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TEMPO AND MODE OF MULTICELLULAR ADAPTATION IN EXPERIMENTALLY EVOLVED *SACCHAROMYCES CEREVISIAE**

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Multicellular complexity is a central topic in biology, but the evolutionary processes underlying its origin are difficult to study and remain poorly understood. Here we use experimental evolution to investigate the tempo and mode of multicellular adaptation during a de novo evolutionary transition to multicellularity. Multicelled "snowflake" yeast evolved from a unicellular ancestor after 7 days of selection for faster settling through liquid media. Over the next 220 days, snowflake yeast evolved to settle 44% more quickly. Throughout the experiment the clusters evolved faster settling by three distinct modes. The number of cells per cluster increased from a mean of 42 cells after 7 days of selection to 114 cells after 227 days. Between days 28 and 65, larger clusters evolved via a twofold increase in the mass of individual cells. By day 227, snowflake yeast evolved to form more hydrodynamic clusters that settle more quickly for their size than ancestral strains. The timing and nature of adaptation in our experiment suggests that costs associated with large cluster size favor novel multicellular adaptations, increasing organismal complexity.

KEY WORDS: **Complexity, development, evo devo, evolutionary dynamics, major transition, multilevel selection.**

Eukaryotic multicellularity arose independently at least 25 times, in some cases leading to the evolution of large, complex organisms such as plants, animals, and multicellular fungi (Grosberg and Strathmann 2007). The first step in the transition to multicellularity was likely the evolution of clustering among conspecific single-celled organisms (Bonner 1998; Fairclough et al. 2010). Cluster formation can provide a direct fitness advantage, such as protection from predation (Boraas et al. 1998) and environmental toxin exposure (Smukalla et al. 2008), or increased efficiency of cooperative feeding (Koschwanez et al. 2011). Key to the evolution of multicellularity from simple clusters of cells is a shift to multicellular-level selection (Buss 1987; Maynard Smith and Szathmáry 1995; Michod 1997; Frank 1998). Multicellular-level adaptation, which can result in the evolution of multicellular traits such as cellular division of labor, may occur when the group-level phenotype affects fitness and is heritable (Lewontin 1970; Griesemer 2001; Okasha 2006), and is promoted by high within-group relatedness (Grosberg and Strathmann 1998; Velicer et al. 2000; Michod and Herron 2006; Diggle et al. 2007; Gilbert et al. 2007).

Despite recent advances, a fundamental question about the transition to multicellularity remains unresolved: How do incipient multicellular organisms increase in complexity, evolving from simple clusters of cells to functionally integrated individuals? Mathematical models (Michod 1997; Bull 1999; Michod and Nedelcu 2003; Pfeiffer and Bonhoeffer 2003; Willensdorfer 2009; Ispolatov et al. 2012) and phylogenetically rooted developmental work (Buss 1987; Kirk 2005; Herron and Michod 2008) have illuminated key steps in this transition, but little empirical

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work has examined multicellular-level evolution in simple undifferentiated clusters of cells (but see Boraas et al. 1998; Ratcliff et al. 2012). Experimental progress has been limited by a lack of suitable model systems. Growth in extant multicellular taxa is developmentally regulated, constraining the routes available for multicellular-level adaptation (Smith et al. 1985). Multicellular development, however, is itself an adaptation that evolved as a consequence of the transition to multicellular-level evolution. How, then, does multicellular adaptation occur after the transition to multicellular-level evolution, but prior to the evolution of development?

We address this question using experimental evolution. *Saccharomyces cerevisiae* rapidly makes the transition to multicellular-level evolution under appropriate conditions (Ratcliff et al. 2012). We selected for rapid settling in initially unicellular *S. cerevisiae*, transferring only individuals that settle to the bottom of a test tube after brief centrifugation. Multicelled "snowflake" yeast evolved and displaced unicellular yeast in all 10 replicate populations within 60 days. Multicelled snowflake clusters become a unit of selection, either settling to the bottom of the tube during settling selection and surviving, or failing to do so and being discarded. The survival of a cluster is dependent on its settling speed, which is higher for larger clusters, so that cluster size is a group-level trait that affects fitness. Under divergent selection, large and small cluster-forming strains evolved in response to strong and weak selection for settling speed, respectively, demonstrating that cluster size is heritable and capable of responding to selection. Snowflake yeast clusters grow exclusively through postdivision adhesion of component cells, ensuring that clusters are uniclonal and minimizing the consequences of within-group selection. These results demonstrate that, once snowflake yeast evolve, selection can operate on differences among clusters.

