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Coevolved mutualists experience fluctuating costs and benefits over time

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Understanding how mutualisms persist over time requires investigations of how mutualist species coevolve and adapt to the interaction. In particular, the key factors in the evolution of mutualisms are the costs and benefits mutualists experience during the interaction. Here, we used a yeast nutritional mutualism to test how mutualists coevolve and adapt in an obligate mutualism. We allowed two yeast mutualists to evolve together for 15 weeks (about 150 generations), and then we tested if the mutualists had coevolved using time-shift assays. We also examined two mutualistic traits associated with the costs and benefits: resource use efficiency and commodity production. We found that the mutualists quickly coevolved. Furthermore, the changes in benefits and costs were nonlinear and varied with evolutionary changes occurring in the mutualist partner. One mutualist initially evolved to reduce mutualistic commodity production and increase efficiency in mutualistic resource use; however, this negatively affected its mutualist partner that evolved reduced commodity production and resource use efficiency. As a result, the former increased commodity production, resulting in an increase in benefits for its partner. The quick, nonlinear, and asynchronous evolution of yeast mutualists closely resembles antagonistic coevolutionary patterns, supporting the view that mutualisms should be considered as reciprocal exploitation.

KEY WORDS: **Coevolution, obligate mutualism, experimental evolution, microbe.**

Mutualisms, or reciprocally beneficial interspecific interactions, are prevalent in every ecosystem and experienced by organisms across the tree of life. The importance of these interactions is unquestionable; thus, we need to understand how mutualisms evolve and persist over time. Mutualists can evolve in response to one another (i.e., coevolve), and such coevolution can have important implications for their persistence. For instance, studies show that coevolution helps mutualisms persist during disturbances (Nuismer et al. 2018) and can drive trait diversification (Thompson 2005; Althoff et al. 2013). Although there are foundational theoretical models that show how mutualisms evolve (e.g., Axelrod and Hamilton 1981; Bull and Rice 1991; Doebeli and Knowlton 1998; Gokhale and Traulsen 2012), we generally lack empirical tests of these models. This is partially because the evolution of long-lived species is difficult to observe. Consequently, to experimentally test coevolution in a feasible period of time, researchers often turn to microbial systems. Experimental evolution with microbial systems has been used extensively to test coevolution in antagonistic interactions, yet experiments examining mutualistic coevolution are relatively rare (reviewed by Brockhurst and Koskella 2013). Furthermore, most of the microbial systems used to study mutualism are by-product mutualisms where the mutualists experience no costs of production of the mutualistic commodities (reviewed by Hillesland 2018). Consequently, studies of by-product mutualisms underestimate a key feature of most multicellular mutualism: the costs of participating in the interaction.

The costs and the benefits experienced by each species involved in mutualisms define the outcomes and effects of mutualists on one another, which in turn determines the prevalence of mutualisms (Bronstein 2001a). In mutualisms, species trade commodities that are relatively cheap to produce (i.e., cost) in exchange for commodities that are either too expensive to produce or that they are unable to make by themselves (i.e., benefit).

Because of this, mutualisms have been largely considered as biological markets (Schwartz and Hoeksema 1998). High costs coupled with low benefits could lead to decreased fitness of mutualists offering the commodity, whereas high benefits with low or no costs could lead to exploitation with decreased fitness of the mutualist partner providing the benefit. Thus, the benefit to cost ratio is instrumental for the maintenance of mutualisms, because it determines the stability of mutualisms, as well as the outcome of these interactions (Bronstein 1994; Schwartz and Hoeksema 1998; Sherratt and Roberts 2002). Although the study of costs and benefits involved in mutualisms is not new, very few studies have experimentally tested how benefits and costs evolve in interacting species and how these traits are related to adaptation of partners to the mutualistic interaction.

Adaptation in mutualisms should involve some form of optimization of the benefit to cost ratio for both partners. The cost in mutualisms is primarily measured by the amount of commodity being offered in the mutualism, which is usually linked to a fitness cost (Bronstein 2001a). One way to reduce the costs is to evolve reduced energetic cost required to produce the same amount of commodity or improve the transfer of commodities (e.g., Pande et al. 2015). However, these changes could be limited by physiological and/or evolutionary constraints. Another way that mutualists can reduce the costs is by reducing the amount of the offered commodity, but this could reduce the population size of its partner, leading to corresponding decreases in the benefits received (De Mazancourt et al. 2005). Indeed, ever-increasing as well as ever-diminishing investments have both been mathematically shown to be evolutionarily unstable (Ferriere et al. 2002). Thus, theory predicts that stabilizing selection constrains the amount of the mutualistic commodities offered in mutualisms (reviewed by Heath and Stinchcombe 2014), although this is not always confirmed by empirical studies (e.g., Rutter and Rausher 2004). Instead of reducing the costs, one way to increase the benefit to cost ratio is to increase the benefits received from the interaction.

Although the benefits received from mutualists are usually shaped by the amount of commodities offered by their partners, there are ways that mutualists can increase their benefits independently of changes in the partner offering the commodity. For instance, the mutualist can become more efficient in resource use, requiring fewer resources to sustain similar population growth (MacLean and Gudelj 2006; Hillesland and Stahl 2010). Theory predicts that increases in efficiency should evolve in symbioses where symbionts become more efficient in using the resources offered by the host (Yoder 2016). Alternatively, the mutualist can evolve to use alternative sources of the commodity and, consequently, become less dependent on the benefits offered by the partner. This latter evolutionary change, however, can be detrimental to mutualism persistence, as it has been shown to cause mutualism breakdown in mycorrhizal systems (Werner et al. 2018). Mutualists may also increase the commodity offered to their partner, resulting in more benefits received if there is a proportional increase in the population size of the partner. However, in this case mutualists will still experience an increase in the cost of producing more commodities in the first place (De Mazancourt et al. 2005). Although there are different mechanisms that can improve the benefit to cost ratio, the underlying factors that favor one versus another or how mutualists evolve in response to changes in their partners remain unclear.

Here, we use a synthetic mutualism between strains of brewer's yeast that form a nutrient exchange mutualism. We genetically engineered one mutualist type to overproduce adenine, which is then released into the liquid medium. This mutualist also lacks the ability to produce lysine (adenine mutualists, hereafter "Ade"). Similarly, we created a second mutualist type that overproduces lysine that is released into the medium, but this mutualist cannot make adenine (lysine mutualists, hereafter "Lys"). In this system, there is a fitness cost to overproducing the exchanged adenine and lysine resources (Fig. S1); thus, this is not a by-product mutualism. Our mutualists were grown together in liquid medium lacking lysine and adenine; thus, the mutualists could not survive without the presence of a mutualistic partner and participate in an obligate mutualism (i.e., partners cannot survive/reproduce without one another). We used this synthetic system to examine coevolutionary processes and changes in the costs and benefits over time in replicate communities of obligate mutualists. We specifically addressed the following questions: (1) Are the mutualists coevolving and what is the pace of coevolution? (2) Are traits related to the costs and benefits evolving in response to the obligate mutualism?

