental conditions is a fundamental driver of life-history evolution (Levins 1968). Uncertain future conditions can favor the evolution of bet hedging strategies in which a single genotype expresses high phenotypic variability (Cohen 1966; Seger and Brockmann 1987; Simons 2009). Diversification bet hedging increases the probability that some individuals of a single genotype are well adapted to their environment, thereby reducing variation in fitness through time and maximizing long-term geometric mean fitness (Seger and Brockmann 1987; Stearns 2000; Simons 2009). Diversification bet hedging, however, is only adaptive if future conditions are insufficiently predictable. If organisms can predict environmental changes before they occur, then genotypes that adopt a more optimal phenotype will be fittest if the cost of this phenotypic plasticity is low (Scheiner 1993). In contrast to diversification bet hedging, when phenotypic plasticity allows a genotype to maintain a position atop shifting fitness peaks, the less phenotypic variance the better. Evolutionary theory demonstrates that in stochastic environments with limited predictability, a strategy that integrates phenotypic plasticity and bet hedging by weighting diversification by the probability of future events can be optimal (Cohen 1966; DeWitt and Langerhans 2004; Wong and Ackerly 2005). While the literature on both bet hedging and phenotypic plasticity is vast, few studies have empirically examined their interaction.

The bacterium *Sinorhizobium meliloti* accumulates large amounts of the storage polyester poly-3-hydroxybutyrate (PHB), apparently as a contingency against starvation (Ratcliff et

al. 2008). Starving *S. meliloti* face a direct trade-off between reproduction and longevity because PHB can be used for either reproduction or for long-term survival (Chapter 4). Short-term starvation favors early use of PHB for reproduction, because PHB that is not used for reproduction by the time food returns is effectively wasted. If starvation turns out to be long-term, conservation of PHB for later use can increase fitness for two reasons. First, reproducing later can increase the number of surviving progeny, because resources return before they starve to death. Second, if starvation causes an overall decline in population size, then future offspring increase parental fitness more than current offspring because each future offspring will constitute a larger fraction of a smaller gene pool (Fisher 1930; Hamilton 1966; Ratcliff et al. 2009).

Initially-high-PHB *S. meliloti* have previously been shown to hedge their bets when starved, forming both high-PHB 'persister' phenotypes that survive long-term starvation and antibiotic exposure, and low-PHB 'grower' phenotypes well-suited for short-term starvation (Chapter 4) . Starvation, however, does not necessarily occur in an information vacuum. Instead, environmental cues such as competitor density may provide information about the probability that an improvement in environmental conditions will end starvation. For example, higher competitor density reduces the per capita share of any food that is encountered, and as a result, influxes of food that would effectively end starvation at low densities may fail to do so at high densities. Thus at higher competitor densities, many of the influxes of food that would have ended starvation at low densities have little effect, resulting in an increased expected duration of

starvation. This explains why diverse taxa respond plastically to high competitor density during resource limitation, forming phenotypes with improved ability to survive starvation (Golden and Riddle 1982; Lazazzera 2000; Viney and Franks 2004). For example, signals produced by starved and crowded juvenile pond snails cause conspecific eggs to delay maturation, doubling the time it takes until they emerge as feeding juveniles (Voronezhskaya et al. 2004). Here we determine whether *S. meliloti* pursues an integrated strategy, hedging their bets against long-term starvation more when competitor density predicts starvation of longer duration.

Materials and Methods

Strains, culture and starvation

Sinorhizobium meliloti strain 1021 was used for all experiments. Rhizobia were cultured in M9 minimal media (Miller 1992) with 10 g L^{-1} mannitol, a media previously shown to result in PHB accumulation (Ratcliff et al. 2008, Chapter 4). Before starvation, rhizobia were cultured for 6 days in 50 mL M9 in 125 mL Erlenmeyer flasks at 22°C, shaken at 100 RPM. Rhizobia were double washed in C-free M9 media (containing no mannitol, 0.1 g L^{-1} thiamine, and HPLC-grade water). To limit carbon contamination, all glassware used in the experiments was first rendered C-free by acid washing in 0.6 M HCl for 1 hour and overnight pyrolization at 550 °C. Rhizobia were starved in 2 mL microcentrifuge tubes as previously described (Ratcliff et al. 2008).