In this article, we examine the tempo and mode of multicellular-level adaptation in snowflake yeast under strong selection for rapid settling through liquid media. Over 227 days of evolution, we measure the rate at which snowflake yeast evolve to settle more rapidly (tempo), and examine the traits responsible for this adaptation (mode). We passaged replicate populations of snowflake yeast for 227 serial transfers. To maintain strong selection for increased settling speed, we periodically increased the strength of settling selection by reducing the time allowed for settling before the bottom fraction of the tube was transferred to fresh media. We measured the settling speed of isolates from 7, 14, 28, 65, and 227 days of evolution using a custom-built microscope, and found a dramatic increase in settling rate. Next, we studied the traits that underlie this adaptation by examining three potential routes to faster settling. In principle, snowflake yeast will settle more rapidly when the force exerted on a cluster from gravity increases relative to fluid resistance. This could occur in several ways, including: if snowflake yeast clusters increase in size, if the cells within a cluster increase their buoyant density, or if the shape of snowflake yeast evolves to be more hydrodynamic.

Materials and Methods

CULTURE CONDITIONS

A single clone of initially unicellular diploid *S. cerevisiae*, strain Y55, was grown as previously described in Ratcliff et al. (2012). Briefly, yeast were cultured in 10 mL YPD media (per L: 10 g yeast extract, 20 g peptone, 20 g dextrose, pH 5.8) at 30°C for 24 h, with 250 rpm shaking. Once per day each population was put through settling selection. Because all replicate populations were composed of initially isogenic unicellular yeast, all changes resulted from de novo mutation, not selection acting on standing genetic variation.

SELECTION REGIME

To select for larger size, we increased the strength of settling selection several times during the experiment (illustrated graphically in Fig. S1). The initial experiment was composed of 10 replicate populations in which the entire population was allowed to settle at 1 g in a 25 × 150 mm culture tube. After 45 min, the bottom 100 μL was transferred to fresh media. On day 7, we changed the transfer regime to make this selection step more time efficient: 1.5 mL of each experimental population was centrifuged at 100 × *g* for 10 s in a 2 mL microcentrifuge tube, then the bottom 100 μL was transferred to fresh media. On day 30, we initiated a divergent selection experiment in which a single founder population was used to establish nine new populations: three settling at 1 g for either 5, 15, or 25 min (Ratcliff et al. 2012). By day 65, there was substantial divergence among these populations for settling speed, with the 5-min treatments settling 20% faster than either the 15- or 25-min treatments (Ratcliff et al. 2012). To continue selecting for increased settling rate, one of the 5-min populations was used to found three new replicate populations, which were given only 1.25 min of settling at 1 g before transfer of the lower 100 μL to fresh media. This selection regime was carried out for 162 days, for a total of 227 days of selection. A representative genotype of snowflake yeast was isolated from these populations after 7, 14, 28, 65, and 227 days of evolution, comprising a single evolutionary lineage exposed to increasingly strong selection for rapid settling. Before analysis, these five isolates were purified by three rounds of single-colony isolation.