Methods

YEAST SYSTEM

Yeast strains were engineered to simulate a nutritional mutualism where one strain produces more lysine than it needs but does not produce adenine (Lys), whereas the other strain produces more adenine than it needs but cannot produce lysine (Ade). The yeast strains were also modified to have pairwise combinations of uracil, histidine, and leucine deletions. These were used as selective markers to monitor the presence/absence of the strains in co-culture. All genetic modifications were produced as described in Vidal et al. (2020). Here, we grew together the Ade strain RY1069 (genotype MATa ste3Δ::kanMX4 lys2Δ0 leu2Δ0 ura3Δ0 ade4^{OP}) with Lys strain RY1051 (MATa ste3Δ::kanMX4 ade8Δ0 his3Δ1 ura3Δ0 LYS21^{OP}), and RY1063 (MATa ste3Δ::kanMX4 lys2Δ0 his3Δ1 ura3Δ0 ade4^{OP}) with RY1039 (MATa ste3Δ::kanMX4 ade8Δ0 his3Δ1 leu2Δ0 lys21^{OP}). We used different combinations of

strains to account for possible effects of the selective markers. Because these strains had different genotypes and were all asexual, they are ecologically similar to different species.

The excess of lysine or adenine produced by each mutualist type is released into the medium, making it a freely available resource. The yeast strains grow together in liquid medium that is constantly mixing; thus, there is no spatial structure. Lysine is stored in the vacuole of the cells and is only made available in the medium after cell death and lysis, whereas adenine is actively released into the medium during the cell's life. In medium lacking adenine and lysine, the mutualists cannot survive without their partner because adenine is required for cell division and lysine is used in cell growth.

Because production of lysine and adenine has fitness costs (Fig. S1), we considered the change in production of these resources as a measure of the evolution of the costs associated with the mutualism. As a measure of the changes in benefits, we used the evolution of resource use efficiency. More specifically, we define efficiency as the ability to convert nutrients into biomass/growth. Another possible direct change in benefits would be by using alternative sources of the mutualistic commodity being exchanged; however, in this system there was no alternative source of lysine or adenine.

COEVOLUTION EXPERIMENT

We used experimental evolution to investigate if yeast mutualists evolve in response to one another and how the benefits and costs might change over time. To set up the coevolution experiment, we grew overnight cultures of single colonies in liquid YPD (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] dextrose). To create an obligate mutualism, we propagated mutualism cultures in synthetic dextrose (SD) medium lacking adenine and lysine (0.15% [w/v] Difco yeast nitrogen base without amino acids or ammonium sulfate, 0.5% [w/v] ammonium sulfate, and 2% [w/v] dextrose, with supplemental amino acids added). To assemble the mutualism cultures, yeast strains were washed in sterile water, and then diluted to a standard density of 0.1 OD_{600} (0.05 OD_{600} of each interacting strain) in 2-ml cultures of SD lacking adenine and lysine. Thus, to sustain population growth, both Lys and Ade mutualists were obligately dependent on each other and both were required for the culture to persist. We replicated each combination of yeast strains (RY1069 with RY1051, and RY1063 with RY1039) 14 times, resulting in a total of 28 independent cultures. All cultures were grown in 48 well deep-well plates at 30°C in liquid medium on a rotating wheel. Every 48 h, we transferred a portion of each culture into fresh medium, resetting the cultures to 0.1 OD_{600}. The cultures were maintained for 15 weeks, and at the end of each week, we plated the cultures on selective media to confirm the presence of each strain. At that time, we also froze a subsample of each co-culture in 25% glycerol and stored them at −80°C.

TIME-SHIFT ASSAYS

To test if the mutualists coevolved to the interaction, we performed time-shift assays (sensu Brockhurst and Koskella 2013) with evolved mutualists paired with ancestral strains. These assays were performed on the evolved mutualists from different time points (weeks 2, 4, 8, 10, and 15 of evolution) such that we could track changes in the strains over time. These time points roughly corresponded to 25, 50, 100, 125, and 150 generations. We cannot precisely calculate the number of generations in this system because the cultures are variable in growth rate both between cultures and between transfers. In addition, the cultures represent two strains together that might differ in generation time. For this reason, we used the time point measured in weeks to analyze and present the results. We used an indirect measure of fitness to assess coevolution. To do this, we determined yield, or the combined population growth of both strains in the co-culture as measured by optical density of the co-cultures. Thus, for the time-shift assay, the yield of the co-cultures represented how well the two strains grew together. Yield measured as optical density can change depending on cell size as well as cell number. A previous assessment showed that cell size can vary slightly in our evolved yeast, but it is difficult to measure because of inherent differences in cell size across life history stages of individuals. We created co-cultures of evolved mutualists with their ancestral partners as well as co-cultures containing both evolved mutualists from the same time point, and co-cultures containing the two ancestral mutualists. By comparing the yield of co-cultures of the ancestral partner growing with an evolved partner from different time points (i.e., time-shift cultures), we were able to obtain an estimate of the temporal dynamics of evolution of each partner (Gaba and Ebert 2009). Additionally, comparison of the yield of co-cultures with both evolved strains to time-shift co-cultures (evolved + ancestral) can provide evidence for coevolution if yield differs (Hillesland 2018).

For the time-shift assays, we used the frozen strains from the selected time points and grew each evolved strain (Lys^{evo} or Ade^{evo}) with its ancestral partner strain (Lys^{anc} or Ade^{anc}), that is, the strain used to set up the evolution experiment. We also had co-cultures with both ancestral partners (Lys^{anc} + Ade^{anc}) and with both evolved partners (Lys^{evo} + Ade^{evo}) for each time point tested. Thus, for each independent culture, we had three combinations of strains (e.g., Lys^{evo} + Ade^{anc}, Lys^{anc} + Ade^{evo}, and Lys^{evo} + Ade^{evo}). For these assays, we focused on a subset of six of the total 28 independent cultures for every time point tested. To set up the time-shift assays, we grew single, overnight cultures of each yeast strain in YPD, washed them in sterile water, and set up pairwise mutualist combinations at a starting density of

0.1 OD_{600} as we did in the initial coevolution experiment. Strains were grown on a rotating wheel for 48 h at 30°C and then we measured OD_{600}.

