INTRODUCTION

Experimental evolution studies with microorganisms such as bacteria and yeast have been an increasingly important and powerful tool to draw long-term inferences of how microbes interact. However, while several strains of the same species often exist in natural environments, many ecology and evolution studies in microbes are typically performed with isogenic populations of bacteria or yeast. In the present study, we firstly perform a genotypic and phenotypic characterization of two laboratory and eight natural strains of the yeast *Schizosaccharomyces pombe*. We then propagated, in a rich resource environment, yeast communities of 2, 3, 4, and 5 strains for hundreds of generations and asked which fitness-related phenotypes—maximum growth rate or relative competitive fitness—would better predict the outcome of a focal strain during the propagations. While the strain's growth rates would wrongly predict long-term coexistence, pairwise competitive fitness with a focal strain qualitatively predicted the success or extinction of the focal strain by a simple multigenotype population genetics model, given the initial community composition. Interestingly, we have also measured the competitive fitness of the ancestral and evolved communities by the end of the experiment (≈370 generations) and observed frequent maladaptation to the abiotic environment in communities with more than three members. Overall, our results aid establishing pairwise competitive fitness as good qualitative measurement of long-term community composition but also reveal a complex adaptive scenario when trying to predict the evolutionary outcome of those communities.

KEYWORDS
adaptation, competitive fitness, growth rates, prediction, propagation, yeast strain communities
environmental, and medical purposes. However, predicting how complex communities of microbes behave over large timescales is difficult since competition for scarce resources and limited space among diverse strains and species can be complex (Durão et al., 2020; Estrela & Brown, 2018; Ghoul & Mitri, 2016; Goldford et al., 2018; Hibbing et al., 2010; Pande & Kost, 2017; Pande et al., 2014). For instance, while competition between microbes is extremely common, positive interactions such as cooperation between species have also been documented (Harcombe, 2010; Rodríguez-Verdugo & Ackermann, 2020; Vidal et al., 2020). Moreover, evolution is likely to occur on timescales relevant for ecological dynamics, and thus, a complete understanding of the maintenance of diversity also requires assimilation of data about the evolutionary forces at work (Celiker & Gore, 2014; Lankau, 2011; Lawrence et al., 2012). On the one hand, novel mutations generate diversity which may be stably maintained (Amicone & Gordo, 2021; Dieckmann & Doebeli, 1999). On the other hand, evolution can also reduce the effectiveness of coexistence mechanisms through selection of the fittest, and thus, competitive exclusion occurs (Geritz et al., 1998; Shoresh et al., 2008). Despite some evidence suggesting that, over time, competition dies down locally, often leading to stable coexistence of genetically distinct lineages, the selective forces acting during competition and the resulting evolutionary fates of the different players depend on ecological conditions in a way that is not yet well understood (Ghoul & Mitri, 2016).

Interestingly, most ecology and evolution studies are still performed in isogenic populations, while several strains of the same species often exist in natural environments and the consequences of such diversity for the evolutionary outcome is poorly known. The intrinsic ability of a strain to adapt depends on its mutation rate (Good et al., 2017; Ramiro et al., 2020), population size (Lanfear et al., 2014), the initial degree of adaptation to the environment (Buckling et al., 2003), and the malleability of its genome (Jouset et al., 2016). However, all these intrinsic factors depend on the biotic environmental context (Castledine et al., 2020; Lawrence et al., 2012; Osmond & de Mazancourt, 2013; Scheuerl et al., 2020; Turcotte et al., 2012). For instance, communities may constrain adaptation by competing for resources and force populations to exploit alternative niches (Hall et al., 2018) or may facilitate adaptation by generating new niches (Calcagno et al., 2017; Emerson & Kolm, 2005; Lawrence et al., 2012) or suppressing competitors (Osmond & de Mazancourt, 2013). Thus, biotic interactions can alter evolutionary responses making it almost impossible to make long-term predictions unless some empirical knowledge is gathered.

*Schizosaccharomyces pombe* is an eukaryotic model organism with wide industrial application, being used to produce distilled spirits such as rum, tequila, or cachaça (Gomes et al., 2002; Pataro et al., 2000), as well as to reduce acidity in wine given its ability to use malic acid (Benito, 2019; Volschenk et al., 2003). Furthermore, *S. pombe*-sequenced genomes and other scientific resources, such as PomBase, are available (Lock et al., 2018). Lastly, it displays short generation times at 30°C (Petersen & Russell, 2016) which allows to observe adaptation in the course of a few months. Here, we study how competition between different strains affects the maintenance of a focal strain by propagating communities of *S. pombe* for almost 400 generations in a rich medium. Planktonic populations were evolved to test, under controlled conditions, if the maximum growth rate or the relative competitive fitness of the commonly studied *S. pombe* laboratory strain (L968) relative to wild isolates could predict its fate in multistrain communities with increasing initial community diversity. We find that the maximum growth rate is not predictive of the long-term fate of a strain, but the relative pairwise competitive fitness qualitatively predicts the strain’s success or extinction. Furthermore, we have also measured the competitive fitness of the ancestral and evolved communities and observed that communities with more than 3 strains often lead to maladaptation to the abiotic environment.