RELATIVE STRENGTH OF SETTLING SELECTION

To determine how changes in the selection protocol (e.g., 5 vs. 1.25 min of settling) affected the strength of selection on settling speed, we assessed the survival of snowflake yeast before

and after each change in settling regime (occurring on days 7, 30, and 65). Ten replicate populations of the 7-, 28-, and 65-day isolates were initiated (30 populations in total). Half were transferred with the last selection regime these isolates experienced in the experiment, whereas the other half was transferred with the new, more stringent selection regime. For example, for the day 28 isolate, five replicate populations were settled at 100 g for 10 s before transfer, whereas the other five replicate populations were settled at 1 g for 5 min. Before collecting samples for analysis, each population was passaged through one 24 h period of growth, then put through settling selection. After a further 24 h of growth, we sampled immediately before and after settling selection, calculating the percentage of clusters surviving settling selection via microscopy. To do this, 10 μL of cell culture (diluted if necessary) was loaded onto a hemacytometer, and nine predetermined fields of view were acquired at 40× magnification on an Olympus IX70 with a Scion CFW-1310C camera. Clusters/mL were determined by counting thresholded clusters in ImageJ.

SETTLING SPEED

The terminal settling speed of individual clusters was measured on a custom built microscope. Five replicate populations of each yeast isolate were grown for 24 h at 30°C with 250 rpm shaking. Each population was diluted 1 : 100 into deionized water and 3 mL placed in a vertical 10 × 10 × 45 mm polystyrene cuvette. The contents of the cuvette were mixed by inversion and then allowed to stand for 60 sec before imaging, so that clusters would reach their terminal velocity. Next, 300 images of the center of the cuvette, 1 cm above the base, were captured at 0.1 sec intervals. The microscope was calibrated before each use to ensure that the same location within the cuvette was imaged. Clusters were thresholded from the background using the *Renyi Entropy* function of the Multithresholder plugin in ImageJ. The average settling speed of individual clusters was determined using the wrMTrck image tracking plugin. Clusters were tracked in at least four separate 300-photo runs per replicate population (Movie S1). For day 7, 14, 28, 65, and 227 isolates, we measured the speed of 821, 1301, 1383, 513, and 938 clusters, respectively.

NUMBER OF CELLS PER CLUSTER

Stationary phase snowflake yeast were flattened into two dimensions by placing 5 μL of cell culture on a slide under a 25 × 25 mm coverslip. For 7-, 14-, 28-, 65-, and 227-day isolates, we manually counted the number of cells in 101, 55, 80, 33, and 45 randomly selected clusters, respectively. When flattening clusters for microscopic analysis, small branches and unicells occasionally break off from large clusters. To avoid counting these, we only photographed clusters with at least 7 cells.

BUOYANT DENSITY

Buoyant density was determined by density gradient centrifugation. Four replicate populations per isolate were grown overnight, then centrifuged at 15,000 × *g* for 30 min in a 70% Percoll gradient. Density was determined with density marker beads (Amersham BioSciences, Buckinghamshire, England). All else being equal, increases in buoyant density should increase settling rates.

CELL SIZE

We homogenized the age structure of the cells within each cluster by digesting cell walls with lyticase and β-glucuronidase / arylsulfatase, resulting in viable single cells (as described in Ratcliff et al. 2012). New clusters grew from these single cells after 6 hours of culture in YPD, at which time 5 μL of media was placed between a slide and a 25 × 25 mm coverslip and the flattened clusters imaged. Cells were generally shaped as prolate ellipsoids. Using the ImageJ command *FitEllipse*, we calculated the major axis (a) and minor axis (b) of the fitted ellipse. Assuming that both minor axes of the ellipsoid were the same (minor axes $b = c$, meaning the cell is round like a cylinder at its center), we estimated cell volume as: $V = 4/3\pi ab^2$.

To avoid measuring only partially grown cells, we excluded all cells without attached daughters from the analysis. For day 7, 14, 28, 65, and 227 isolates, we measured the volume of 142, 144, 142, 136, and 122 cells, respectively. Cell mass (in picograms) was determined by multiplying cell volume by the strain's average buoyant density.

CLUSTER SHAPE

We examined the shape of 361 day-65 and 267 day-227 snowflake yeast clusters by growing 5 replicate 10 mL tubes for 24 h, then imaging cells diluted 1 : 5 at 100× magnification in a hemocytometer. The hemocytometer chamber has a 100-μm depth, allowing us to image clusters in their native shape. To avoid measuring the shape of small branches or unicells, we considered only full-sized ($>1875\ \mu m^2$) clusters in the analysis. Images were manually curated to separate any adjacent clusters that were touching, so that they could be measured separately. Two-dimensional (2D) roundness of clusters (a proxy for 3D sphericity) was determined in ImageJ with the *Round* measurement function.