To compare the yield after 48 h of growth, we used the natural logarithm of the ratio of co-cultures with evolved strains and co-cultures with only ancestral strains that were grown in the same deep-well plate (Hedges et al. 1999):

$$\ln\left[\frac{(\text{Lys}^{\text{evo}}+\text{Ade}^{\text{evo}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right], \quad (1)$$

$$\ln\left[\frac{(\text{Lys}^{\text{evo}}+\text{Ade}^{\text{anc}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right], \quad (2)$$

$$\ln\left[\frac{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{evo}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right]. \quad (3)$$

To test how the yield of co-cultures with both evolved mutualists differed from the ancestral co-culture, we used a linear model with the natural logarithm of the ratio between evolved co-cultures and ancestral co-cultures (eq. 1 above) as the response variable. First, we compared how these co-cultures with both evolved mutualists differed from ancestral co-cultures. In this case, "time point" was the fixed effect, and we set the intercept to zero. Second, to understand how these co-cultures changed over time, we compared how the yield of co-cultures from each time point differed from one another using glht from the R package multcomp (Hothorn et al. 2008). Third, to examine differences between co-cultures with both evolved mutualists (Lys^{evo} + Ade^{evo}) and time-shift co-cultures (Lys^{evo} + Ade^{anc} or Lys^{anc} + Ade^{evo}), we compared their ratios (eqs. 1, 2, and 3 above) using ANOVA and Tukey's HSD tests for each time point. In this case, the ratios were the response variable, and the type of co-culture (Lys^{evo} + Ade^{evo}, Lys^{evo} + Ade^{anc}, or Lys^{anc} + Ade^{evo}) was the independent variable.

To estimate the temporal dynamics of evolutionary change in each partner, we compared the time-shift co-cultures (Lys^{evo} + Ade^{anc} or Lys^{anc} + Ade^{evo}) to the ancestral co-cultures (Lys^{anc} + Ade^{anc}). We first tested how the yield of time-shift cultures evolved, with the ratio (eqs. 2 and 3 above) as the response variable, and time point tested as a continuous fixed effect. Strain identification was included as a covariate for the model with Lys^{evo} but not for Ade^{evo}, as this variable contributed to the variance of the former. We then determined whether the ratios differed from zero (representing difference from ancestral) at each time point for each evolved mutualist time-shift. Because the strain identity had an effect on cultures containing the Lys^{evo}, we analyzed how each time point differed from ancestral (ratio $\neq 0$) using *t*-tests. For the time points in which there were differential responses by the two Lys strains (RY1039 and RY1051), we compared the strains separately. For the co-cultures with Ade^{evo}, we used a linear model as before, with time point as a nominal variable and the intercept set to zero. We compared how the yield of co-cultures from each time point differed from each other using Tukey's HSD with the function *glht* from the R package multcomp.

The time-shift assays also allowed us to test for the possible negative or positive effect that the evolved mutualist partners could have on one another, because the evolutionary changes could lead to either increases or decreases in mutualistic benefits. To test if the effect of the Ade^{evo} and Lys^{evo} on one another is antagonistic or synergistic, we used theory based on epistatic effects of mutational load. We assumed that each additional mutation (in our case, evolutionary change of the mutualists when grown together) would cause a disproportionate increase in fitness if the interaction was synergistic. Alternatively, if the evolutionary changes of the evolved mutualists had an antagonistic effect, fitness would be disproportionally lower (Bohannan et al. 1999; Gao et al. 2010). To test for this potential disproportional fitness effect, we first calculated expected fitness values based on null models calculated by adding or multiplying the effect of the time-shift assays:

$$\text{Additive}: \left[\left(\frac{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{evo}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right)+\left(\frac{(\text{Lys}^{\text{evo}}+\text{Ade}^{\text{anc}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right)\right], \quad (4)$$

$$\text{Multiplicative}: \left[\left(\frac{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{evo}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right)\times\left(\frac{(\text{Lys}^{\text{evo}}+\text{Ade}^{\text{anc}})}{(\text{Lys}^{\text{anc}}+\text{Ade}^{\text{anc}})}\right)\right]. \quad (5)$$

The expected fitness (or yield) values of these null models assume no interaction between the evolutionary changes of the two mutualist species. We used both additive and multiplicative models, as suggested by Hillesland and Stahl (2010) and Bohannan et al. (1999). These models differ in the biological expectations of the scale of the interaction between mutations, depending on the trait being considered (Gao et al. 2010). As we do not have an a priori expectation of the scale of the effect of one evolved mutualist on another, we used both additive and multiplicative models. We subtracted the yield of Lys^{evo} + Ade^{evo} co-cultures by each of the null models, and compare how these subtractions differed from zero at each time point using *t*-tests. If the subtractions differ from zero, this shows that the evolved mutualists together have a disproportionate fitness change than would be expected under these null models, signifying a synergistic interaction if the value is positive, or antagonistic if negative.

MEASUREMENT OF THE EVOLUTION OF BENEFIT AND COST TRAITS

To test if there was a change in the mutualistic traits that could explain the patterns of coevolution between partners, we examined changes in resource use efficiency (benefits) and production

of mutualistic resources (costs). Here, we define resource use efficiency as the conversion of a limited amount of resource into biomass, and we measured it as the carrying capacity (i.e., yield) of each strain growing alone in medium with a low amount of adenine and lysine. Thus, strains that have evolved increased benefits can convert small amounts of adenine or lysine into higher yield as compared to the ancestral strain (MacLean and Gudelj 2006; Hillesland 2018). To assess the costs associated with the interaction, we indirectly measured production of the mutualistic resources (lysine or adenine). To test for changes in production and efficiency, we used the ancestral strain and the same evolved strains as explained above. To measure efficiency, we grew single-strain cultures by growing overnight cultures in YPD medium (yeast extract, peptone, dextrose), washing them in sterile water, and diluting in synthetic dextrose (SD) medium to 0.1 OD_{600}. We used SD medium with 0.05% (w/v) adenine and lysine to simulate an environment with low availability of mutualistic resources. By comparing the yield of evolved cultures from different time points growing in low-resource environment, we tested if mutualists evolved to have increased efficiency. We replicated each independent evolved culture at least twice and after 48 h, we measured yield as OD_{600}.

To test if production of adenine and lysine changed over the course of the experiment, we grew each strain separately in SD containing 1% adenine (40 mg/L) and lacking lysine for the Lys mutualists and 1% lysine (90 mg/L) and lacking adenine for the Ade mutualists. The mutualists were grown alone in medium with either added lysine or adenine so that we could measure changes in the production of the commodities. After 48 h of growth, we used sterile filters to remove the Ade mutualists from 200 μl of medium where the Ade mutualists were growing. To obtain lysine produced by Lys mutualists, we first lysed the cells because lysine is only released after the cell dies. To do this, we shocked cells at 100°C for 5 min immediately followed by incubation in a dry ice, absolute alcohol bath for 2 min, and then filtered the medium to remove the Lys mutualists. To indirectly measure the amount of lysine or adenine produced by each mutualist, we used the filtered media to grow a test strain that cannot produce lysine or adenine (SY9915 from Euroscarf, MATa ade2Δ0 ade8Δ0 his3Δ1 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0). We added the test strain to 1950 μl of fresh SD medium lacking adenine and with 1% lysine plus 50 μl of filtered medium from the Ade mutualist; thus, the only adenine available was that present in the filtered medium. Similarly, lysine production was tested by combining 100 μl of filtered medium from the Lys mutualists with 1900 μl of SD medium lacking lysine with 1% adenine. The volumes of filtered medium necessary to sustain growth were tested in pilot studies, and we found that 50 μl of adenine filtered medium and 100 μl of lysine filtered medium were sufficient to sustain measurable growth of the test strain. We measured yield (OD_{600}) of the test strain after 48 h.

