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# Seasonal resource oscillations maintain diversity in bacterial microcosms

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## ABSTRACT

**Hypothesis:** Seasonal variation in availability of resources maintains co-existence between different ecological types due to frequency-dependent interactions.

**Organism:** *Escherichia coli*.

**Methods:** When populations containing two ecotypes of *E. coli* that evolved from a common ancestor under sympatric conditions grow in batch culture on a mixture of glucose and acetate, they first use up the available glucose and then switch to acetate consumption, thus creating a fluctuating environment with two different 'seasons'. We removed the alternation between the seasons so that polymorphic populations only experienced the first (glucose) season or the second (acetate) season and monitored the frequency of the ecotypes. We subsequently removed the stationary phase of the batch culture to determine its contribution to competitive interactions between the two ecotypes. (During the stationary phase, a population stops growing because it has depleted its resources and produced too high a level of toxins.)

**Results:** We show that for two stable, heritable ecotypes of the bacterium *E. coli* co-existing on two seasonally available resources, the removal of either resource season erodes the stable co-existence equilibrium and results in dominance by one or the other ecotype, depending on which season is removed. We observed similar shifts from co-existence to dominance in three evolutionarily independent populations. In two of these, the stationary phase had no effect on competition between the types, but in the third population, stationary phase favoured one type. Because resources are depleted in each batch, in each season the growth rate of one ecotype depends on the growth rate of the other ecotype. Our results thus strongly support the claim that co-existence between the two ecotypes is maintained by frequency-dependent competition in a seasonal environment.

*Keywords*: ecological diversification, environmental variability, frequency dependence, seasonality, seasonal resource availability, trade-off.

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## INTRODUCTION

Theories of niche assembly and dynamics have identified avoidance of competition as a key factor for the origin and maintenance of diversity (Armstrong and McGehee, 1980; Brew, 1982; Tilman, 2004). When environmental variation is modelled as a spectrum of different resources that remains constant through time (Dieckmann and Doebeli, 2004), resource- or niche-partitioning generates resource specialists. By specializing, different types avoid each other's competition, and this reduction in competition allows co-existence. A different type of environmental variation occurs when the availability of different resources varies with time (Chesson, 2000). In the extreme case when only a single resource is available at any given time, specialists cannot usually avoid each other's competition and instead compete for the same resources. In this situation, a classical result for the case in which ecological interactions are frequency-independent is that co-existence of specialists is not expected, and that the generalist type with the highest geometric mean fitness across different resource seasons out-competes all other types (Kimura, 1954; Dempster, 1955; Dean, 2005). If competition is frequency-dependent, so that in a given environmental season the growth rate of one type depends on the growth rate of the other types present, seasonal variation in resource abundance can maintain co-existence of two different types if there are trade-offs between performances at different points of the environmental cycle (Dempster, 1955; Hutchinson, 1961; Stewart and Levin, 1973; Armstrong and McGehee, 1980; Hsu, 1980; Namba and Takahashi, 1993; Dean, 2005; Leimar, 2005). Such frequency dependence can occur, for example, when temporal environmental variation in resource availability is generated through depletion and subsequent replenishment of these resources. In this case, the amount of resources available to one type at any given time depends on the amount of resources used at that time by other types, so that a trade-off between adaptations to high and low resource abundancy can generate the frequency dependence necessary to maintain co-existence between types lying on different points along the trade-off curve (Dempster, 1955; Stewart and Levin, 1973; Leimar, 2005).

Even though intrinsically generated seasonal cycles created by the depletion of resources should occur frequently in nature, empirical evidence for the evolution and maintenance of diversity due to temporal environmental fluctuation is rare, in contrast to the case of contemporaneously available niches. The best examples of diversity maintained by seasonality are from competition experiments among lake phytoplankton, where pulses of resource enable co-existence of subdominant species with the dominant species with the result that more species co-exist in pulsed environments than in similar constant environments (Sommer, 1984, 1985; Grover, 1988). Contrary to this pattern, experimental evidence from microbial microcosms shows that while co-existing specialist types often evolve in constant environments with different contemporaneous niches, this diversity is much harder to maintain in temporally fluctuating environments, which typically contain a single dominant generalist type (Kassen, 2002; Elena and Lenski, 2003). In spite of this result, batch cultures of microorganisms provide very good examples of intrinsically established seasonal cycles caused by resource depletion and subsequent replenishment. Here we present direct experimental evidence that seasonality in batch cultures can indeed be the causative agent maintaining the co-existence of different and competing bacterial ecotypes.