Assays for PHB and reproduction

For all experiments, the fraction of rhizobia with high vs. low PHB was determined by staining with the PHB stain Nile Red and analyzing rhizobia on a Benton Dickinson FACSCalibur flow cytometer as previously described in (Ratcliff et al. 2008) and in Chapter 4 of this thesis. High and low-PHB rhizobia were identified by gating (Figure A1). Reproduction in the long-term experiment was determined by drop-plating rhizobia onto yeast mannitol agar plates (Somasegaran and Hoben 1994). Reproduction for all other experiments was determined flow cytometrically, using the live/dead fluorescent probe YO-PRO-1 as previously described in Chapter 4 of this thesis.

Statistics

Two-phase segmented ordinary least squares linear regressions (Toms and Lesperance, 2003) were fit to the short-term (0-108 h) starvation experiment by solving linear regressions for each replicate (12 per treatment), and then using two-way ANOVA and Tukey's HSD to determine if the slopes of the first and second phases of each treatment were significantly different at $\alpha = 0.05$. If they were not significantly different, a single linear regression was fit for the treatment. All other statistical tests are as stated in the text. Assumptions of all parametric tests were checked and met for all data with the exception of the experiment in which we diluted previously starved cells; for this we used the non-parametric Wilcoxon rank-sum test as an alternative to a 1-way t-test because of our small sample sizes. Statistics were computed in JMP 8.0.

Results

We first evaluated PHB use and reproduction in initially high-PHB rhizobia starved at low ($5 \cdot 10^5$ mL^{-1}), medium ($5 \cdot 10^6$ mL^{-1}) and high ($5 \cdot 10^7$ mL^{-1}) densities (Figure 6-1). When starved, rhizobia reproduced, differentiating into high and low-PHB phenotypes. During the first 36 hours of starvation, rhizobia at the lowest density reproduced 9.6-fold more quickly than the rhizobia starved at the higher densities, which did not differ (Figure 6-1a; main effect of density, $p < 0.0001$, $F_{5,48} = 333.9$, two-way ANOVA, differences between treatments assessed with Tukey's HSD). This resulted in a faster decrease in the frequency of high-PHB cells in the population starved at low density (Figure 6-1b; main effect of density, $p < 0.0001$, $F_{5,48} = 274.2$, two-way ANOVA, differences between treatments assessed with Tukey's HSD). *S. meliloti* thus respond to competitor density plastically, delaying reproduction when the density of competitors was high.

For this plasticity to be adaptive, rhizobia that delay use of PHB for reproduction must have a higher fitness during long-term starvation than those that use PHB for reproduction (Stearns 1989). We therefore performed a similar experiment over a long time frame, starving initially high-PHB rhizobia at 10^5, $5 \cdot 10^5$, or $5 \cdot 10^6$ cells mL^{-1} (Figure 6-2a), and examined rhizobial fitness after short- and long-term starvation. Three replicate populations of rhizobia were destructively sampled for each density of starvation, and population size determined by plate-counting. Reproduction, as measured by the population's increase in size, was averaged over the first period of starvation (14-

42 days) or over the latter period (56-154 days) of starvation, and a two-way ANOVA conducted to determine if there was a significant interaction between competitor density and reproductive timing. For all three treatments there was significant difference in rhizobial fitness for early and late starvation. Rhizobia starved at 10^5 and $5 \cdot 10^5$ cells mL^{-1} reproduced early, which came at a cost to fitness during long-term starvation. This early reproduction would have increased their fitness if sufficient food were encountered to end starvation within the first 40 days. In contrast, rhizobia starved at $5 \cdot 10^6$ cells mL^{-1} reproduced late, sacrificing early starvation fitness for late starvation fitness (Figures 6-2b and A2; interaction term, $p < 0.0001$, $F_{2, 90} = 48.34$, differences assessed with Tukey's HSD). This delay would have increased fitness if sufficient food were encountered to end starvation during the latter 100 days of the experiment, but would be maladaptive if starvation ended within the first 40 days. Thus if competitor density predicts the duration of starvation, the phenotypic plasticity it induces is adaptive.