## 2 MATERIAL AND METHODS

### 2.1 Strains and growth conditions

The L968mCherry strain was constructed by amplifying the his1::3nmt1-mcherry-hphMX6 from plasmid pFa6a-3nmt1-mcherry-hphMX6 (Avelar, 2012), using primers in which where 80 base pairs were homologous to the his1 gene and 20 were homologous to the pFa6a backbone (Bähler et al., 1998). The primers used were 5’-agctgacctgcttaatattatatggttaaatgtgaaggctcttacctcgaattcgcgattgcag-3′ and 5′-ttttgacttcgagtcagtaatttt-3′. These were confirmed to have the mating type h+. The resulting fragment was used to transform strains L975 matP:nat (mating type h+) and L972 matM:nat (mating type h−) (Bähler et al., 1998) using the lithium acetate method (Keeney & Boeke, 1994) and selected in hygromycin plates. Fluorescence was confirmed by flow cytometry and fluorescence microscopy. In order to minimize artifacts produced during the transformation process, the resulting strains were mated to L972 (mating type h+) and L975 (mating type h−). From these two crosses, two strains were obtained: L972mCherry and L975mCherry. These were confirmed to have the mating loci of the parental L972 and L975. Finally, we have crossed L972mCherry (h−) with the L698 (h90) strain to obtain the L698mCherry reference strain. The chosen reference strain was hygromycin resistant, had h90 mating type, and was fluorescent. L968, L972, and L975 were obtained from the Yeast Genetic Resource Center, and the natural yeast isolates are described in Avelar et al. (2013). Strains were grown in YES medium (5% yeast extract, 3% glucose, 225 mg/L histidine, 225 mg/L adenine, 225 mg/L leucine, and 225 mg/L uracil, and 2% Bacto agar for YES agar) supplemented with 100 μg/ml ampicillin at 30°C without shaking, unless otherwise stated. Ampicillin was added to the medium throughout the experiment to avoid bacterial contaminations. For ethanol tolerance, 10% (v/v) was added to the medium.
2.2 | Growth curves

Yeast strains were streaked individually onto YES agar plates and incubated overnight at 30°C. The next day, at least three independent colonies from each strain were inoculated separately in YES medium (200 µl per well) in a 96-well plate and incubated overnight at 30°C without shaking. The following day, yeast was quantified by optical density at 600 nm wavelength (OD$_{600}$nm), and numbers of cells correlation obtained by plating cultures of the L968mCherry strain. Approximately $=1 \times 10^4$ cells were inoculated in 100-well plates containing YES medium and incubated at 30°C with shaking only before each measurement in a Bioscreen C (Oy Growth Curves Ab Ltd.) benchtop microplate reader, measuring OD$_{600}$nm every 20 min for 48 h. Antagonistic interactions such as toxin production or the presence of beneficial waste metabolites were assessed by measuring the growth rate of single strains growing in a YES-altered medium.

To make YES altered media, the supernatant of a community of five S. pombe strains (L968mCherry, L968, SPW23, R435, andNCYC132) grown for 24h in YES medium was collected by centrifugation and 10% of this supernatant was applied to fresh YES medium.

2.3 | Long-term propagations and Flow cytometry

Propagations were carried over in 96-well plates with YES media incubated at 30°C with no shaking and with 5 daily bottlenecks followed by a three-day incubation step. The bottleneck was a 1:500 dilution of grown cells into fresh media (200 µl total volume) and represents around 9 generations. The relative frequency of the L968mCherry strain on the communities during the propagations was measured sporadically resourcing to LSR Fortessa flow cytometer using a 96-well plate autosampler. Briefly, flow cytometry samples consisted of 190 µl of PBS and 10 µl of 10-fold dilution of the yeast culture in PBS. The mCherry fluorescent protein was excited with a 561-nm laser and measured with a 630/75-nm pass filter.

2.4 | Prediction analysis

In order to predict the long-term fate of our focal strain, we used a multilocus population genetics model based on the fitness of each strain relative to the focal one.

In a community with N strains, let f be the focal strain (L968mCherry in our study). We define the fitness relative to f as $w_{f_i} = 1 - s_i$, where $s_i$ is estimated from the pairwise competitions (Figure 2A), and $w_{f_i} = 1$.

If $x_i(t)$ is the frequency of the strain i at time t, then its frequency in the next generation is given by $x_i(t+1) = \frac{x_i(t) + \sum j \neq i x_j(t) w_{f_i} x_j(t) x_{ji} \Delta t}{\sum j \neq i x_j(t) w_{f_i} x_j(t) x_{ji} \Delta t}$, which for the focal strain simply becomes $x_f(t+1) = \frac{x_f(t) + \sum j \neq f x_j(t) w_{f_i} x_j(t) x_{jf} \Delta t}{\sum j \neq f x_j(t) w_{f_i} x_j(t) x_{jf} \Delta t}$. The initial frequency of the focal strain $x_f(0)$ was computed from the L968mCherry frequency in each experiment while the initial frequencies of the remaining strains were assumed to be equally distributed ($x_i(0) = \frac{1}{N-1}$). Following the model described above, we update the frequency of each strain over 400 generations and obtain the prediction for the focal strain (as shown in Figures 2 and 3).

2.5 | Isolation of evolved clones

Evolved clones of the L968mCherry (369 generations) from the different communities propagated were isolated with YES agar plates supplemented with hygromycin (50 µg/ml) and ampicillin (100 µg/ml).

2.6 | Competitive fitness assays

Pairwise competitions to determine the relative fitness of the ancestral strains in comparison with the focal strain S. pombe L968mCherry were carried out by mixing in a proportion of 1:1 the fluorescent strain with one of the nonfluorescent strains and allowed for the competition to proceed for 48 h at 30°C. The initial and final frequencies of the fluorescent and nonfluorescent strains were obtained by counting their cell numbers in the flow cytometer as described above. Fitness per generation ($S_{ng}$) was calculated as $S_{ng} = \ln(R_f/R_i)/t$, where t is the number of generations for the fluorescent strain and $R_f$ and $R_i$ are the final and initial ratios between fluorescent and nonfluorescent strains, respectively. Number of generations of the fluorescent strain was calculated by $t = \ln(X_f/X_i)$, where $X_f$ and $X_i$ stand for the absolute number of reference strain in the end and in the beginning of the competition, respectively.

The relative fitness of the initial and evolved communities at the end of the experiment was measured by competitive growth against the fluorescent ancestral focal strain L968mCherry. Firstly, we have calculated the competitive fitness of the nonfluorescent strains present in the initial communities through the use of the frequencies of fluorescent L968mCherry against the nonfluorescent strains in the first 3 bottlenecks (≈27 generations). Secondly, we have mixed ancestral L968mCherry in a proportion of 1:1 to the evolved communities (≈370 generations) where the focal strain had gone extinct and measured the frequencies of mCherry fluorescence and nonfluorescent after 48 h. The initial and final frequencies of the fluorescent and nonfluorescent strains were obtained by counting their cell numbers in the flow cytometer as described above. Final fitness per generation ($S_{ng}$) of the communities was calculated as described for the pairwise competitions.