In our experimental serial batch cultures, strains of the bacterium *Escherichia coli* are inoculated each day into a new virgin environment containing a mixture of glucose and acetate, a preferred and a non-preferred source of carbon, respectively. Wild-type genetic regulation to repress the consumption of non-preferred resources forces the bacteria

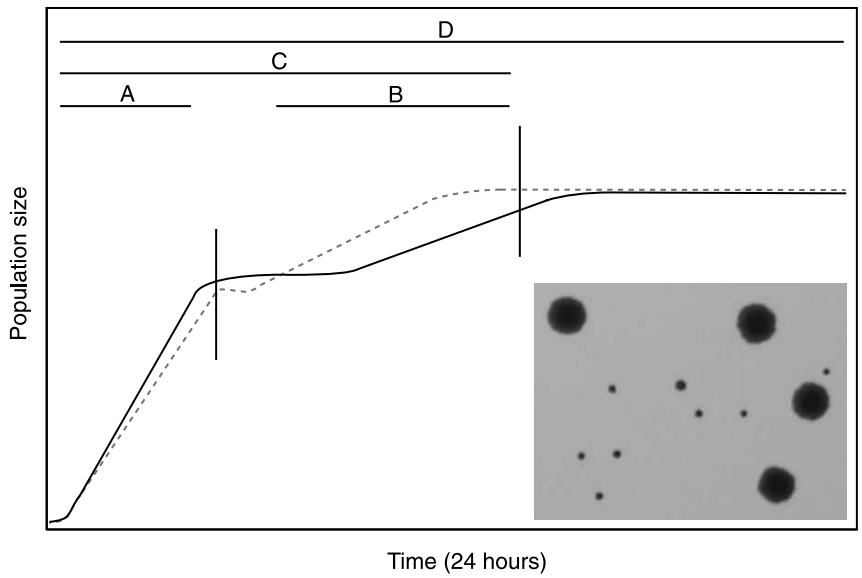


**Fig. 1.** Schematic seasonal cycles for Large and Small resource use in glucose-acetate batch culture. Redrawn from Figure 4 in Friesen *et al.* (2004). Curves represent population growth of Larges (solid line) and Smalls (dashed line) with horizontal bars delineating the (A) first season, (B) second season, (C) first + second seasons, (D) full seasonal cycle including stationary phase, which represents the evolutionary environment and was used as the experimental control. Vertical dividers roughly identify switch points partitioning the growth curves into the first and second seasons. In the inset, Large and Small colony growth phenotypes of evolutionarily diversified *E. coli* from population 31 are plated on tryptone agar supplemented with tetrazolium dye.

to consume the glucose before the acetate (A in Fig. 1). Once the glucose is exhausted, subsequent expression of previously repressed genes allows *E. coli* to consume the non-preferred carbon source acetate (B in Fig. 1) (O'Beirne and Hamer, 2000). This genetic regulation of resource use establishes two resource seasons in each batch: a first season when glucose is metabolized, and a second season when acetate is consumed. Onset of the second season is precipitated by resource depletion during the first season, even though acetate is available throughout the first season.

If there is a trade-off in competitive abilities in the two seasons, increased relative performance during the glucose season leads to decreased relative performance during the acetate season. Because resources are limiting, performance in both seasons is relative to that of other types that are present, and hence selection is frequency-dependent. In theory, such frequency dependence can generate co-existence between different types (Leimar, 2005).