Results

PERIODIC INCREASES IN SELECTION INTENSITY

During the course of the experiment, we changed the settling selection regime three times, modifying both the duration of settling and intensity of gravitational acceleration (Fig. S1). We quantified the effect of these changes on the intensity of settling selection by measuring snowflake yeast survival in the new versus old selective conditions. The first change on day 7, from 45 min of

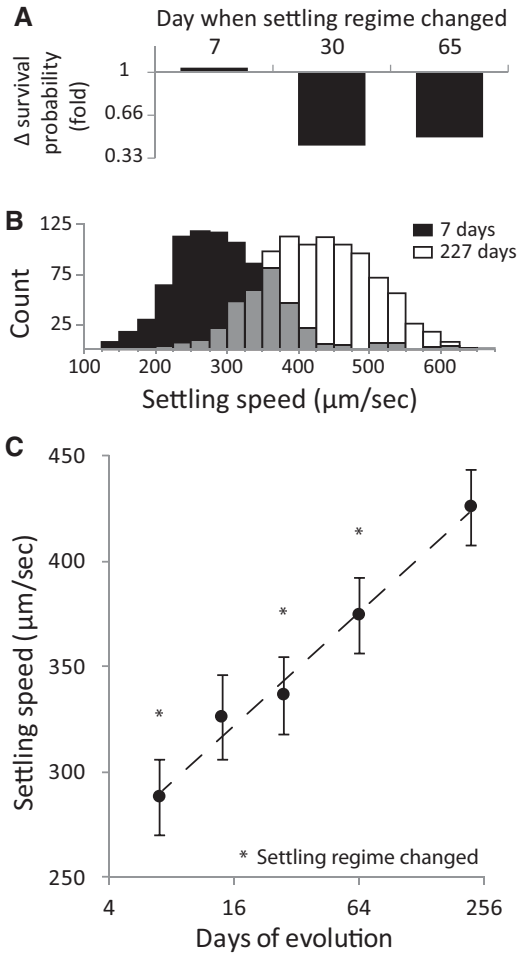

Figure 1. Strength of selection and settling speed over the course of the experiment. The settling selection regime was changed several times during the experiment (marked by asterisks in C) to maintain strong selection for fast settling. (A) The effect of the new settling selection regime, imposed on days 7, 30, and 65, on the probability of cluster survival for yeast isolated from days 7, 28, and 65. The new settling regimes imposed on days 30 and 65 were more stringent, each reducing cluster survival by about half. (B) Settling speed distributions for the first snowflake yeast to evolve (7 days), and those from the end of the experiment (227 days). (C) Although the settling speed of snowflake yeast increased over the course of the experiment, their rate of increase declined exponentially with time. Plotted are least squares means and associated standard errors for each strain from the nested restricted maximum likelihood-analysis of variance (REML-ANOVA) analysis (see text). The dashed line is a logistic regression to strain means, $y = 38.3 \ln(x) + 216.2$.

settling at 1 × *g* to 10 sec of settling at 100 × *g*, was done to make settling selection more time efficient. This had no effect on the survival of day-7 clusters ($P = 0.74$, significance assessed with preplanned contrast after analysis of variance [ANOVA]; Fig 1A). We anticipated that once snowflake yeast evolved to survive settling selection, further increases in settling rate would cease. To continue selecting for increased settling speed, we increased the strength of selection on day 28 and again on day 65. On day 28, yeast were allowed to settle for 5 min at 1 × *g* before transfer, reducing survival during the settling step by 2.3-fold ($P < 0.0001$ preplanned contrast; Fig. 1A). On day 65, settling selection was shortened to 1.25 min at 1 × *g*, reducing cluster survival by twofold ($P = 0.0039$ preplanned contrast; Fig 1A).