2.7 | Whole-genome sequencing analysis and genetic distances

Genomic DNA was extracted using standard procedures based on bead lysis followed by phenol/chloroform extraction and ethanol
precipitation of genomic DNA. The concentration and purity of DNA were quantified using Qubit and NanoDrop devices, respectively. DNA library construction and sequencing were performed by the IGC genomic facility. Each sample was paired-end using an Illumina MiSeq benchtop Sequencer. Standard procedures generated datasets of Illumina paired-end 250 bp read pairs. Sequencing adapters were autodetected from the raw reads and removed using fastp (Chen et al., 2018), in which case reads were trimmed from both sides, using window sizes of 4 bases across which the average quality had met a minimum threshold of 20 to be retained. The minimum read length for a read to be maintained was set to 100 bps with at least 50% quality phred score 20 content. The maintained reads were aligned to the Saccharomyces pombe reference genome L968mCherry strain (version 2020-03-01) via BWA-sampe (Li & Durbin, 2010) with default parameters. To identify genetic distances, single nuclear polymorphisms were called against the L968mCherry reference genome using a naive pipeline employing the mpileup utility within SAMtools (Li et al., 2009) and a custom filter script written in python. This script filtered base calls to ensure a minimum read mapping quality of 20 and a base call quality of at least 30 for variant calling. Among these high-quality positions, initially at least 80% of reads and a minimum of 5 quality reads had to support a putative SNP or indel on both strands with a strand bias (pos. strand/neg. strand) above 0.2 and below 5.0 for this mutation to be considered further. As a second substitution calling approach, Freebayes (Garrison & Marth, 2012) was used with the same quality requirements as those used for the naive variant calling script. Once substitutions passing filters by both approaches were used to calculate the genetic distances between the eight genomes. Pairwise genetic distances were calculated as the number of base differences between the pair of genomes divided by the total number of bases in the division of the genome considered (nuclear chromosomes combining the I, II, and III chromosomes, mitochondrial, or mating type). The script ensures that only genes present in the reference genome and in the sequence reads of the other genomes are accounted for the genomic distance analysis. To determine the phylogenetic tree, the reads of the ancestral genomes were firstly assembled by SPAdes 3.11 (Bankevich et al., 2012), and then, we used the DNAML package from PHYLIP 3.6 (Felsenstein, 1993) to infer phylogenies from nucleotide sequences by maximum likelihood.

2.8 | Spot assays

Single colonies of the yeast strains were grown in YES medium for =24 h at 30°C until they reached saturation. As before, number of cells were adjusted by absorbance whereas all strains were considered to follow the rule OD600nm = 1 corresponds to =2.97 × 10^7 cells/ml. Serial dilutions were performed in PBS to adjust populations to have the absolute number of 10^3, 10^4, 10^5, 10^6, and 10 cells in 5µl drop. Spots were done in freshly prepared YES plates supplemented with 10% ethanol. Plates were quickly dried to allow spots to soak in quickly before being incubated at 30°C for 3 days. The assay was performed in triplicate.

3 | RESULTS

3.1 | Genomic and phenotypic characterization of Schizosaccharomyces pombe across two environments

We performed a genotypic and phenotypic characterization of a collection of S. pombe strains (see Table S1), including the L968 laboratory strain (with and without the mCherry marker cloned at the his1 locus) and eight natural strains (UFMG-SPW23, UFMG-A1153, UFMG-R435, NCYC132, UFMG-A1263, UFMG-A826, UFMG-A571, and UFMG-R418). Firstly, we sequenced the whole genome of all strains and built a phylogenetic tree to assess their genetic relatedness (Figure 1A). We then chose five strains for long-term propagation based on their growth rates in standard laboratory medium (YES medium) being similar (0.34 h^{-1} ± 0.01 2SEM; Figure 1B) and calculated their genetic differences at the level of single nucleotide polymorphisms (SNPs) per base on the genome (considering chromosomes I, II, and III) (Figure 1C) and on the mating region distance.

Due to the application of yeasts in traditional biotechnologies such as baking, brewing, distiller’s fermentations, and wine making (Attfield, 1997), we have also phenotypically characterized the strains concerning their growth rates in YES media supplemented with 10% and 20% ethanol (Figure 1B). While 20% ethanol is a highly stressful environment for these strains and complete inhibition of growth of all S. pombe occurs, 10% ethanol is a milder stress under which fitness differences are revealed. When analyzing the effect of environment and genotype on the growth rate of the yeast strains, we observed that there was a significant interaction between these two factors (F = 3.14, p = .0022; ANOVA). All of the 10 genotypes tested showed a significant decrease in growth rate when exposed to 10% ethanol (Figure 1B, p < .0001 to all genotypes), highlighting the major impact of the environment (F = 795.9, p = 2.2 × 10^{-16}) in all the genotypes (F = 3.65, p = .00054). StrainNCY132 was particularly sensitive to this concentration of ethanol when compared to the laboratory strain L968mCherry (p = .0056, ANOVA with Dunnett’s multiple comparisons), a result that was further corroborated by a spot assay in plates supplemented with 10% ethanol (Figure 1D). For UFMG-R435, the growth rate was not significantly lower than L968mCherry (p = .203, ANOVA with Dunnett’s multiple comparisons), but the spot assay showed its higher sensitivity (Figure 1B–D). The NCYC132 strain was first described in East African millet beer in 1893, while L968, the standard laboratory strain, was isolated from French wine in 1924 (Jeffares et al., 2015). All other natural isolates from our collection have also been collected from cachaça (a sugarcane spirit) in Brazil (Jeffares et al., 2015). While wine and cachaça can have ethanol concentrations ranging from 5.5% to 20%, the millet beer is thought to have a concentration of ethanol of around 3%.
Thus, the natural ecology of the NCYC132 strain could be associated with the high susceptibility of this strain to ethanol. Interestingly, we noticed that in the absence of stress caused by ethanol, the strains L968\textsuperscript{mCherry}, L968, UFMG-SPW23, UFMG-R435, and NCYC132 show similar growth rates when competing in YES medium supplemented with glucose (Figure 1B), and therefore, one can predict that these strains will be able to coexist when propagated in communities large enough that random drift would only cause extinction in extremely large timescales (Lankau, 2011). Due to the scarcity of studies of evolution in the context of strain diversity, we have thus selected these five strains to study how the presence of other strains could affect long-term evolution of a focal strain (fluorescently mCherry marked for ease of measurements).