Earlier we proposed that this type of frequency dependence due to seasonal environments has facilitated sympatric evolutionary diversification of a single ancestral *E. coli* strain into two co-existing ecotypes (Doebeli, 2002; Friesen *et al.*, 2004). After 1000 generations of evolution in serial batch culture in the glucose–acetate two-resource environment, several replicate *E. coli* populations diversified into two ecomorphs, named 'Larges' and 'Smalls' according to the morphology of the colonies they form when plated on agar (Fig. 1, inset) (Friesen *et al.*, 2004). Larges and Smalls differ in how they use the resources: isolated Large genotypes

exhibit a high growth rate on glucose, a lower growth rate on acetate, and a long time to switch to acetate once the glucose is exhausted. Relative to Larges, isolated Small genotypes grow less rapidly on glucose, more rapidly on acetate, and switch more quickly to consume acetate. Derived Larges and Smalls from three independently evolved populations (29, 31, and 33) showed consistent differences in switching times and growth rates on glucose versus acetate (Friesen *et al.*, 2004).

Friesen *et al.* (2004) also showed that during a single seasonal cycle, the three populations showed an initial increase in the proportion of the Large ecotype, followed by a decrease that corresponded to an increase in the Small ecotype (Figure 6B in Friesen *et al.*, 2004). These frequency fluctuations across a single day of batch culture tracked with glucose and acetate exhaustion in the media, and therefore suggested that temporal environmental variation caused by batch culture could be crucial for the maintenance of diversity in this system. Here we explicitly test whether two-season batch culture maintains diversity by performing competition experiments over many consecutive batches in environments in which one of the two seasons was missing.

Co-existence of Larges and Smalls in these populations is an ideal testing ground for the idea that the maintenance of this diversity is due to the seasonality of the environment generated by two-resource batch culture. We therefore analysed the importance of the two resource seasons for co-existence between random sub-samples of Larges and Smalls from three populations by removing the alternation between the seasons so that populations only experienced the first season or the second season. Of special importance is that in the experiments reported here, the populations did not experience the switch from one season to the next, and so the experiments address the effects of two different seasons, rather than of switching between those seasons. In an additional experiment, we tested the effect of the stationary phase on co-existence. In batch culture, bacterial population growth comes to a halt as a result of depletion of available resources and accumulation of toxic products. The resulting stationary phase is generally thought to be an important determinant of the ecological and evolutionary dynamics of bacteria (Matin *et al.*, 1989). Removing the stationary phase to determine its potential to influence competitive interactions between Larges and Smalls showed that stationary phase is unlikely to be the main driving force maintaining diversity in our microcosms. Instead, we present conclusive evidence that temporal fluctuation in resource availability is crucial for maintaining evolutionarily stable dimorphisms in three independently evolved populations in which diversity arose in sympatry from a common ancestral genotype.

## MATERIALS AND METHODS

### Strain evolution, media, and storage

Populations 29, 31, and 33 were evolved from a single ancestral *E. coli* B strain (REL606) over 1000 generations in a two-resource environment consisting of Davis Minimal medium (DM) supplemented with 205 $\mu g \cdot ml^{-1}$ glucose and 205 $\mu g \cdot ml^{-1}$ acetate (glucose-acetate) (Friesen *et al.*, 2004; Tyerman *et al.*, 2005). All three populations are stably diversified into the Large and Small ecotypes. For all experiments described here, we garnered whole-population samples from these populations by scraping ~50 µl of frozen culture from the top of each population sample, which was preserved in 20% glycerol at −80°C. This sampling procedure is stochastic and yields variable inoculation proportions of the two ecotypes. For each

experiment, we cultured the populations from the freezer in 10 ml glucose-acetate for 24 h at 37°C and 250 rev · $min^{-1}$ using 18-mm (diameter) culture tubes.

### Assay conditions and controls

For each experiment, the populations were inoculated into the appropriate medium. We varied the media, numbers of replicates and transfers, and the dilution per transfer for each experiment, as described below. Three replicates of a single Large and single Small colony isolate from population 33 were used as controls to ensure that strains would not evolve to or become contaminated by the opposite ecotype during the course of the experiment. To assay the proportion of Larges for each transfer, we diluted each culture and plated the colonies on tryptone agar with a tetrazolium indicator dye; plates were incubated at 37°C after inoculation. We counted the number of colonies that grew within 24 h ('Larges') and the number of colonies that grew between 24 and 48 h ('Smalls').