To understand how efficiency and production changed over time, we considered the differences of each time point relative to the ancestral state, using the natural logarithm of the ratio of evolved mutualist by ancestral mutualist as

$$\ln\left(\frac{\mathrm{Lys}^{\mathrm{evo}}}{\mathrm{Lys}^{\mathrm{anc}}}\right), \tag{6}$$

$$\ln\left(\frac{\mathrm{Ade}^{\mathrm{evo}}}{\mathrm{Ade}^{\mathrm{anc}}}\right). \tag{7}$$

We tested how efficiency changed over time by comparing the yield of evolved mutualists at 48 h with that of ancestral strains. Similarly, we examined changes in production of adenine and lysine by measuring population growth of the test strain and then comparing growth between filtered medium from the evolved mutualists and filtered medium from the ancestral mutualist. The residual of the models in efficiency and production indicated that the data were normally distributed; therefore, we used a Gaussian distribution in our linear models. Strain identity did not influence yield in any of the measures of efficiency and production; thus, we excluded this effect from the model. In all models, the natural logarithm of the ratio of yield (eqs. 6 and 7) was the response variable and time point was the fixed effect. We set the intercept of the model to zero; thus, significance indicates that the evolved strains differ from ancestral (ln of ratio $\neq$ 0). The traits associated with the costs and benefits could evolve linearly if there is runaway selection; however, it is unlikely that these traits would increase or decrease indeterminately. Thus, we fit a quadratic model and compared it to a linear model using time point as a continuous variable, and tested the best fit using Akaike Information Criteria (AIC) and ANOVA comparisons. For our models, we used the function lmer or lm in the R package lme4 (Bates et al. 2015). All statistical analyses were performed using R environment version 3.6.0 (R Core Team 2020).

Results

All of the cultures had both mutualists persist to the end of the experiment. The 28 independent cultures had similar growth of OD_{600} ~7 at week 15. All of the results below used a subset of the strains that were stored during the evolution experiment, as explained in the methods.

ARE THE MUTUALISTS COEVOLVING AND WHAT IS THE PACE OF COEVOLUTION?

To determine if the mutualists coevolved, we took three approaches. First, we compared the yield of $\mathrm{Lys}^{\mathrm{evo}} + \mathrm{Ade}^{\mathrm{evo}}$ to the yield of $\mathrm{Lys}^{\mathrm{anc}} + \mathrm{Ade}^{\mathrm{anc}}$. We found that from weeks 8 to 15 of