SETTLING SPEED

Snowflake yeast evolved to settle more rapidly during the course of the experiment, with the terminal velocity of a cluster increasing from 296 μm/sec after 7 days to 428 μm/sec by 227 days ($F_{4, 19.05} = 0.0005$, main effect of strain in a restricted maximum likelihood [REML]-ANOVA, with replicate population [random effect] nested in strain, and microscope run [random effect] nested in replicate population; $r^2 = 0.73$; Fig. 1B,C). Variance among replicate populations of each genotype accounted for 16% of the total variance, and variance among replicate 300-photo settling runs for each population accounted for 49% of the total variance. The large effect of the latter is likely because of differences in the carrier water's mass flow between replicate runs, brought into motion by the settling of the snowflake yeast. Settling speed versus time is best described by logistic regression (Fig. 1C), demonstrating that the rate at which snowflake yeast evolve to settle more quickly decreased exponentially with time.

Snowflake yeast can evolve to settle more rapidly by simply evolving larger cluster sizes. All else equal, increasing cluster size results in a larger increase in mass, and downward force from gravity, than it does the cluster's surface area, which impedes settling because of friction and hydrodynamic drag. For spherical objects, such as approximately spherical clusters of snowflake yeast, enlarging the radius *d*-fold increases the mass : surface area ratio by *d*-fold.

EVOLUTION OF LARGER CLUSTERS: INCREASED CELL COUNT

Snowflake yeast may evolve larger size, and hence faster settling, by forming clusters that contain more cells. We examined this in our five isolates of snowflake yeast by counting the number of cells per cluster using a microscope. Before analysis, we log transformed these data to normalize the size distributions. The number of cells per cluster increased dramatically during the course of the experiment (logistic regression, $P < 0.0001$, $r^2 = 0.15$, $y = 0.28 \cdot \mathrm{Ln}(x) + 3.17$; Fig. 2), from an initial mean of 42.6 cells per cluster in day-7 yeast to a mean of 114.5 cells per cluster in day-227 yeast (reported are back-transformed means from the Ln-transformed distributions).

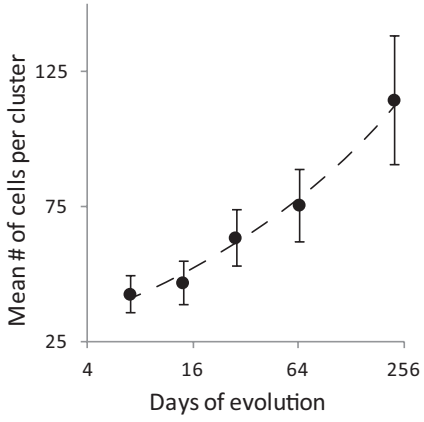

Figure 2. The number of cells per cluster increased in response to strong selection for faster settling. Shown are back-transformed means and 95% confidence intervals from log-transformed data. The dashed line is a power function fit to strain means, $y = 23.2x^{0.29}$.

EVOLUTION OF LARGER CLUSTERS: INCREASED CELL MASS

Alternatively, snowflake yeast may evolve larger size through an increase in the mass of each cell making up the cluster. To investigate this, we measured the average size and buoyant density of individual cells for each isolate. Between day 28 and 65, there was a 2.21-fold increase in the volume of individual cells ($F_{4,687} = 215.75$, $P < 0.0001$, differences assessed with Tukey–Kramer honestly significant difference (HSD) with $\alpha = 0.05$; Figs. S2A and 3B,C). However, this increase in cell volume was accompanied by a 2% decline in cell buoyant density ($F_{4,19} = 134.94$, $P < 0.0001$, differences assessed with Tukey–Kramer HSD with $\alpha = 0.05$; Fig. S2B). As a result, these larger cells contained 2.16-fold as much mass ($F_{4,687} = 205.35$, $P < 0.0001$, significance assessed with Tukey–Kramer HSD with $\alpha = 0.05$; Fig. 3A). A simple model confirms that larger cells, despite their slightly lower density, should increase settling speed substantially (Fig. S2). Cell mass declined between 65 and 227 days, but day-227 yeast cells still contained 54% more mass than the 7, 14, and 28 days snowflake yeast cells.