### Media and transfer conditions for each season

To address how seasonal resource availability affected the maintenance of diversity, we altered the media and the serial transfer schedule for each experiment. For the first season experiment, we deleted the second season by propagating populations through serial transfer before glucose was exhausted. We grew five replicates for each of three populations to late log phase on glucose and, while the cells were still doubling at a high rate (at approximately 4 h), we transferred 1/32 individuals into fresh medium. (Population 33 received 1/100 cells per transfer for the initial three transfers.) We repeated this transfer process for 31–44 generations, such that the competing ecotypes nearly exhausted their glucose supply 6–7 times without ever being allowed full access to the acetate supply, which was present but unavailable because of catabolite repression.

For the second season experiment, we used each population to exhaust the 205 $\mu g \cdot ml^{-1}$ glucose content of glucose-acetate medium, then filtered the bacteria from the glucose-exhausted media using a 0.2-μm vacuum filter. Second season media were tested for glucose and acetate content and found to contain $212 \pm 0.003$ $\mu g \cdot ml^{-1}$ acetate and less than $3.6 \pm 0.001$ $\mu g \cdot ml^{-1}$ glucose (means ± standard errors). We inoculated five replicates of each sterile, conditioned medium with its appropriate population using a 1/32 serial dilution every 15 h, on average. The second season resources were seasonally depleted eight times over 36 generations of competition.

The first 12 h of growth in batch culture include the full glucose season, the full acetate season on average, but only a small fraction of the stationary phase, a period when cells enter metabolic stasis to survive starvation after resource depletion in 24-h batch culture (Matin *et al.*, 1989). In their normal batch culture regime of one transfer every 24 h, our microcosms typically spend slightly more than 12 h in stationary phase, and so it is conceivable that processes during this phase impinge on the co-existence of the strains. To test for such effects, we reduced the time spent in stationary phase by serially transferring five replicates through glucose-acetate medium every 12 h at a 1/100 dilution, shortening stationary phase by 12 h, on average (C in Fig. 1). In the control experiment, we grew six replicates to determine the equilibrium ratio of 'Larges' to 'Smalls' in the evolutionary medium (glucose-acetate) via five successive transfers at a 1/100 dilution and 24 h between transfers.

### Data analysis

For first and second season data, the inoculation proportion of the Large colony type was compared with the final time point within each experiment to detect changes in the proportion of Larges over time using two-tailed *t*-tests in JMP (SAS Institute, 2001). To determine how changes to the seasonal cycle affected the ratio of the Large to Small ecotypes, the final time points for all four experiments were contrasted using *F*-tests in proc GLM in SAS. We compared the means of the final time points of each experiment with Ryan-Einot-Gabriel-Welsh (REGWQ) multiple range tests (SAS Institute, 1989). Proportional data were arcsine transformed for all analyses.

## RESULTS

When we cycled the populations through the first season experiment, Larges outcompeted Smalls in all three populations. After the final bout of the first season, the frequency of Larges had increased significantly relative to their initial frequency and relative to the final frequency in the control population (Fig. 2, Table 1). In all replicates, both ecotypes persisted at the final assay. However, the composition of the microbial communities changed significantly, and the weaker competitors became very rare: Smalls comprised only 5.7, 14.2, and 0.7% of the first season populations after 43, 44, and 32 generations, respectively.

In the second season experiment, Smalls outcompeted Larges in all three populations. After 36 generations, the frequency of Larges was significantly lower than each population's initial and control values (Fig. 2, Table 1). In a single replicate of population 31, the Large ecotype was extirpated or reduced to less than 0.49%. In all other population replicates, both ecotypes persisted. After 35–36 generations, Larges comprised only 1.1, 0.4, and 23% of the second season for populations 29, 31, and 33, respectively.