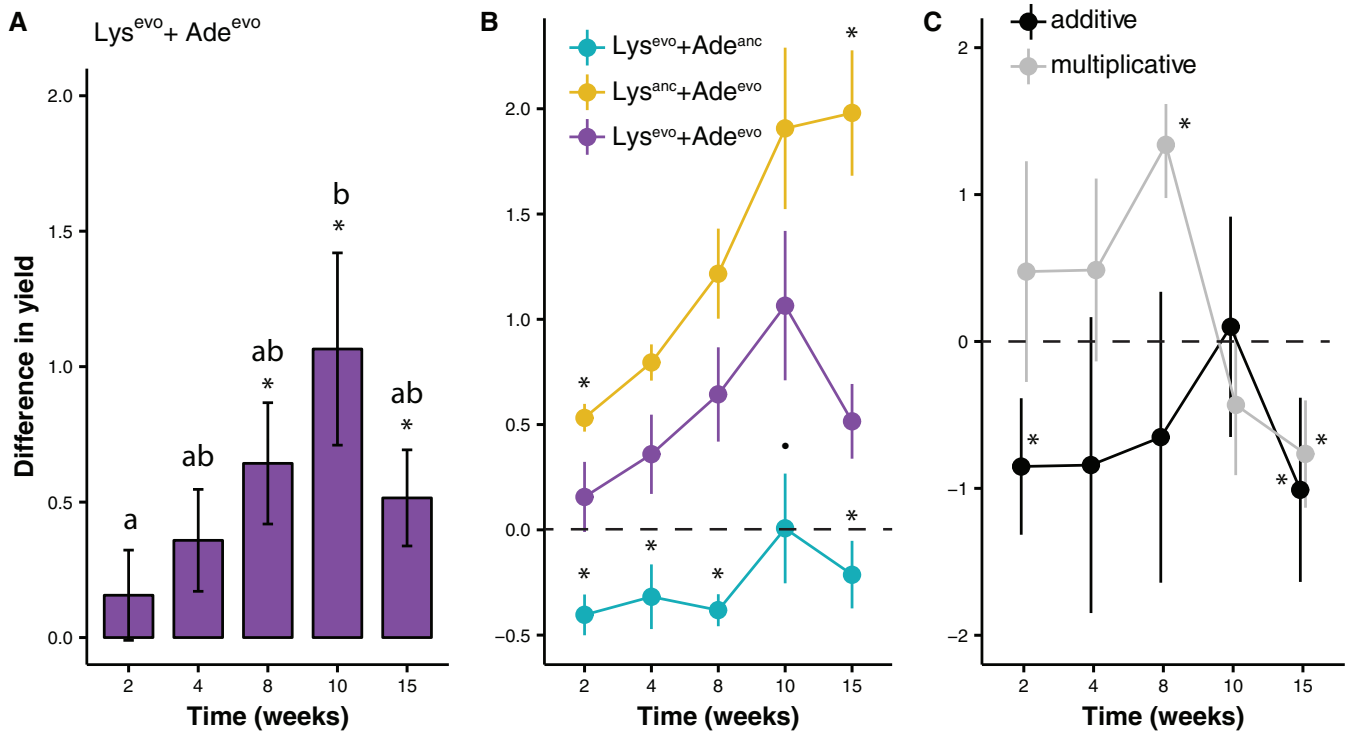

Figure 1. Coevolution of mutualists. (A) Difference in yield of Lys^{evo} + Ade^{evo} co-cultures in comparison with Lys^{anc} + Ade^{anc} co-cultures (mean ± SE). Asterisks indicate significant differences from zero; letters represent comparisons between time points. (B) Side-by-side comparison of yield (mean ± SE) from the time-shift assays (Lys^{anc} + Ade^{evo} or Lys^{evo} + Ade^{anc}) and Lys^{evo} + Ade^{evo} co-cultures, all relative to Lys^{anc} + Ade^{anc}. Black dashed line represents no difference from Lys^{anc} + Ade^{anc}. Positive values show increases in yield relative to Lys^{anc} + Ade^{anc}, whereas negative values are decreases in yield relative to Lys^{anc} + Ade^{anc}. *$P < 0.05$ and •$0.1 > P > 0.05$ for comparisons of time-shift co-cultures (Lys^{anc} + Ade^{evo} or Lys^{evo} + Ade^{anc}) to Lys^{evo} + Ade^{evo} co-cultures. For graphs A and B, we used the ratios −1 without ln-transforming them to show how the evolved strains differ from ancestral. (C) Test for synergistic or antagonistic effects of the evolved mutualists on one another. Values are the difference in the observed yield of evolved mutualists in comparison with expected yield from null models of additive and multiplicative effects. Black dashed line indicates no difference between observed and expected yield under the null models. Negative values indicate that the observed co-cultures had lower yield than expected, showing antagonistic effects of mutualists on one another. Positive values show synergistic effects caused by mutualists having great yield than expected. Errors are confidence intervals; asterisks indicate significant differences from zero. The time points roughly correspond to 25 (2 weeks), 50 (4 weeks), 100 (8 weeks), 125 (10 weeks), and 150 (15 weeks) generations.

evolution, Lys^{evo} + Ade^{evo} had higher yield than the Lys^{anc} + Ade^{anc} ($F_{5,31} = 7.76$, $P < 0.0001$; Fig. 1A), whereas yield from weeks 2 and 4 of evolution did not differ from ancestral. The difference in yield of the Lys^{evo} + Ade^{evo} relative to ancestral was the greatest at week 10 as compared to week 2, reaching 100% improvement in yield. Second, we compared the yield of Lys^{evo} + Ade^{evo} to the time-shift cultures (Lys^{evo} + Ade^{anc} and Lys^{anc} + Ade^{evo}). We found that overall the yield of Lys^{evo} + Ade^{evo} was higher than Lys^{evo} + Ade^{anc} for all time points (Fig. 1B). In contrast, the yield of Lys^{evo} + Ade^{evo} was generally similar to Lys^{anc} + Ade^{evo}, except for weeks 2 and 15 when Lys^{evo} + Ade^{evo} had lower yield than Lys^{anc} + Ade^{evo} (Fig. 1B).

Finally, we examined if the evolved mutualists had a synergistic or antagonistic effect on one another by comparing the observed yield of Lys^{evo} + Ade^{evo} to expectations following additive or multiplicative null models (Fig. 1C). This analysis showed that the effect of the evolved mutualists on one another was not consistent across time (Fig. 1C). For instance, at 4 and 10 weeks of evolution, the observed yield of Lys^{evo} + Ade^{evo} matched expectations of independent effects of the mutualist species (i.e., observed was not different from that expected under additive or multiplicative null models). At 8 weeks of evolution, however, the yield of evolved mutualists was greater than expected from the multiplicative null model, suggesting synergism when the evolved mutualists were together. In contrast, at 2 and 15 weeks of evolution, the observed yield of Lys^{evo} + Ade^{evo} was lower than expected under the additive and/or multiplicative null models, suggesting antagonistic effects.

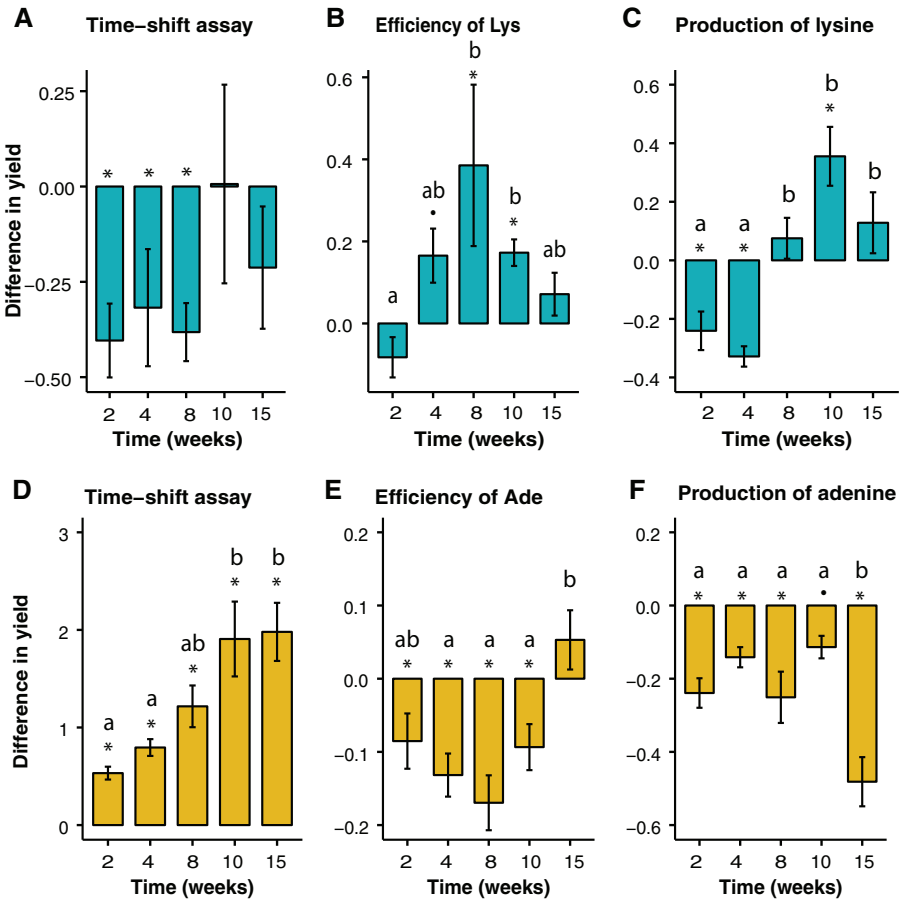

Figure 2. Changes in the lysine and the adenine mutualists over a 15-week experiment (mean ± SE). (A) Time-shift assay of co-cultures containing Lys^{evo} and Ade^{anc} shown as the difference in yield of these co-cultures from yield of Lys^{anc} + Ade^{anc} co-cultures. (B) Efficiency of the Lys^{evo} shown as the difference in yield of Lys^{evo} in comparison with yield of Lys^{anc} in poor medium. (C) Production of lysine by the Lys^{evo} shown as the difference in yield of a test strain when growing in filtered medium of Lys^{evo} in comparison with filtered medium of Lys^{anc}. (D) Time-shift assay of Lys^{anc} + Ade^{evo} co-cultures, shown by the difference in yield of these co-cultures in comparison with Lys^{anc} + Ade^{anc} co-cultures. (E) Efficiency of Ade^{evo}, shown as the difference in yield of the Ade^{evo} with the yield of the Ade^{anc} in poor medium. (F) Production of adenine by the Ade^{evo}, shown as the difference in the yield of a test strain when growing in filtered medium extracted from cultures of the Ade^{evo} as compared with filtered medium taken from the Ade^{anc}. The time points roughly correspond to 25 (2 weeks), 50 (4 weeks), 100 (8 weeks), 125 (10 weeks), and 150 (15 weeks) generations.