3.2 | Competitive fitness of the L968\textsuperscript{mCherry} focal strain in pairwise propagations

We performed pairwise competitive fitness assays between the focal strain L968\textsuperscript{mCherry} strain and the other four chosen strains, which had similar growth rates, in YES medium for 48h at 30ºC. From the pairwise competitions, a relative fitness of the L968\textsuperscript{mCherry} strain against each strain was calculated (Figure 2a). Considering that the strains had similar absolute fitness, as measured by their maximum growth rates, it is remarkable that L968\textsuperscript{mCherry} showed stark fitness differences when competing against each of the other strains. Competition with L968, its closest relative, shows that the mCherry and hygromycin resistance constitutive expression has a significant cost ($s = -0.05 \pm 0.03$ 2SEM). The relative fitness of L968\textsuperscript{mCherry} is much smaller when competing with...
UFMG-SPW23 or UFMG-R435 ($s = -0.14 \pm 0.03$ and $-0.18 \pm 0.05$, respectively; Figure 2a). On the other hand, L968mCherry outcompetes the most divergent strain NCYC132 ($s = 0.18 \pm 0.05$ per generation). This wide variance of competitive fitness of the L968mCherry strain leads to diverse predictions about the long-term outcome of this strain in cultures given the different initial community compositions. While solely considering the measured growth rates, prolonged coexistence between pairs of strains is expected, the measured relative fitness predicts extinction of the L968mCherry strain when competing with strains L968, UFMG-SPW23, or UFMG-R435, but fixation of this strain when competing with NCYC132 during a long-term pairwise propagation, if no mutations occur in either background during the propagation process, that is, if no evolutionary rescue occurs. Plus, the timescales of extinction should also differ according to the competitor.

To test these predictions on the fate of the L968mCherry strain over long periods of time, we have propagated for $\approx 370^{th}$ generations four pairwise communities (L968mCherry/L968; L968mCherry/SPW23; L968mCherry/R435; L968mCherry/NCYC132; Figure 2b). We used flow cytometry to follow the mCherry frequency corresponding to the L968mCherry strain over time in pairwise propagations (Figure 2c–f). And the pair where L968mCherry has a higher fitness, the focal strain swept to fixation (> 99% for more than 2 sequential time-points; Figure 2d). Yet, the time to extinction or to fixation was longer than predicted by the short-term pairwise competitions (full lines in Figure 2c–f). This difference suggests that
some compensatory mutations reducing the fitness difference between L968mCherry and the other strains may have occurred during the first 100 generations. Accumulation of beneficial mutations is known to occur rapidly in populations of Saccharomyces cerevisiae (Johnson et al., 2021; Lang et al., 2013) and would result in a delay of the extinction of L968mCherry. The extinction was further confirmed by plating the communities after 369 generations in YES plates supplemented with hygromycin B that selects the L968mCherry strain: In only three out of six replicates of the L968/L968mCherry, we were able to isolate a few L968mCherry clones (Table 1).

3.3 | Competitive fitness of the L968mCherry focal strain in communities with higher diversity

To understand how the dynamics of our focal strain is affected by the strain diversity in the community, we propagated communities with 3, 4, and 5 members for ≈370th generations (Figure 2b). Firstly, we used a multigenotype population genetic model to calculate the expected outcome of long-term competitions where the focal strain starts at ≈50% frequency assuming that no mutations occur in any of the strains of the community (Figure 3, full lines). Given its marginal fitness, the focal strain is predicted to go extinct even when the community has the strain NCYC132, which is less fit than the focal strain. Consistent with this expectation, we observe a quick decrease in the mCherry fluorescent cells in all propagations with more than two strains in less than 200 generations (Figure 3). Again, this indicates that competitive fitness is starkly more accurate than maximum growth rate in predicting the long-term outcome of the more complex communities. Extinction was again confirmed by plating in plates supplemented with hygromycin and occurred in 52 out 55 propagations (≈95% of the propagations; Table 1). Interestingly, as observed for the communities with just two strains, in the more diverse communities the time to extinction is longer than theoretically predicted. The fact that the relative pairwise fitness of the strains could also qualitatively predict the observed outcome of the communities with 3 or more members (Figure 3) suggests both an absence of higher order interactions between strains and that very few successful beneficial mutations capable of changing the relative fitness of the strains emerged in the propagations over this period. The few cases where we were able to isolate the focal strain by the end of the propagations (5% of the cases) suggest a few events of evolutionary rescue (see Table 1 and Figure 3, panels b, f, and k, where resistance to extinction occurs in some replicates).

3.4 | Adaptation during the propagation of the Schizosaccharomyces pombe communities

To assess evolution during the propagation, we searched for signs of adaptation in the communities by measuring the competitive fitness of the ancestral and evolved populations against the ancestral L968mCherry strain (see Methods for details; Figure 4A). For the six replicas of the propagation L968mCherry/NCYC132, the only cases when the L968mCherry went to fixation, we competed the evolved populations against the ancestral L968 strain instead. From all the communities, only one of the initial pairwise populations (NCYC132/L968mCherry) showed clear signs of increased competitive fitness over the period of evolution studied (17 ± 10% SD increase, p = .0005; Wilcoxon signed-rank test) suggesting strong adaptation to the abiotic environment. While the communities with the starting

| Community | Replica | # isolated colonies |
|-----------|---------|---------------------|
| L968/L968mCherry | A, C, F | 0 |
| | B | 393 |
| | D | 1572 |
| | E | 9 |
| SPW23/L968mCherry | A, B, C, D, E, F | 0 |
| R435/L968mCherry | A, B, C, D, E, F | 0 |
| NCYC132/L968mCherry | A | 342 |
| | B | 332 |
| | C | 901 |
| | D | 500 |
| | E | 286 |
| | F | 1128 |
| L968/NCYC132/L968mCherry | A | 52 |
| | B, C | 0 |
| | D | 8 |
| L968/SPW23/L968mCherry | A, B, C, D | 0 |
| L968/R435/L968mCherry | A, B, C, D | 0 |
| SPW23/R435/L968mCherry | A, C, D | 0 |
| | B | 11 |
| SPW23/NCYC132/L968mCherry | A, B, C, D | 0 |
| R435/NCYC132/L968mCherry | A, B, C, D | 0 |
| L968/SPW23/R435/L968mCherry | A, B, C, D | 0 |
| SPW23/R435/NCYC132/L968mCherry | A, B, C, D | 0 |
| L968/SPW23/NCYC132/L968mCherry | A, B, C, D | 0 |
| L968/R435/NCYC132/L968mCherry | A, B, C, D | 0 |
| All 5 strains | A, B, C, D, E, F, G, H, I, J, K, L, M, N, O | 0 |