Control strains remained uncontaminated and true-breeding except in a single instance when Small colonies appeared in the Large control at 1.3% after the fourth transfer, lower than the 6% minimum change noted in the experimental strains. These contaminant (or newly evolved) small colonies may represent the true Small ecotype, or they may be small

**Table 1.** Means comparisons of the proportion of Large colony types for the first and last time points for each population in the first season, second season, and stationary experiments as well as comparisons of the final time points across all experiments

| | Population 29 | | | Population 31 | | | Population 33 | | |
|---|---|---|---|---|---|---|---|---|---|
| Experiment | *t* | d.f. | *P* | *t* | d.f. | *P* | *t* | d.f. | *P* |
| First season | −14.376 | 7 | <0.0001 | −5.598 | 5 | 0.0025 | −10.648 | 8 | <0.0001 |
| Second season | 38.746 | 8 | <0.0001 | 42.589 | 8 | <0.0001 | 8.714 | 7 | <0.0001 |
| Stationary experiment | 1.810 | 9 | 0.1037 | −14.225 | 9 | <0.0001 | −1.27 | 9 | 0.2890 |
| | *F* | d.f. | *P* | *F* | d.f. | *P* | *F* | d.f. | *P* |
| Final time points | 365.48 | 3,16 | <0.0001 | 600.54 | 3,15 | <0.0001 | 57.12 | 3,16 | <0.0001 |

*Note*: Data were arcsine transformed proportion of Large colony types.