*$P < 0.05$ and •$0.1 > P > 0.05$ for comparisons with zero. Letters are for comparisons between time points. For the graphs, we used the ratios −1 without log transforming to show how the evolved strains differ from ancestral.

ARE TRAITS RELATED TO THE COSTS AND BENEFITS EVOLVING IN RESPONSE TO THE OBLIGATE MUTUALISM?

In the previous section, we analyzed the changes in co-cultures with both evolved mutualists together, which confounds the changes in each mutualist. Here, to see how each mutualist is evolving and how the benefits and costs are changing in each mutualist, we analyzed each mutualist type separately.

LYSINE OVERPRODUCERS

To understand the pattern of coevolution we observed in the Lys^{evo} + Ade^{evo} co-cultures, we examined the yield of Lys^{evo} + Ade^{anc} co-cultures and how the mutualistic traits evolved. Time shift assays showed that the Lys^{evo} changed in comparison to the Lys^{anc}; however, the difference in yield between evolved and ancestral strains did not differ among time points ($F_{4,24}$ = 1.4, P = 0.265; Fig. 2A). For the first 8 weeks of evolution, the co-cultures of the Lys^{evo} + Ade^{anc} grew about 40% less than Lys^{anc} + Ade^{anc} (Fig. 2A). After 10 weeks of evolution, this pattern shifted such that co-cultures of the Lys^{evo} + Ade^{anc} had similar yield to Lys^{anc} + Ade^{anc}. For weeks 2 and 15 of evolution, we observed a significant effect of the identity of the Lys mutualists used in the experiment: for both time points, strain RY1051 evolved to have a negative effect on the yield of co-cultures with Ade^{anc}, whereas co-cultures with Lys^{evo} strain RY1039 did not differ from Lys^{anc} + Ade^{anc} co-cultures.

The observed patterns from the time-shift assays were coupled with changes in the efficiency ($F_{4,67} = 3.72$, $P = 0.008$) and production of lysine ($F_{4,30} = 9.07$, $P < 0.0001$) by the Lys^{evo}. Lys mutualists evolved to be marginally more efficient in resource use after 4 weeks of evolution, and they were the most efficient at week 8 with a 40% increase in efficiency (Fig. 2B). However, at week 15, efficiency returned to the ancestral level (Fig. 2B). In contrast, the production of lysine decreased in weeks 2 and 4 of evolution. Similar to efficiency, changes in production were also nonlinear: there was a reversal in production that started at week 8, resulting in an increase in production at 10 weeks of evolution, but returning to the ancestral production level at week 15 (Fig. 2C). The changes in efficiency and production of the Lys^{evo} more closely followed a quadratic model than a linear model (Fig. S2).

ADENINE OVERPRODUCERS

To understand both the pattern of coevolution and changes in Lys^{evo}, we also tested how Ade^{evo} + Lys^{anc} differed from ancestral co-cultures and how the mutualistic traits evolved. Time shift assays showed that the Ade^{evo} changed in comparison with the Ade^{anc}, with the difference between evolved and ancestral increasing over time ($F_{4,25} = 8.09$, $P = 0.0002$). The Lys^{anc} + Ade^{evo} co-cultures had consistently higher yield than Lys^{anc} + Ade^{anc} co-cultures (Fig. 2D). At weeks 10 and 15, this difference in yield was higher than after 2 and 4 weeks of evolution, reaching up to 200% higher yield than Lys^{anc} + Ade^{anc}. In contrast with the Lys^{evo}, there was no difference between the two Ade mutualist strains in how yield evolved with respect to the ancestral strain ($F_{1,28} = 0.88$, $P = 0.356$).

Although the co-cultures with Ade^{evo} had increased yield, the Ade^{evo} from weeks 2-10 were less efficient than Ade^{anc} (Fig. 2E). This means that with limited resources, Ade^{evo} produced fewer (or smaller) cells than the Ade^{anc}. After 15 weeks of evolution, efficiency returned to the ancestral level. For all time points tested, there was a reduction in the amount of adenine produced by Ade^{evo} in comparison with Ade^{anc}, reaching the greatest reduction at week 15 (Fig. 2F). The changes in efficiency and production of the Ade^{evo} were a better fit to a quadratic model than a linear model (Fig. S3).

Discussion

Mutualisms are often considered reciprocally exploitative interactions because selection should favor mutualists that maximize the gain of benefits while minimizing the costs. Indeed, in nature we see exploitative strategies emerging in every type of mutualism (Bronstein 2001b); however, we also have evidence of mutualisms that have persisted for millions of years (e.g., Pellmyr and Leebens-Mack 1999). To understand how mutualisms persist over time despite the potential pressure to exploit their partners, we need to understand how mutualists coevolve and how traits related to the costs and benefits change over time. However, testing both coevolution and trait evolution is challenging in natural systems, especially due to the constraints of manipulating natural systems. By using a tractable, laboratory-based mutualism, we tested how mutualists evolved in response to one another. With these experiments, we found evidence for coevolution after only 25-30 generations (2 weeks). Furthermore, we showed that the changes in the benefits and costs were nonlinear and varied with evolutionary changes occurring in the mutualist partner (Fig. 3). Together, our results demonstrate that coevolution in mutualisms can occur quickly, nonlinearly, and asynchronously.

We found three lines of evidence indicating that coevolution occurred in our synthetic mutualism. First, we observed that both the Lys^{evo} and Ade^{evo} changed relative to their ancestral forms (Figs. 2A and 2D). This result provides evidence of coevolution following Brockhurst and Koskella (2013) who suggested that one way to demonstrate coevolution is to show that both interacting species have changed relative to the ancestral pair. Second, time-shift assays can show coevolution if the fitness varies among co-cultures depending on which partners are paired (Hillesland 2018). Indeed, we found that the fitness of Lys^{evo} growing with Ade^{evo} is different than its fitness when grown with Ade^{anc} for all time points tested. Similarly, Ade^{evo} growing with Lys^{evo} was also different than with Lys^{anc} at weeks 2 and 15 (Fig. 1B). Surprisingly, at these two time points, Ade^{evo} had greater yield with Lys^{anc} than with Lys^{evo}, which suggests that Ade^{evo} was negatively affected by Lys^{evo}. This possible negative effect of Lys^{evo} on Ade^{evo} at weeks 2 and 15 is further evidenced by the comparison of the evolved mutualists with multiplicative and additive null models, which indicated an antagonistic effect of the mutualists on one another. Finally, our third piece of evidence demonstrating coevolution is that for at least three time points, the effects of the evolved mutualists on one another were different from predictions based on the independent effects of the mutualists together. Thus, we have ample evidence of coevolution in this system, although the effect of the coevolved mutualists on one another was not always synergistic, as we discuss below.