Note: Frozen stocks were grown overnight in rich medium before 100 µl was plated in the hygromycin plates. Exception made for the NCYC132/L968mCherry competition (marked by ***) where the mCherry strain fixed and a 10−2 dilution were plated in plates with ampicillin which did not have hygromycin. All isolated colonies had mCherry fluorescence.
compositions L968/L968>mCherry and SPW23/L968>mCherry had no significant signs of adaptation, the R435/L968>mCherry community displayed an unexpected 11 ± 10% decrease in fitness by the end of the experiment (p = .0034, Wilcoxon signed-rank test). Additionally, 9 out of the 11 (82%) communities with 3 or more initial members also showed a significant decrease in fitness by the end of the propagation (Figure 4A). We have then grouped the communities by their initial number of strains, independently of their genotype. For communities with two initial members, the average fitness change was 2 ± 13% SD. However, for communities with three or more initial members, we observe a consistent and significant 11–14% average decrease in the competitive fitness at the end of the propagation (p < .0001 for the communities with 3 and 5 initial members and p = .0015 for communities with 4 initial members, Wilcoxon signed-rank test; Figure 4B), suggesting maladaptation to the medium.

Additionally, we tested for evolution of the focal strain by measuring growth rate. We isolated clones of evolved L968>mCherry from propagations L968>mCherry/NNCYC132, where the fluorescent yeast swept to fixation, and compared them with the growth rate of the ancestral strain (Figure 4C). We found a significant increase in growth rate of the focal strain in replica D (≈19 ± 6% SD increase, p = .0092, Dunnett’s multiple comparisons test), in agreement with the increase in competitive fitness (Figure 4A). Similarly, when testing for evolutionary rescue of the evolved focal strain isolated by plating in with hygromycin from propagations where the nonfluorescent yeast took over by the end of the experimental evolution, we did not find any significant increase in growth rate of remaining focal strain (Figure 4C). Overall, both results from the competitive fitness and growth rates suggest that in most propagations the focal strain did not accumulate strong beneficial mutations. Moreover, in the few propagations where strong beneficial mutations did occur, the populations of the focal strain swept to fixation, and thus were at higher absolute numbers (≈10^7 cells per well). This leads to the prediction that the isolated mCherry clones from propagations where the nonfluorescent strain(s) swept to fixation would eventually disappear if the propagation would be maintained for a longer period of time.

Afterward, we tested for the presence of beneficial waste products (cross-feeding metabolites) or further direct antagonist interactions among the five yeast strains by measuring the growth rate of any strain was not significantly affected by the presence of spent media (Figure 4D), we concluded that an interference competition effect (Hibbing et al., 2010) as, for instance, production of toxins, is unlikely. This result partially explains the high predictability of the L968>mCherry strain frequency in communities over time.
DISCUSSION

Ecological and evolutionary dynamics of adaptation in the context of diverse communities is seldom studied to evaluate how a focal strain would evolve in different ecological contexts. We propagated yeast populations with different degrees of initial genomic and phenotypic diversity, but similar growth rates in YES medium, during hundreds of generations. Our results show that community composition influences the fate of a focal strain. Its survival or extinction could be qualitatively predicted from the outcomes of pairwise competitions between strains when measured in the same environment. This is in agreement with the proposed qualitative assembly rule predicting the community structure from the outcomes of pairwise competitions between bacterial species (Friedman et al., 2017).
Moreover, our results are in line with previous studies showing that maximum growth rate can be a poor estimator of competitive fitness (Concepción-Acevedo et al., 2015; Durão et al., 2015; Lax et al., 2020), and thus unlikely to predict the outcome of community composition in the long-run. Frequent loss of strain coexistence has also been previously observed in rich media, presumably because a high nutrient concentration leads to more and stronger negative interactions between microbes (Ratzke et al., 2020). Overall, previous results obtained for interspecific competition between different bacterial species still hold true to intraspecific competition between different strains of the same yeast species, despite the higher degree of competition expected in the latter case. Since all of the strains used in this work are homothallic (Table S1), it is theoretically possible that they could be switching and mating (Avelar et al., 2013) during the propagations. We expect both mating types to be present in all of the experiments, as switching happens during mitosis and it only takes a few generations to reach an equilibrium where half of cells are of each mating type. However, to initiate mating S. pombe requires nitrogen starvation (Egel, 1971), which makes it unlikely to occur in the rich medium used to perform the propagations. YES medium contains abundant nitrogen both in the yeast extract and in the added amino acids. Even though we cannot completely exclude that mating was initiated by a small fraction of yeast cells during the experiment that would also be highly deleterious for the strain as it would take longer for cells to resume growth after stationary phase.

Interestingly, our results show that the level of previous adaptation to the environment is a poor predictor of the outcome of the pairwise competitions since two out of three natural strains could outcompete the laboratory strain L968mCherry which is routinely grown in the YES medium (Figure 2). Alternatively, a slightly better predictor of differences in competitive fitness was the genomic distances between the strains (Figures 1C and 2a). For instance, the L968 strain was genetically closest to the L968mCherry reference strain and also displayed the most similar competitive fitness ($S_{tg} = -0.05 \pm 0.03$), while theNCYC132 strain was the most divergent strain when compared to the reference strain and showed a major difference of fitness both in the absence (0.18 ± 0.05) or presence of 10% ethanol (Figure 1B-D).