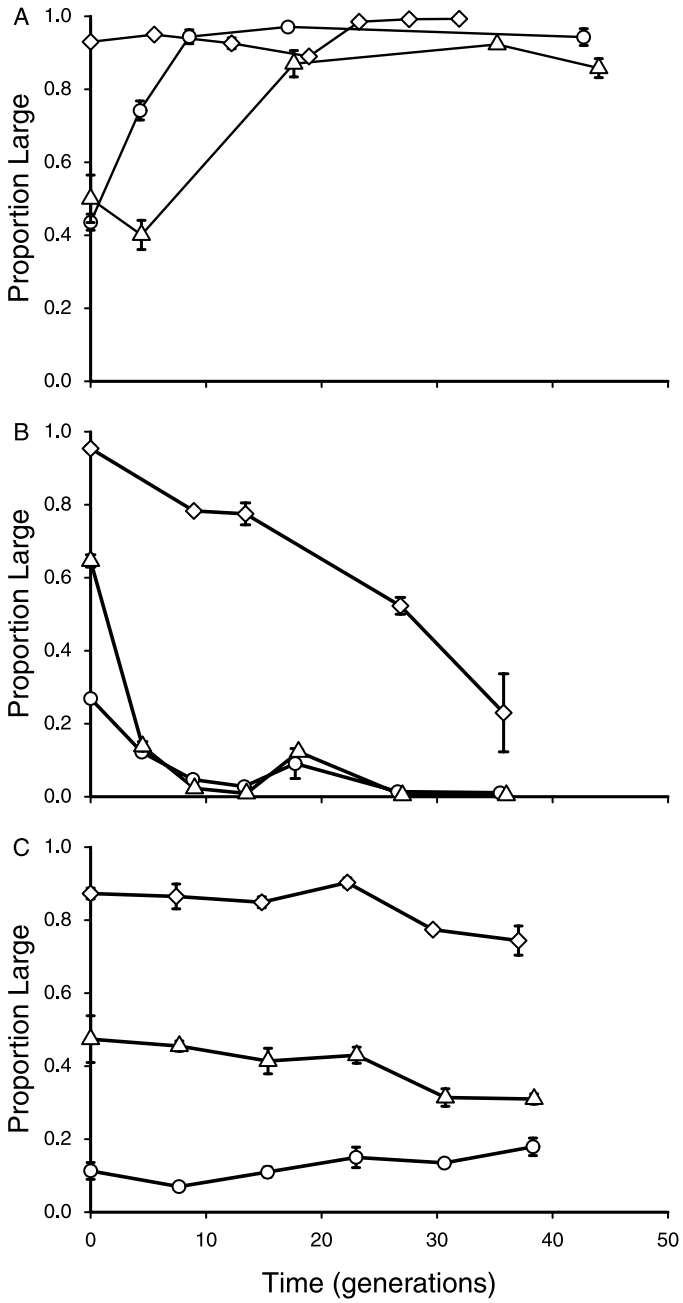


**Fig. 2.** The competitive ability of Larges versus Smalls when restricted to a single season for three independently evolved populations. Populations 29 (circles), 31 (triangles), and 33 (diamonds) were cycled through the first resource season (A), second resource season (B), and the control dynamics given a full seasonal cycle (C). For each population, the final proportion Large from the first and second seasons differs significantly from the final control proportion Large (Table 1). Data represent the proportion of the Large colony type averaged across 5–6 replicates (± 1 standard error) (see 'Methods').

for an unrelated reason. They were not tested to determine if their acetate use corresponded to the typical Small ecotype. Because invasion by the opposite ecotype into the control Large and Small replicates is rare and in accord with pilot work and previous experiments, we are confident that the shifts in ecotypic frequency in our results reflect selection pressures of each seasonal environment.

To determine whether stationary phase contributes to the negative frequency dependence that maintains diversity in our microcosms, we assayed the proportion of each type after two resource seasons with and without stationary phase (D vs. C in Fig. 1). When Larges and Smalls from populations 29 and 33 were grown through both resource seasons but not stationary phase, the dynamics of the two types equilibrated at the control frequency (Fig. 3), implying that stationary phase does not confer a competitive advantage to either type. In contrast, Larges in population 31 reached a final assay frequency greater than the control (Fig. 3) but less than the first season frequencies (Fig. 2), indicating that stationary phase confers a sizeable advantage to Smalls in this population only.

## DISCUSSION

Co-existence of different types competing for the same resources requires frequency dependence with a trade-off in resource use ability (Armstrong and McGehee, 1980), so that rarity confers an advantage. Therefore, examples of the evolution of intrinsically caused seasonal fluctuations that are maintained by negative frequency-dependent competitive interactions should be commonplace, yet they are not. Here we have provided an example in an experimental evolution model where seasonal fluctuations can be important for maintaining diversity if the fluctuations are a consequence of the competitive interactions, as when resources are depleted periodically. In this case, frequency dependence can occur if

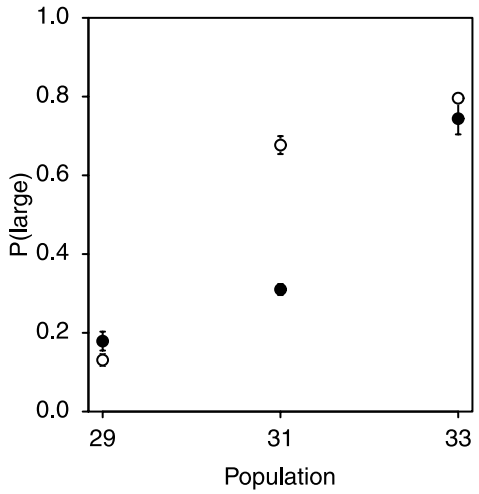


**Fig. 3.** Ecotypic diversity with and without stationary phase. Comparison of the final time points for the control proportion of Larges with stationary phase (solid circles) and reduced stationary phase (open circles) shows that stationary phase favours the persistence of Smalls in population 31 but does not influence the ratio of ecotypes in populations 29 and 33. Data represent the proportion of the Large colony type averaged across 5–6 replicates (± 1 standard error) (see 'Methods').

performance in each season depends on the phenotypic composition of the population, as is often the case if the traits under investigation affect competition for limiting resources. A trade-off between performance in different seasons can then lead to co-existence between different types along the trade-off curve (Armstrong and McGhee, 1980; Chesson, 2000; Leimar, 2005). While our earlier work hypothesized that such seasonal resource use maintained diversity in this system, here we have explicitly demonstrated that this mechanism is likely to maintain the diversity that had evolved in our *E. coli* populations. Each batch is supplied with a mixture of glucose and acetate, whose sequential depletion results in a resource environment that alternates between a glucose (first) season and an acetate (second) season. This sets the stage for frequency-dependent interactions between the Large and Small *E. coli* strains, along the trade-off between performance on glucose and acetate.