Our assays showed that the interaction between mutualists can swing from synergistic to antagonistic in a few generations. The interaction is always mutualistic because the partners cannot survive without the provision of adenine or lysine by the other partner. However, the magnitude of the net benefit received varies over time, in some cases being more beneficial than expected from their combined independent effects (Fig. 1C, week 8), whereas at other times, the mutualists are less beneficial than expected (Fig. 2C, weeks 2 and 15). One possible reason for this antagonistic (less beneficial) effect is that changes related to the mutualistic interaction could also influence competition

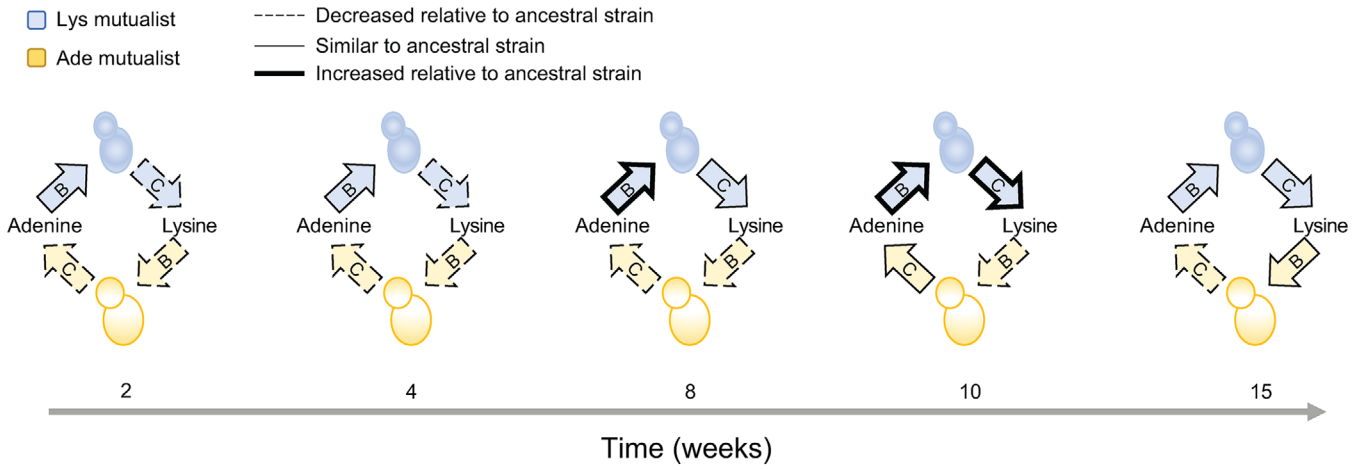

Figure 3. Summary of changes related to benefits (B) and costs (C) observed in the Ade^{evo} and the Lys^{evo} over time. The time points roughly correspond to 25 (2 weeks), 50 (4 weeks), 100 (8 weeks), 125 (10 weeks), and 150 (15 weeks) generations.

for shared resources. In addition to exchanging the mutualistic commodities that they need, these mutualists also compete for other resources such as sugar and nitrogen available in their common environment. For example, if a mutualist is less limited by the resource provided by its partner (i.e., adenine or lysine) and grows more than its partner, then this mutualist would potentially consume more of the shared resources (e.g., sugar), limiting the growth of its partner and causing an antagonistic effect. At week 2, Lys^{evo} produced less lysine and thus had a lower cost associated with the interaction (Fig. 3), which could have resulted in greater population growth of Lys^{evo}, consuming more of the shared resources and limiting the growth of Ade^{evo}. However, the growth of these mutualistic partners also depends on the population density of one another. For instance, the potential negative effect of Lys^{evo} on Ade^{evo} could also have negative consequences for Lys^{evo} because with fewer Ade^{evo} there would be less adenine to sustain population growth of the Lys^{evo} mutualist. As a result, the Lys^{evo} mutualists became more efficient and produced more lysine at week 8 than at weeks 2 and 4 of evolution (Fig. 3), when we also observed a synergistic interaction between the partners. The changes we observed in just a few generations of the Lys mutualists suggest that there is possibly selection against antagonistic effects.

This nonlinear pattern of coevolution corresponds with nonlinear changes in traits associated with the benefits (i.e., resource use efficiency) and costs (i.e., production of adenine or lysine). We hypothesize that the nonlinearity in trait evolution is caused by changes in one partner's traits having indirect negative effects on the other partner's responses. For instance, we found that the Lys^{evo} initially decreased the costs associated with the interaction by reducing lysine production. Because reduction of lysine likely led to smaller population sizes of their Ade partners, as supported by the initial negative effect of Lys^{evo} on co-cultures with Ade^{anc}, there was a concomitant decrease in the availability of adenine for the Lys^{evo}. As a result, selection favored an increase in the production of lysine by the Lys^{evo} and a reduction in adenine dependency as shown by the increase in resource use efficiency of Lys^{evo}. However, increased production of lysine is costly, leading to an eventual return to ancestral levels of production and efficiency. These changes in production and efficiency suggest that stabilizing selection is acting to minimize the costs of lysine overproduction while still producing enough lysine to secure the receipt of commodities from its mutualist partner. One way to test this hypothesis would be to isolate the evolved strain before we observed the increase in production and allow it to evolve in an environment with adenine artificially provided. In the case where adenine availability to Lys is not dependent upon the population size of the Ade producers, we might observe no changes or further reductions in lysine overproduction.

Because lysine is more limiting than adenine in this obligate mutualism, the evolution of mutualistic traits by Ade^{evo} was more constrained. Selection to reduce adenine production seems likely because production of adenine by Ade is inherently greater than production of lysine by the Lys mutualist (Vidal et al. 2020). Indeed, we observed a reduction in adenine production by Ade^{evo} for all weeks tested (Fig. 3). Additionally, our results show that the efficiency of Ade^{evo} was reduced for the first 10 weeks of evolution. The Ade mutualist is strongly limited by the amount of lysine made available by the Lys mutualist that releases lysine only when the cells die (Shou et al. 2006; Vidal et al. 2020). Therefore, at the start of the mutualism as well as periodically after each transfer, little lysine is available (Vidal et al. 2020). In addition to this already low availability of lysine, Lys^{evo} also initially evolved to produce less lysine, so the amount of available lysine was even lower than the ancestral state. It is possible that traits other than efficiency were under strong selection for

the Ade mutualist to deal with the low lysine availability at the onset of the mutualism. An important trait, for instance, would be starvation resistance, which was previously shown to be an competitive trait for strains that depend on lysine (Vidal et al. 2020). Thus, the evolution of the Ade mutualist is potentially restricted to changes that could sustain population growth in response to the low availability of lysine produced by the Lys mutualist.