EVOLUTION OF MORE HYDRODYNAMIC CLUSTERS AND MORE EFFICIENT SETTLING

Increased cluster size is not the only possible evolutionary route to faster settling in snowflake yeast. Changes in the multicellular form of the snowflake yeast could, for example, result in the evolution of clusters with tighter cellular packing or a more hydrodynamic profile, allowing clusters of a given size to settle more rapidly. Assessing settling rate as a function of cluster size, we find that the day-227 yeast settle more quickly for their size than do the other four isolates ($F_{4,4} = 12.43$, $P < 0.0001$, interaction between log-transformed cluster area and strain in an ANCOVA with settling speed as the response variable. Day-227 yeast had the largest slope in 98/100 bootstrap simulations; Fig. 4A).

Because the faster settling of day-227 yeast is not due to an increase in cellular buoyant density (Fig. S2), we examined overall cluster shape. Snowflake yeast are generally spherical, but frequently possess elongate branches of cells that disrupt the hydrodynamic profile of the cluster. The presence of these branches can be detected as a reduction in the roundness of a 2D image of the cluster (see Fig. S3A,B for the 2D profile of representative clusters). Day-227 yeast were 5.7% more round than day-65 yeast ($t_{631.8} = 4.03$, $P < 0.0001$; Fig. 4B–D), an effect not caused by their larger average size (Fig. S3C). By reducing drag, day-227 yeast should use their biomass more efficiently, settling more rapidly than their less hydrodynamic ancestors.

Discussion

Direct investigation of the transition to multicellularity demonstrates the centrality of multilevel selection in organismal evolution (Maynard Smith and Szathmáry 1995; Michod 1997). We previously found that, once snowflake yeast evolve from

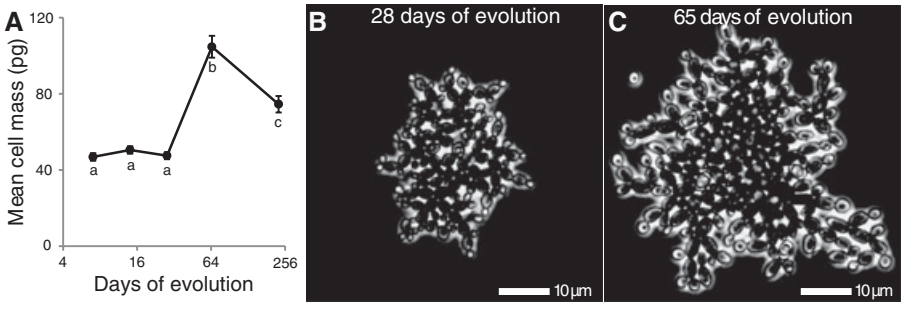

Figure 3. The mass of individual cells increased sharply between day 28 and day 65. (A) Mean cell mass for each strain, calculated by multiplying cell volume by the strain's average buoyant density. Error bars are 95% confidence intervals, different letters denote significantly different means ($\alpha = 0.05$, Tukey–Kramer HSD). The large increase in cluster mass at day 65 was because of an increase in the size of individual cells between (B) day 28 and (C) day 65 yeast. Clusters shown have been flattened before imaging.