Our results show that destruction of the standard two-season cycle critically affects the co-evolved co-existence between the two strains because Larges and Smalls were superior competitors to each other in the glucose and acetate season, respectively. The removal of either season made the ratio of ecotypes less equitable along a trend towards complete loss of a disfavoured ecotype. The complete loss of one type was almost never fully realized over the short time-course of this experiment because once we disrupted the alternation of the two-season cycle, selection against the non-favoured type becomes very weak when that type is rare. Our current results indicate that season-specific adaptations could be sufficient to maintain diversity in the seasonal environment. In these microbial test tube communities, diversity was lost with the removal of seasonal oscillations, and hence the seasons contribute significantly to the maintenance of diversity in this system.

Some diversified microbial systems exhibit cross-feeding (Rosenzweig *et al.*, 1994; Vasi *et al.*, 1994; Rainey *et al.*, 2000), where one type persists by specializing on the waste products of the other type. Because Smalls consume glucose readily (Friesen *et al.*, 2004; Tyerman *et al.*, 2005; Spencer *et al.*, 2007), they cannot be considered traditional cross-feeders. However, while Larges generate an accumulation of acetate during growth on glucose, acetate does not accumulate during glucose growth for Smalls (Spencer *et al.*, 2007). During competition in the first season, the Smalls can potentially access acetate, which might allow them to persist in the face of competition with Larges for glucose.

Recent work has brought much needed attention to the stationary phase, historically considered a time of stasis for a microbial population. As others found with changes in mutation rates and selection during stationary phase, we deemed it possible that stationary phase affected the co-existence of Larges and Smalls in our experimental microcosms. In particular, the stationary phase reached after exhaustion of both resources might constitute a third, cannibalistic season in the serial batch culture environment. For two populations, stationary phase does not differentially favour survival of Larges or Smalls. Population 31, however, showed significant decreases in the Large ecotype during stationary phase. In population 31, the resource seasons were sufficient to maintain two types, but the weighting towards the small type in stationary phase could indicate that the stable dimorphism is short-lived over evolutionary time. As the Smalls becomes favoured in the second season and the stationary phase, they have the evolutionary potential to outcompete the Larges and come to dominate in this population. The differences between population 31 and the others with respect to stationary phase also reinforce the notion that parallel ecological selection pressures generate similar phenotypic solutions that are potentially mediated by different genetic mechanisms, such that Larges and Smalls from the different populations are not exactly identical, despite their similar ecological behaviours (Tyerman *et al.*, 2005).

Furthermore, extrinsic factors have been proposed to maintain co-existence when one type can buffer its population against the competitive effects of the other, such as over-wintering in plants and early entry into stationary phase in bacteria (Chesson, 2000; Descamps-Julien and Gonzalez, 2005). This mechanism of co-existence, called 'storage', would not maintain two types in our system because neither Larges nor Smalls enter stasis during the second season. Rather, both ecotypes have positive growth rates in the second season regardless of the presence of the competing ecotype (Friesen *et al.*, 2004).

Our findings extend previous work in microbial populations that examined the potential for seasonal effects to maintain diversified ecotypes. Assays of the 24-h dynamics of seasonal (batch) culture had previously identified seasonal resource use as a potential source of stability between diversified ecotypes in single-resource (Rozen and Lenski, 2000) and two-resource environments (Friesen *et al.*, 2004; Tyerman *et al.*, 2005), but no conclusive tests had been performed. Competition assays in exhausted medium, as described in our second season experiment, have also been used to test for allelopathic and cross-feeding strains in continuous (chemostat) culture (Helling *et al.*, 1987; Rosenzweig *et al.*, 1994), but again this did not specifically constitute a test for the maintenance of variation. When resources were provided alternately instead of simultaneously to competing genotypes in a chemostat, single types won, except within a very limited range of stability (Dean, 2005; Lunzer *et al.*, 2002; Suiter *et al.*, 2003). In contrast to all of these studies, we have competed populations of co-evolved ecotypes in the two different resource seasons in which they diversified. We restricted their resource base to a single resource and augmented the effects of that restriction by extending the number of generations they could grow on each resource. Our manipulation of seasonality altered the stability of dimorphisms that had co-evolved in sympatry from a common genotype, which provides a strong test of how temporal resource availability affects diversity derived by natural selection.