While high density during starvation induces delayed reproduction, we do not know if this behavior is fixed after this initial diversification, or if starving rhizobia respond to changes in competitor density during starvation. We thus starved a population of high-PHB rhizobia at $5 \cdot 10^6$ cells mL^{-1} for 10 days, generating a population bimodal for PHB per cell, and then reduced the number of competitors 10-fold by dilution with fresh starvation media. These rhizobia were starved for 3 additional days and assayed for population size and PHB content. A reduction in competitor density caused an average of 66% of the high-PHB rhizobia to break dormancy, consuming PHB for reproduction

and becoming low-PHB cells ($p = 0.031$, $W_+ = 10.5$, $n = 6$, Wilcoxon signed-rank test against 0%). As a result, the overall population size increased by 2.8-fold ($p = 0.031$, $W_+ = 10.5$, $n = 6$, Wilcoxon signed-rank test against 1). High-PHB persister cells thus resume growth when environmental conditions improve, even if this change is not a return of food itself, but rather a cue predicting a shorter-term return of food or reduced competition for resources when they return.

Evolutionary theory predicts that in a stochastic environment with limited predictability, bet hedging should be integrated with phenotypic plasticity linked to sensing of environmental cues (DeWitt and Langerhans 2004; Wong and Ackerly 2005). So far, our experiments have demonstrated that such an interaction exists for the bacterium *S. meliloti*: when competitor density predicts a shorter duration of starvation, rhizobia form fewer high-PHB persister cells and invest more PHB in short-term reproduction. However, our experiments have only examined a few levels of competitor density. To quantitatively determine the relationship between plasticity and bet hedging, we generated a reaction norm of the interaction (Stearns 1989) by starving initially high-PHB rhizobia at $2 \cdot 10^5$ to $2 \cdot 10^7$ cells mL^{-1} in 11 different densities distributed $\log_{10}$-linearly. After 7 days rhizobia were assayed for reproduction and PHB content flow cytometrically. The reaction norm was nonlinear (Figure 6-3; $p < 0.0001$, $F_{2,30} = 132.2$, $r^2 = 0.9$, ANOVA of the logistic regression). First and second-degree components of the regression equation were highly significant ($p < 0.0001$, 2-way t-tests). *S. meliloti* were

thus sensitive to changes in competitor density when density was low ($2{\cdot}10^5$ - $5{\cdot}10^6$), but insensitive to further increases in competitor density.

Discussion

Previous work has shown that when *S. meliloti* are starved, a single genotype hedges its bets by reproducing and differentiating into high-PHB 'persister' and low-PHB 'grower' phenotypes. The persister phenotype delays reproduction, retaining PHB, and has improved survival of long-term starvation and ampicillin exposure (Chapter 4). Here we show that the extent of bet-hedging is contingent on rhizobial social context. When the density of conspecific competitors is high, clonal populations of *S. meliloti* reproduced less and formed more phenotypically-diverse populations containing a larger fraction of high-PHB cells. These results are consistent with the hypothesis that *S. meliloti* integrates bet hedging and phenotypic plasticity, using competitor density as a cue predicting the duration of starvation. There were no conditions tested in which phenotypic plasticity was complete; rhizobia starved at all densities formed bimodal populations containing high and low-PHB cells, and in all experiments rhizobia starved at greater than $5{\cdot}10^6$ cells mL^{-1} responded minimally to further increases in competitor density.

It is puzzling that all rhizobia, regardless of competitor density, use a significant amount of PHB for reproduction when starved. Why invest resources in offspring that have a high probability of starving to death? We have two hypotheses. First, this

may reflect the limited precision of competitor density as a cue predicting the duration of starvation. Some cues, like photoperiod, predict seasonality with a high degree of precision. Unlike day length which is directly linked to the season, competitor density provides a probabilistic estimate of the duration of starvation, but the precision of this estimate may be low. Precision will depend on the specific resource distribution encountered by *S. meliloti* in the field. When cues are unreliable, strategies that integrate plasticity and bet hedging are substantially better than phenotypic plasticity alone (DeWitt and Langerhans 2004). Some reproduction, regardless of density, may thus be favored due to limited cue accuracy. Alternatively, the production of some grower cells in the face of long-term starvation may reflect a game-theoretic adaptation to their competitor's expected behavior. If most rhizobia go dormant when starved at high density, then the difference between perceived competition and actual competition for resources may be large. *S. meliloti* that use some PHB to produce grower cells may thus be able to take advantage of small amounts of food that would be insufficient to end starvation for the entire population.