Our results show that in communities where the focal strain went extinct, the times to extinction are longer than predicted by a simple model of constant selection. These could be expected if some de novo mutations occur to compensate for deleterious effects of the mutations it carries. However, in only a few cases a sign of adaptation could be detected, suggesting other causes for the observed delay. Indeed, even though evolutionary changes can occur on timescales relevant for ecological dynamics (Barber et al., 2021; Lankau, 2011), evolutionary rescue by a strong adaptive mutation was an extremely rare event in the focal strain. Another possible explanation for the observed delay is abrupt population size changes, as the bottlenecks involved a 1:500 dilution not included in the model to predict the focal strain dynamics using the relative fitness computed from a 48-h competition assay. The decrease in population size during the bottlenecks could lead to significant periods of time where the yeast strains could coexist in an environment with abundant glucose making selection weaker. More relaxed bottlenecks or propagations in chemostats where the community population sizes can be constant could lead to different outcomes.

The reduction of competitive fitness of the remaining strains against the L968mCherry strain observed in most of the evolved communities with three or more initial members suggests an incidental maladaptation of the strains of these yeast communities to the abiotic environment, that is, to the growth media. These results have been observed recently in bacteria (Castledine et al., 2020; Lawrence et al., 2012; Scheuerl et al., 2020) and suggest that biotic adaptation, where the individual strains of the communities are adapting to the (transient) presence of each other, may be constraining adaptation to the abiotic environment as a result of of trade-offs (Briscoe Runquist et al., 2020; Lawrence et al., 2012). For instance, it has been observed that bacterial species diverge in their use of resources when in communities and frequently evolve to use waste products generated by other species, leading to a trade-off between adaptation to either the carbon resources initially present in the medium or produced by the other species (Lawrence et al., 2012). However, in our case we have not detected any harmful or beneficial significant effect on the growth rates of the single strains when mixing waste product medium with the fresh medium (Figure 4D).

Another factor which may explain a slower adaptation to the environment is a high nutrient concentration leading to more and stronger negative interactions between microbes (Egel, 1971; Castledine et al., 2020; Scheuerl et al., 2020) and suggest that biotic adaptation, where the individual strains of the communities are adapting to the (transient) presence of each other, may be constraining adaptation to the abiotic environment as a result of of trade-offs (Briscoe Runquist et al., 2020; Lawrence et al., 2012). For instance, it has been observed that bacterial species diverge in their use of resources when in communities and frequently evolve to use waste products generated by other species, leading to a trade-off between adaptation to either the carbon resources initially present in the medium or produced by the other species (Lawrence et al., 2012). However, in our case we have not detected any harmful or beneficial significant effect on the growth rates of the single strains when mixing waste product medium with the fresh medium (Figure 4D).

Additionally, if a beneficial mutation occurs in a less competitive genetic background (i.e., the L968mCherry background in the three or more-member communities), then their decreasing effective population size during the propagation reduces the chances of ever been selected. Indeed, the more strains there are in a community, the smaller the population size of each strain and the less likely to get selection of beneficial mutations and more likely to get accumulation of deleterious mutations.

Overall, our results aid establishing pairwise competitive fitness as good qualitative measurement of long-term community composition but they also reveal a complex adaptive scenario when trying to predict the evolutionary outcome of those communities.

**ACKNOWLEDGMENTS**

The authors acknowledge Anke Konrad for aiding in the genetic distances’ calculation, Ricardo S. Ramiro for the support with the genotype by environment analysis, and Beatriz Abreu by helping...
with the long-term propagation. We are grateful for the technical support of IGC’s Flow Cytometry facility and, in particular, to Denise Brito for technical assistance. This work and PD salary were supported by Portuguese Science Foundation (FCT) Grant PTDC/BIA-EVL/31528/2017.

CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTION
Paulo Durão: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (lead); Methodology (lead); Validation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). Massimo Amicone: Data curation (equal); Formal analysis (equal); Writing-review & editing (equal). Lília Perfeito: Funding acquisition (equal); Methodology (supporting); Writing-review & editing (equal). Isabel Gordo: Conceptualization (equal); Funding acquisition (equal); Project administration (lead); Supervision (lead); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT
Whole-genome sequence data for all strains are available in the Sequence Read Archive (SRA) database (BioProject accession no. PRJNA744270). Data available at https://doi.org/10.5061/dryad.280gb5mq