Our results indicate that this loss of diversity was due to a change in the nature of the selective regimes operating in the microbial communities. In the original two-season glucose-acetate environment, negative frequency dependence caused each ecotype to increase in frequency when rare in the population, thereby maintaining diversity at an intermediate equilibrium (Friesen *et al.*, 2004). When we removed the regular seasonal oscillations between glucose-dominated and acetate-dominated environments, an ecotype that became rare was no longer restored to higher frequency but rather remained rare. Thus, the removal of the seasons eliminated or irreplaceably altered the negative frequency dependence that had maintained the two ecotypes at stable, intermediate equilibria in their evolutionary environment. In the long run, the consequences of the removal of seasonal oscillations will be either a complete loss of the weaker competitor or the maintenance of diversity at significantly altered frequencies. In the first instance, the significant shift in the proportions of the two ecotypes could indicate the slow but inexorable collapse of diversity. In this case, seasonal resource depletion could have slowed the complete competitive exclusion of an ecotype (Chesson, 1986). Alternatively, long-term maintenance of the two types after removal of one of two resource seasons could come about from a co-evolutionary response where selection is rapid enough to overcome the dramatic shift in resource environments. Rapid co-evolutionary adaptation of each ecotype to the altered environment might force the pairs to new, stable equilibria. Although the ecotypes do not exhibit complete specialization, their specialist attributes might facilitate their adaptive response to shifts in resource availability (Whitlock, 1996).

In fact, in some sense it is surprising that the glucose-alone season cannot sustain the two

types, as suggested by theories of niche assembly that focus on qualitative avoidance of resource competition (Brew, 1982). This is because during this season both resources are present, and hence there is the potential for niche expansion from the glucose resource onto the acetate resource. Such expansion could be facilitated by selection for a mutation that disrupted wild-type catabolite repression, thereby enabling a mutant type to consume acetate in the presence of glucose (Spencer *et al.*, 2007). This would lead to co-existence of two resource specialists according to classical partitioning of contemporaneous niches. Thus, our first season should be able to maintain two specialized ecotypes, but our results show that it does not, at least not the two ecotypes that have evolved in our microcosms. It is possible that in the selective environment, a complete acetate specialist would grow more slowly during the first season than the observed acetate specialist that switches from glucose to acetate. Due to the difference in quality between the two resources, glucose and acetate, we observed the evolution of a glucose specialist and a glucose-acetate generalist. A selective environment with nutrients more similar in quality may promote more classical niche differentiation. Regardless, this again underlines the potential importance of seasonality for the maintenance of diversity.

Many of the ecological factors that promote the origin and maintenance of diversity remain elusive, but here we provide direct experimental evidence that one way to maintain biological diversity is seasonal fluctuations in availability of limiting resources. Empirical disruption of the seasonal cycle caused a subsequent shift in the frequencies of the types and loss of diversity, a pattern that was repeatable in ecologically similar populations with co-evolved ecotypes. The process of intrinsically generated seasonal resource depletion is likely to be a widespread phenomenon in natural systems as disparate as desert mice (Brown, 1989) and cactus-tending ants (Morris *et al.*, 2005), and could therefore be a very general mechanism to maintain diversity. Resource depletion always creates new environments, and if the resource is regularly renewed, these new environments become a seasonal selective force. Adaptation to an environment with temporal resource availability may facilitate the evolution of other types through trade-offs in competitive ability at different times in the temporal cycle. In fact, each pair of ecotypes in this experiment was co-evolved from a common ancestor in a seasonal environment, implying that seasonality is not only an ecological phenomenon but can also drive the evolution of diversity by selecting for rare mutants that use the seasonal environment in a complementary manner to the resident ecotype.

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