While this paper has focused specifically on the use of the storage polyester PHB for current vs. future reproduction by *S. meliloti*, the evolutionary benefits of reproductive delay during long-term starvation should apply generally. Starvation is a common cause of death and has long been known to drive population decline (Malthus 1807; Pianka 1999). Because the fitness benefit of reproduction increases

as overall population size decreases (Fisher 1930), delayed reproduction can be adaptive if an individual faces a trade-off between reproduction and survival (Ratcliff et al. 2009). This trade-off is considered a widespread constraint in life history evolution (Williams 1966), and is supported by experimental manipulations of mating frequency (Zwaan et al. 1995; Oliver and Cordero 2009) and fecundity (Yanagi and Miyatake 2003), selection experiments (Rose 1984; Zwaan et al. 1995), and an observed negative correlation between longevity and fecundity (Tinkle 1969; Ricklefs 2000). As a result, reproductive delay during periods of starvation and population decline may be a nearly universal adaptation. Organisms as diverse as bacteria (Chung et al. 1994), yeast (Roth 1970), rotifers (Gilbert and Schreiber 1998), nematodes (Houthoofd et al. 2002; Fielenbach and Antebi 2008) and mammals (Ball et al. 1947) either become dormant or refrain from reproducing when dietary cues predict resource exhaustion, gaining longevity at the expense of reproduction.

The nematode *Caenorhabditis elegans* and *S. meliloti* react similarly to competitor density during food limitation. Food limitation causes *C. elegans* to form the long-lived and nonreproductive 'dauer' phenotype. When competitor density during food limitation is high, *C. elegans* enters the dauer state earlier, and once a dauer, is inhibited from resuming normal development by high densities of competitors (Golden and Riddle 1982). *C. elegans* responds to competitor density by sensing the concentration of a chemical pheromone (Butcher et al. 2009). While we do not know what the specific chemical cue used by *S. meliloti* is, the mechanism may be similar.

S. meliloti produces and responds to quorum sensing chemicals, N-acyl homsoserine lactones, which may allow them to assess the local density of conspecific competitors. The expression of many genes (Hoang et al. 2004), as well as behaviors such as motility (Hoang et al. 2008) and exopolysaccharide production (Marketon et al. 2003) are regulated by quorum sensing in *S. meliloti*, but further work will be required to determine if it underlies reproductive delay. Standard spent-media assays for quorum sensing cannot be used in this context, because spent media from high-density starvations contains resources that allow small populations of rhizobia to divide, not starve (data not shown).