The observation that resource use efficiency evolved to both increase and decrease over a short time shows that this important trait associated with the benefits of the interaction can be instrumental for mutualism persistence and evolution. Resource use efficiency can be considered as the acquisition and conversion of mutualistic commodities into population growth, which are usually fixed terms in density-dependent theoretical models (e.g., Holland and DeAngelis 2010). However, as we have shown here, conversion of mutualistic commodities into population growth (measured here as resource use efficiency) is a trait that can evolve, and as such could have important implications for mutualism dynamics. Because population size of mutualists is often associated with the benefits received, incorporating the evolution of resource efficiency in mutualism models would advance our understanding of mutualism evolution and stability.

The nonlinearity and asynchrony of evolutionary changes in Ade and Lys mutualists closely resemble expectations based on the coevolutionary dynamics of antagonistic interactions. Antagonistic coevolution is often described by the Red Queen Hypothesis, or the idea that ever-changing biotic environments lead to rapid evolution of interacting species (reviewed in Brockhurst et al. 2014). Gandon et al. (2008) argue that Red Queen dynamics can result in two patterns of coevolution: arms race dynamics and fluctuating selection dynamics, the latter being characterized by frequency-dependent selection and the former by mutation accumulation and directional selection. With arms race dynamics, we expect that adaptation between interacting species would result in a continuous increase (or decrease) through time; however, this is not what we found (e.g., Fig. 2A). In contrast, fluctuating selection dynamics can be harder to demonstrate because it is dependent on the timeframe tested. Although we cannot definitively say, our results are suggestive of fluctuating dynamics. In addition to the coevolutionary dynamics, the rapid pace of evolutionary changes observed in our mutualisms is also consistent with the fast pace assumed by the Red Queen Hypothesis. In contrast, the Red King Hypothesis was proposed as an alternative model predicting that slow evolutionary rates are favorable in mutualisms (Bergstrom and Lachmann 2003). However, Gokhale and Traulsen (2012) showed theoretically that a slow evolutionary rate can be detrimental when there are more individuals from each species participating in the mutualistic interaction, which might explain why our results using a system with large population sizes did not follow the Red King's prediction of a slow evolutionary pace. Our results underscore the view that mutualisms should be considered as a reciprocal exploitation (Doebeli and Knowlton 1998; Bronstein et al. 2006) with constant conflict between interacting species, similar to what is expected under the Red Queen Hypothesis.

As with any experiment, the patterns we observed could be impacted by experimental artifacts. One potential artifact of our experimental design is the transfer regime: we transferred a fraction of the co-culture to fresh medium every other day. This transfer could potentially lead to bottleneck effects and drift. To avoid this issue, we transferred volumes that returned each culture to a standard density of 0.1 OD_{600}, creating a large founding population that would be less prone to bottleneck effects. A second challenge to this experiment is interpreting the measure of the yield of co-cultures because optical density cannot distinguish the two strains in co-cultures and will be affected by changes in cell size and cell number. We argue, however, that because the differences in yield were large, these differences represent changes in population size more so than changes in cell size, making yield a good approximation of fitness. Additionally, we also note that there was variation in outcomes among the independent co-cultures that we analyzed. The cultures did not all follow the same evolutionary dynamics, as would be expected in any experimental evolution experiment. The patterns we observed were strong enough to not be obscured by the variation among cultures; however, we acknowledge that this variability makes the results more prone to type II errors. When these errors were more likely, we repeated the assays to confirm the absence of a pattern. Furthermore, the time-shift, efficiency, and reduction of production shown here are patterns that we have also observed in other independent experiments using this yeast system, whereas the pattern of increase in lysine production is indeed a unique outcome of this experiment. We argue that this pattern matches our expectations based on changes in the costs and benefits. When the Lys mutualist evolved reduced lysine overproduction, this resulted in a reduced population size of the Ade mutualist that reduced the benefits received by Lys. As a result, increased production of lysine was likely favored. Thus, the results we present here most likely demonstrate real evolutionary changes rather than experimental artifacts.

Our results corroborate findings from other studies of microbial mutualisms that have shown that mutualists can adapt to the interaction to have increased fitness. For example, Harcombe (2010) showed in the interaction between *Escherichia coli* and *Salmonella* that cultures with a higher percentage of cooperators had higher yield than cultures with noncooperators. Similarly, Hillesland and Stahl (2010) showed that even though the mutualism between a bacterium and an archeon is initially erratic, it achieved stability and higher yield after ∼300 generations. Although these studies document how the fitness of species

involved in the interaction can increase over time, studies rarely show how traits associated with the costs and benefits evolve. One of the rare studies to test evolution of mutualistic traits used a bacterial mutualism to show that mutualists can quickly increase production of resources to exchange with one another (Hosoda et al. 2011). Our study is novel in demonstrating that mutualists can change both the production of the commodity exchanged, as well as the benefits obtained in the mutualism through changes in efficiency. However, the response of the mutualists to alter the benefit to cost ratio is dependent on how limited they are by mutualistic resources.

Understanding how mutualisms persist requires consideration of the evolution of mutualistic traits that can drive coevolution between partners. Our experiment used a microbial mutualism to show that coevolved partners adapted in different ways to the interaction based on their mutualistic traits. These differences between partners in the evolution of costs and benefits were mainly due to how limited each partner was by the commodities being exchanged and by the time lag of responses to changes in their partner's traits. Exploration of the evolution of the costs and benefits involved in mutualisms should be further conducted with other systems and under differing conditions to show if these outcomes are context dependent or if there are general rules of the coevolutionary process as it pertains to mutualisms. Future research should also explore how environmental context and resource availability can influence coevolution and adaptation of mutualists to the interaction.

AUTHOR CONTRIBUTIONS

KAS modified the yeast strains and designed and conducted the coevolution experiment. KAS and MCV designed and MCV conducted the efficiency and production assays. MCV analyzed the data and wrote the first draft. KAS and MCV contributed equally to draft revisions.

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DATA ARCHIVING

We have included the data as supplementary information.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Cost of overproduction of mutualistic commodities.
Figure S2: Quadratic fit for resource use efficiency (A) and production of lysine (B) of Lys^{evo}.
Figure S3: Quadratic fit for resource use efficiency (A) and production of adenine (B) of Ade^{evo}.