ORCID
Paulo Durão https://orcid.org/0000-0002-4411-2958

REFERENCES
Amicone, M., & Gordo, I. (2021). Molecular signatures of resource competition: Clonal interference favors ecological diversification and can lead to incipient speciation. Evolution, https://doi.org/10.1111/evo.14315
Attfield, P. V. (1997). Stress tolerance: The key to effective strains of industrial baker’s yeast. Nature Biotechnology, 15, 1351-1357. https://doi.org/10.1038/nbt1297-1351
Avelar, A. T. (2012). Chromosomal structure: A selectable trait for evolution. PhD thesis
Avelar, A. T., Perfeito, L., Gordo, I., & Ferreira, M. G. (2013). Genome architecture is a selectable trait that can be maintained by antagonistic pleiotropy. Nature Communications, 4, 2235. https://doi.org/10.1038/ncomms3235
Bähler, J., Wu, J.-Q., Longtine, M. S., Shah, N. G., lii, A. M., Steever, A. B., Wach, A., Filippsen, P., & Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in <i>Schizosaccharomyces pombe</i>. Yeast, 14, 943–951. https://doi.org/10.1002/(SICI)1097-0041(19980714):10<943::AID-YEAA2923.0.CO;2-Y
Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology, 19, 455–477. https://doi.org/10.1089/cmb.2012.0021
Barber, J. N., Sezms, A. L., Woods, L. C., Anderson, T. D., Voss, J. M., & McDonald, M. J. (2021). The evolution of coexistence from competition in experimental co-cultures of <i>Escherichia coli</i> and <i>Saccharomyces cerevisiae</i>. ISME Journal, 15, 746–761. https://doi.org/10.1038/s41396-020-00810-z
Benito, S. (2019). The impacts of <i>Schizosaccharomyces</i> on winemaking. <i>Applied Microbiology and Biotechnology</i>, 103, 4291–4312. https://doi.org/10.1007/s00253-019-09827-7
Briscoe Runquist, R. D., Gorton, A. J., Yoder, J. B., Deacon, N. J., Grossman, J. J., Kothari, S., Lyons, M. P., Sheth, S. N., Tiffen, P., & Moeller, D. A. (2020). Context Dependence of Local Adaptation to Abiotic and Biotic Environments: A Quantitative and Qualitative Synthesis. The American Naturalist, 195, 412–431. https://doi.org/10.1086/707322
Buckling, A., Wills, M. A., & Colegrave, N. (2003). Adaptation limits diversification of experimental bacterial populations. Science, 302, 2107–2109. https://doi.org/10.1126/science.1088848
Calcagno, V., Jarne, P., Loreau, M., Mouquet, N., & David, P. (2017). Diversity spurs diversification in ecological communities. <i>Nature Communications</i>, 8, 15810. https://doi.org/10.1038/ncomms15810
Castledine, M., Padfield, D., & Buckling, A. (2020). Experimental (co)evolution in a multi-species microbial community results in local maladaptation. <i>Ecology Letters</i>, 23, 1673–1681. https://doi.org/10.1111/ele.13599
Celiker, H., & Gore, J. (2014). Clustering in community structure across replicate ecosystems following a long-term bacterial evolution experiment. <i>Nature Communications</i>, 5, 4643. https://doi.org/10.1038/ncomms5643
Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastq: An ultra-fast all-in-one FASTQ preprocessor. <i>Bioinformatics</i>, 34, 1884–1890. https://doi.org/10.1093/bioinformatics/bty560
Concepción-Aucedo, J., Weiss, H. N., Chaudhry, W. N., & Levin, B. R. (2015). Malthusian parameters as estimators of the fitness of microbes: A cautionary tale about the low side of high throughput. <i>PloS One</i>, 10, e0126915. https://doi.org/10.1371/journal.pone.0126915
Dieckmann, U., & Doebeli, M. (1999). On the origin of species by sympatric speciation. <i>Nature</i>, 400, 354–357. https://doi.org/10.1038/22521
Durão, P., Ramiro, R. S., Pereira, C., Jurič, J., Pereira, D., & Gordo, I. (2020). Radial expansion facilitates the maintenance of double antibiotic resistances. <i>Antimicrobial Agents and Chemotherapy</i>, 64(9), e00668-20. https://doi.org/10.1128/AAC.00668-20
Durão, P., Trindade, S., Sousa, A., & Gordo, I. (2015). Multiple resistance at no cost: Rifampicin and streptomycin a dangerous liaison in the spread of antibiotic resistance. <i> Molecular Biology and Evolution</i>, 32, 2675–2680. https://doi.org/10.1093/molbev/msv143
Egel, R. (1971). Physiological aspects of conjugation in fission yeast. <i>Planta</i>, 98, 89–96. https://doi.org/10.1007/BF00387025
Emerson, B. C., & Kolm, N. (2005). Species diversity can drive speciation. <i>Nature</i>, 434, 1015–1017. https://doi.org/10.1038/nature03450
Estrela, S., & Brown, S. P. (2018). Community interactions and spatial structure shape selection on antibiotic resistant lineages. <i>PLOS Computational Biology</i>, 14, e1006179. https://doi.org/10.1371/journal.pcbi.1006179
Felsenstein, J. (1974). The evolutionary advantage of recombination. <i>Genetics</i>, 78, 737-756. https://doi.org/10.10393/genes78.2.737
Felsenstein, J. (1993). PHYLIp (phylogenetic inference package) version 3.6 (pp. 188–192). Department of Genetics, University of Washington.
Friedman, J., Higgins, L. M., & Gore, J. (2017). Community structure follows simple assembly rules in microbial microcosms. <i>Nature Ecology & Evolution</i>, 1, 109. https://doi.org/10.1038/s41559-017-0109
Garrison, E. E., & Marth, G. (2012). Haplootype-based variant detection from short-read sequencing. arXiv:1207.3907 [q-bio].
Geritz, S., Kisdi, É., Meszéna, G., & Metz, J. (1998). Evolutionarily singular strategies and the adaptive growth and branching of the evolutionary tree. <i>Evolutionary Ecology</i>, 12, 35–57. https://doi.org/10.1023/A:1006554906681
Gomes, F. C. O., Pataro, C., Guerra, J. B., Neves, M. J., Corrêa, S. R., Moreira, E. S. A., & Rosa, C. A. (2002). Physiological diversity and trehalose accumulation in Schizosaccharomyces pombe strains isolated from spontaneous fermentations during the production of the artisanal Brazilian cachaça. Canadian Journal of Microbiology, 48, 399–406. https://doi.org/10.1139/w02-032

Good, B. H., McDonald, M. J., Barrick, J. E., Lenski, R. E., & Desai, M. M. (2010). Novel cooperation experimentally evolved between species. Evolution Letters, 2, 580–589. https://doi.org/10.1002/evl2.138

Harcombe, W. (2010). The ecology and evolution of microbial communities. Nature, 551, 45–50. https://doi.org/10.1038/nature24287

Hall, J. P. J., Harrison, E., & Brockhurst, M. A. (2018). Competitive species interactions constrain abiotic adaptation in a bacterial soil community. Evolution Letters, 2, 580–589. https://doi.org/10.1002/evl2.138

Harcombe, W. (2010). Novel cooperation experimentally evolved between species. Evolution, 64, 2166–2172. https://doi.org/10.1111/j.1558-5546.2010.00959.x

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: Surviving and thriving in the microbial jungle. Nature Reviews Microbiology, 8, 15–25. https://doi.org/10.1038/nrmicro2259

Hill, W. G., & Robertson, A. (1966). The effect of linkage on limits to artificial selection. Genetics Research, 8, 269–294. https://doi.org/10.1017/S0016672300010156

Jeffares, D. C., Ralls, C., Rieux, A., Speed, D., Převorovský, M., Mourier, T., Marsellach, F. X., Iqbal, Z., Lau, W., Cheng, T. M. K., Pracana, C. W., Humphrey, P. T., Jagdish, T., Jerison, E. R., Kosheleva, K., Lawrence, K. R., Min, J., Moulana, A., Phillips, A. M., Purkanti, R., Rego-Costa, A., McDonald, M. J., Nguyen Ba, A. N., & Desai, M. M. (2021). Phenotypic and molecular evolution across 10,000 generations in laboratory budding yeast populations, eLife, 10, e63910. https://doi.org/10.7554/eLife.63910