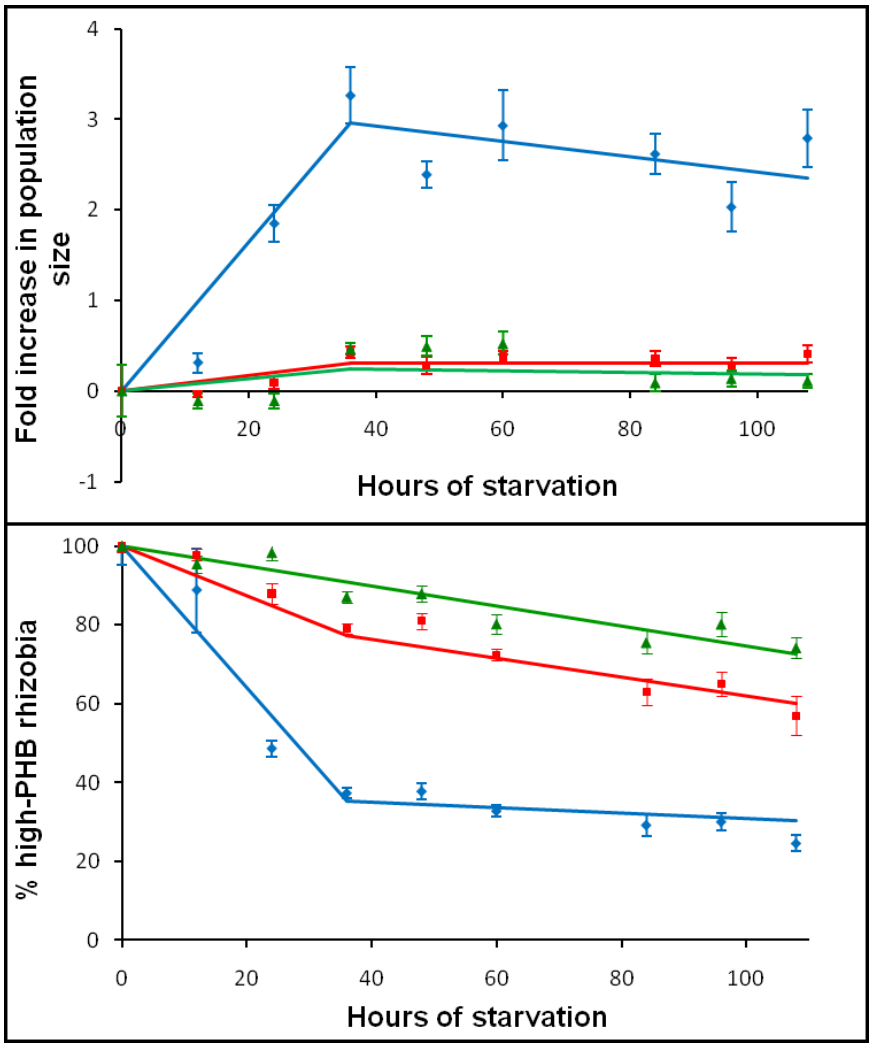

Figure 6-1. High density during starvation inhibits use of PHB for reproduction. High-PHB *S. meliloti* starved at $5{\cdot}10^7$ cells mL^{-1} (green triangles) or $5{\cdot}10^6$ (red squares) delayed (**a**) reproduction and (**b**) PHB use relative *to S. meliloti* starved at $5{\cdot}10^5$ cells mL^{-1} (blue diamonds). Two-phase segmented linear regressions were fit to each treatment (see methods for details). Plotted are means ± SEM of 12 replicates.

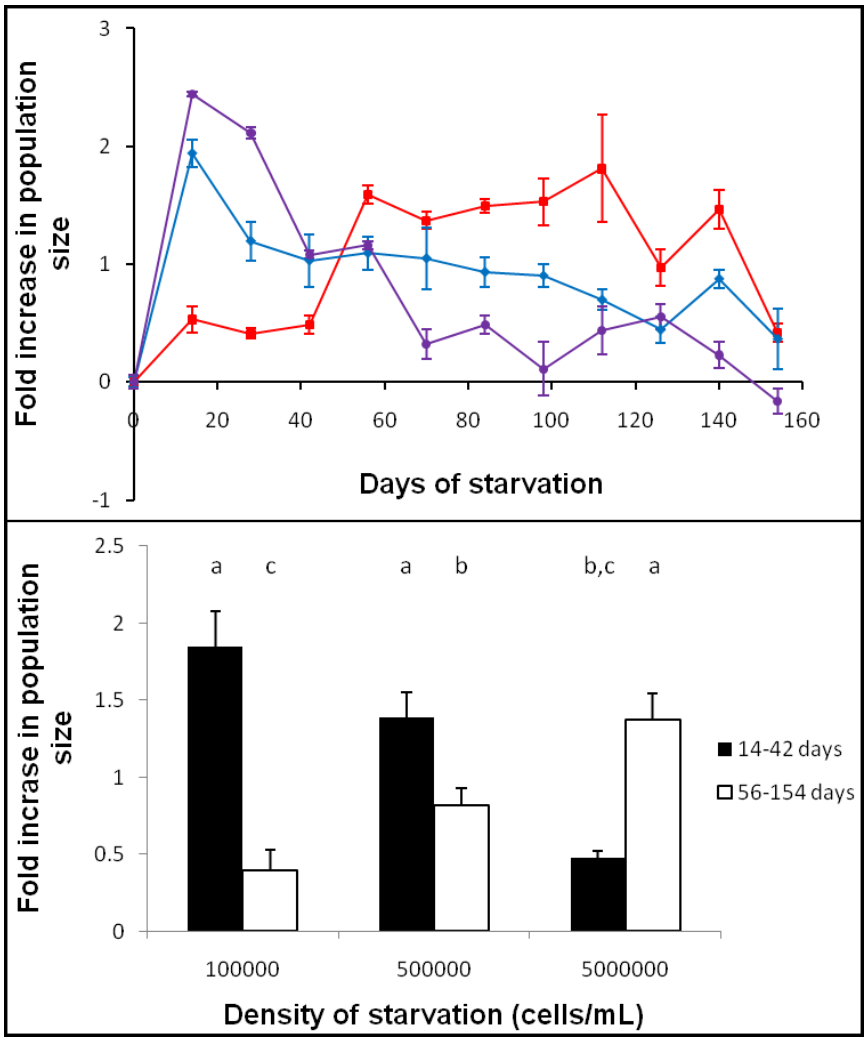

Figure 6-2. High competitor density during starvation induces reproductive delay. (**a**) *S. meliloti* starved at lower densities (10^5 cells mL^{-1}, purple circles, or $5 \cdot 10^5$ cells mL^{-1}, blue diamonds) reproduced early, maximizing fitness during short-term starvation, but paid a fitness cost during long-term starvation. In contrast, rhizobia starved at a higher density ($5 \cdot 10^6$ cells mL^{-1}, red squares) delayed reproduction, sacrificing early starvation fitness for late starvation fitness. Rhizobial population size stayed above initial for the duration of the experiment, with the exception of the rhizobia starved at the lowest density after

154 days of starvation. (**b**) Population size was averaged for each treatment over the first phase of starvation (short-term, 14-42 days) and the second phase of starvation (56-154 days). The two populations starved at low density reproduced significantly earlier than those starved at the high density. Values with different letters are significantly different at $\alpha = 0.05$. Plotted are means ± SEM.

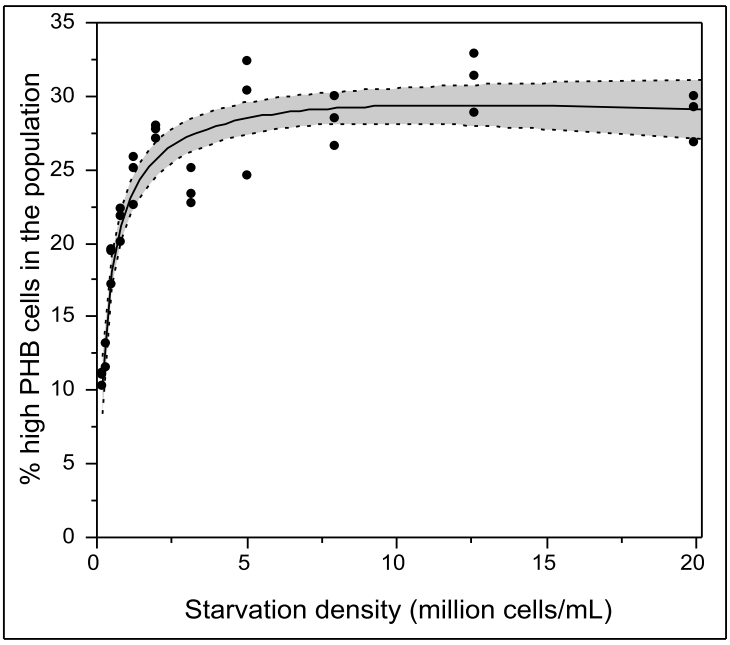

Figure 6-3. Reaction norm of diversification into high and low-PHB phenotypes as a function of competitor density. *S. meliloti* starved at higher densities formed populations with more high-PHB cells, but were far more sensitive to changes in competitor density when overall density was low. The regression function is % high PHB = $-1.3 \cdot \text{Ln}(\text{density})^2 + 5.6 \cdot \text{Ln}(\text{density}) + 22.4$. Plotted are the regression function and its 95% confidence interval (shaded).