Jousset, A., Eisenhauer, N., Merker, M., Mouquet, N., & Scheu, S. (2016). High functional diversity stimulates diversification in experimental microbial communities. Science Advances, 2, e1600124. https://doi.org/10.1126/sciadv.1600124

Keeney, J. B., & Boeke, J. D. (1994). Efficient targeted integration at leu1-32 and ura4-294 in Schizosaccharomyces pombe. Genetics, 136, 849–856. https://doi.org/10.1093/genetics/136.3.849

Konstantinidis, K. T., & Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. Proceedings of the National Academy of Sciences of the United States of America, 102, 2567–2572. https://doi.org/10.1073/pnas.0409727102

Lanfear, R., Kokko, H., & Eyre-Walker, A. (2014). Population size and the rate of evolution. Trends in Ecology & Evolution, 29, 33–41. https://doi.org/10.1016/j.tree.2013.09.009

Lang, G. I., Rice, D. P., Hickman, J. M., Sodergren, E., Weinstock, G. M., Botstein, D., & Desai, M. M. (2013). Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. Nature, 500, 571–574. https://doi.org/10.1038/nature12344

Lankau, R. A. (2011). Rapid Evolutionary Change and the Coexistence of Species. Annual Review of Ecology, Evolution, and Systematics, 42, 335–354. https://doi.org/10.1146/annurev-ecolsys-102710-145100

Lawrence, D., Fiegna, F., Behrends, V., Bundy, J. G., Phillimore, A. B., Bell, T., & Barracough, T. G. (2012). Species interactions alter evolutionary responses to a novel environment. PLoS Biology, 10, e1001330. https://doi.org/10.1371/journal.pbio.1001330

Lax, S., Abreu, C. I., & Gore, J. (2020). Higher temperatures generally favour slower-growing bacterial species in multispecies communities. Nature Ecology & Evolution, 4, 560–567. https://doi.org/10.1038/s41559-020-1126-5

Li, H., & Durbin, R. (2010). Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics, 26, 589–595. https://doi.org/10.1093/bioinformatics/btp698

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R., 1000 Genome Project Data Processing Subgroup (2009). The sequence alignment/map format and SAMtools. Bioinformatics, 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Lock, A., Rutherford, K., Harris, M. A., & Wood, V. (2018). PomBase: The scientific resource for fission yeast. Methods in Molecular Biology, 1757, 49–68. https://doi.org/10.1007/978-1-4939-7737-6_4

Osmond, M. M., & de Mazancourt, C. (2013). How competition affects evolutionary rescue. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 368, 20120085. https://doi.org/10.1098/rstb.2012.0085

Pande, S., & Kost, C. (2017). Bacterial unculturability and the formation of intercellular metabolic networks. Trends in Microbiology, 25, 349–361. https://doi.org/10.1016/j.tim.2017.02.015

Pande, S., Merker, H., Bohl, K., Reichelt, M., Schuster, S., de Figueiredo, L. F., Kaleta, C., & Kost, C. (2014). Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. The ISME Journal, 8, 953–962. https://doi.org/10.1038/ismej.2013.211

Pataro, C., Guerra, J. B., Petrilho-Peixoto, M. L., Mendonça-Hagler, L. C., Linardi, V. R., & Rosa, C. A. (2000). Yeast communities and genetic polymorphism of Schizosaccharomyces cerevisiae strains associated with artisanal fermentation in Brazil. Journal of Applied Microbiology, 89, 24–31. https://doi.org/10.1046/j.1365-2672.2000.01092.x

Petersen, J., & Russell, P. (2016). Growth and the Environment of Schizosaccharomyces pombe. Cold Spring Harbor Protocols, 2016(3), pdb.top079764. https://doi.org/10.1101/pdb.top079764

Ramiro, R. S., Durão, P., Bank, C., & Gordo, I. (2020). Low mutation load and high mutation rate variation in gut commensal bacteria. PLoS Biology, 18, e3000617. https://doi.org/10.1371/journal.pbio.3000617

Ratze, C., Barrere, J., & Gore, J. (2020). Strength of species interactions determines biodiversity and stability in microbial communities. Nature Ecology & Evolution, 4, 376–383. https://doi.org/10.1038/s41559-020-1099-4

Rodriguez-Verdugo, A., & Ackermann, M. (2020). Rapid evolution destabilizes species interactions in a fluctuating environment. The ISME Journal, 15(2), 450–460. https://doi.org/10.1038/s41396-020-00787-9

Scheuerl, T., Hopkins, M., Nowell, R. W., Rivett, D. W., Barracough, T. G., & Bell, T. (2020). Bacterial adaptation is constrained in complex communities. Nature Communications, 11, 754. https://doi.org/10.1038/s41467-020-14570-z

Shoresh, N., Hegreness, M., & Kishony, R. (2008). Evolution exacerbates the paradox of the plankton. PNAS, 105, 12365–12369. https://doi.org/10.1073/pnas.080302105

Turcotte, M. M., Corrin, M. S. C., & Johnson, M. T. J. (2012). Adaptive evolution in ecological communities. PLoS Biology, 10, e1001332. https://doi.org/10.1371/journal.pbio.1001332

van Rossum, T., Ferretti, P., Maistrenko, O. M., & Bork, P. (2020). Diversity within species: Interpreting strains in microbiomes. Nature Reviews Microbiology, 18, 491–506. https://doi.org/10.1038/s41579-020-0368-1

Vidal, M. C., Wang, S. P., Rivers, D. M., Althoff, D. M., & Segraves, K. A. (2020). Species richness and redundancy promote persistence of
exploited mutualisms in yeast. Science, 370, 346–350. https://doi.org/10.1126/science.abb6703
Volschenk, H., van Vuuren, H. J. J., & Viljoen-Bloom, M. (2003). Malo-ethanolic fermentation in Saccharomyces and Schizosaccharomyces. Current Genetics, 43, 379–391. https://doi.org/10.1007/s00294-003-0411-6

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.