APPENDIX: SUPPLEMENTARY FIGURES

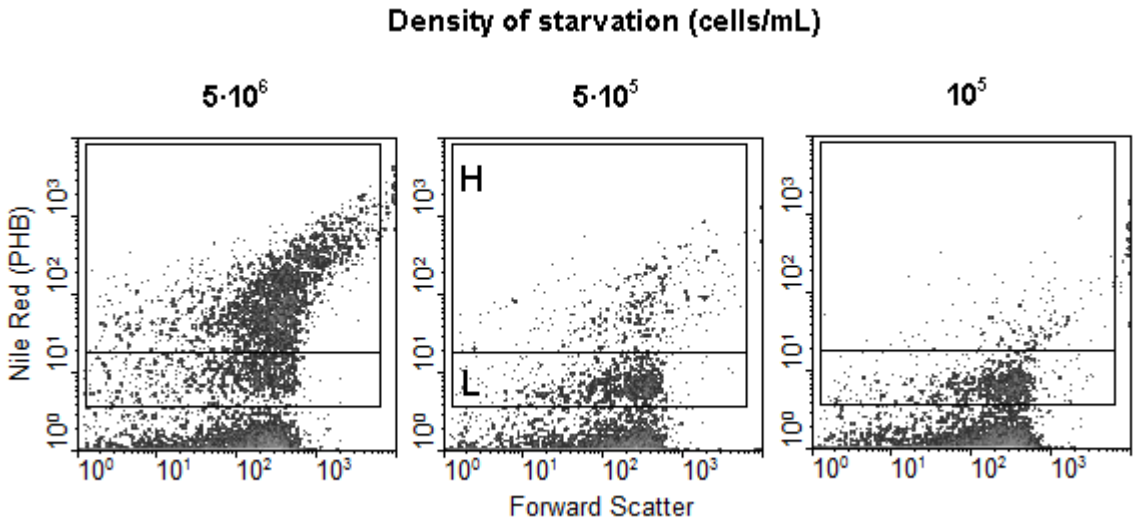

Figure A1- A single high-PHB population of *S. meliloti* was starved at high ($5 \cdot 10^6$), medium ($5 \cdot 10^5$) or low (10^5 cells mL^{-1}) density for 72 hours. PHB/cell was determined by staining with the fluorescent probe Nile Red and analyzing rhizobia flow cytometrically. High and low-PHB phenotypes were determined by gating: high-PHB cells were those from the region marked H, low-PHB cells those from the region marked L.

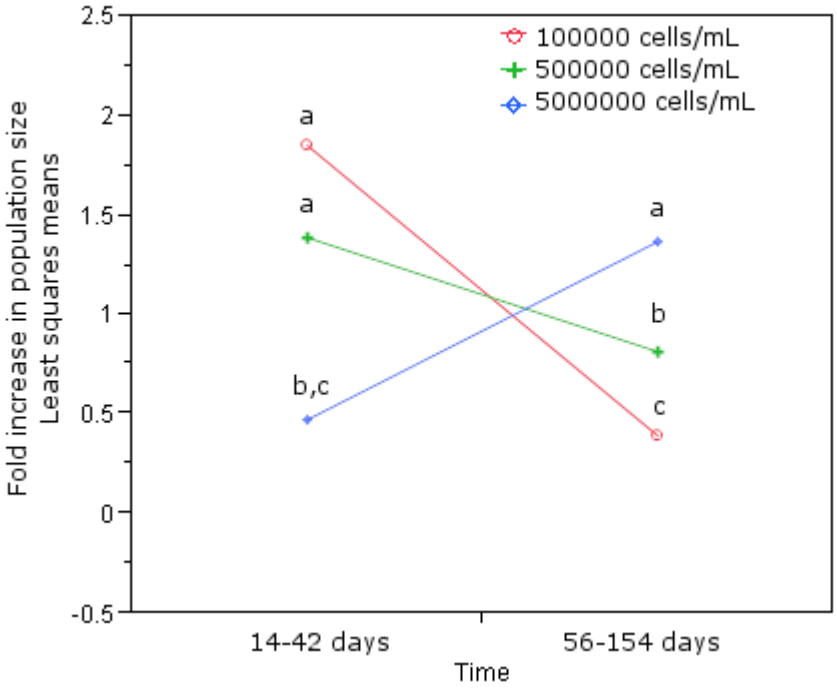

Figure A2. Interaction plot from the two-way ANOVA of population size during short and long-term starvation. Values with different letters are significantly different at $\alpha = 0.05$. This figure presents similar data to Figure 6-2b, but provides an alternative method of presentation that may be more familiar to some readers.

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