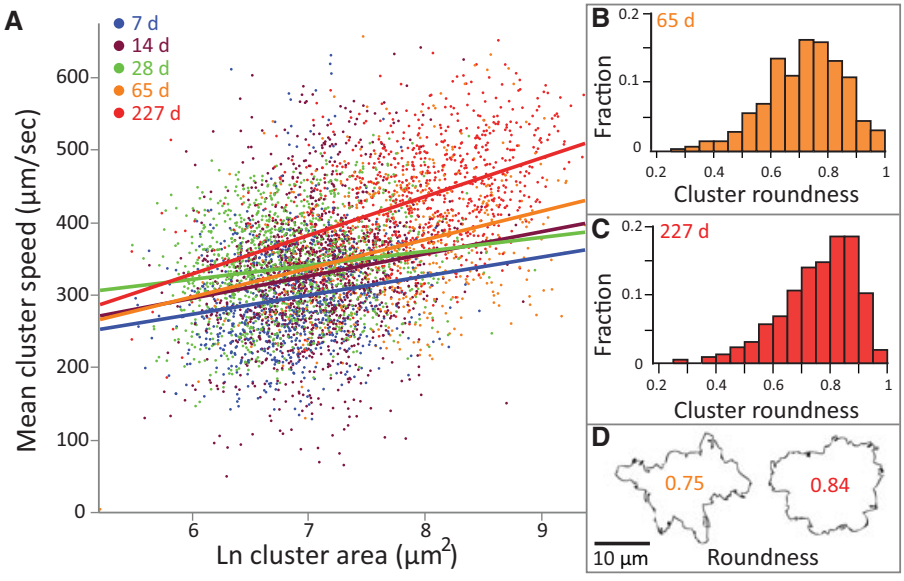

Figure 4. **Evolution of faster settling, more hydrodynamic clusters. (A) Day 227 snowflake yeast (red) settle more quickly for a cluster of a given size than do earlier isolates. (B,C) These yeast have also evolve a more hydrodynamic form, making less "branchy," more round clusters than their day 65 ancestor. (D) Outlines of similarly sized clusters from days 65 (left) and 227 (right).**

unicellular ancestors, they precipitate a rapid switch from selection acting at the unicellular level, to selection acting at the multicellular level (Ratcliff et al. 2012). In this article, we focus on the tempo and mode of multicellular adaptation after the transition to multicellular-level evolution, during the very first steps in the evolution of multicellular development, a nearly unexplored ancestral phase in the evolution of all extant multicellular lineages.

Multicellular adaptation occurred in three distinct modes (Fig. 5). In period 1 (7–28 days), snowflake yeast evolved faster settling solely by increasing the number of cells per cluster. This mode of adaptation continued for the duration of the experiment. In period 2 (28–65 days), snowflake yeast begin modifying the nature of the cell, building larger clusters by increasing the mass of individual cells. In period 3 (65–227 days), snowflake yeast evolve to settle not just more rapidly, but also more efficiently, forming more hydrodynamic clusters that settle more quickly for their size. Determination of the precise temporal boundaries for each phase was limited by the sampling scheme, but their order was unambiguous.

Evolutionary radiations are often spurred by the acquisition of a key trait that opens up novel routes for subsequent adaptation (Simpson 1953; Schluter 2000). Examples of this are endodsymbiotic precursors to mitochondria and chloroplasts (Cavalier-Smith 2002), adhesive proteins in metazoans (King et al. 2003) and extracellular polysaccharides in algae (Prochnik et al. 2010), the angiosperm flower (Friis et al. 2006), and the tetrapod limb (Shubin et al. 1997). The snowflake body plan appears to be such a trait. Once the snowflake body plan evolved, novel routes for subsequent adaptation became available, involving both quantitative (Figs. 2 and 3) and qualitative (Fig. 4) structural modifications.

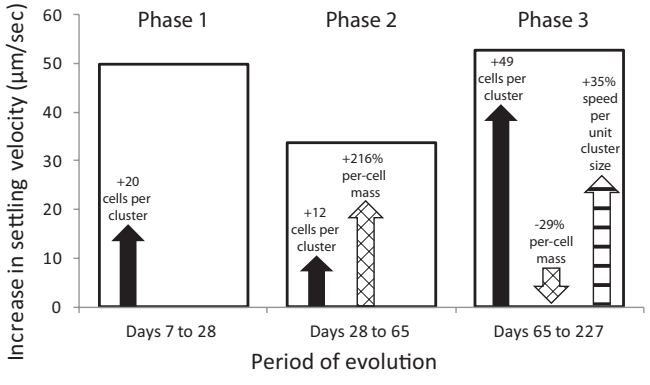

Figure 5. **Three phases of adaptation for faster settling in snowflake yeast. The open bar graph reports changes in mean settling speed (y-axis), whereas the enclosed arrows report changes in the mean number of cells per cluster (solid), the size of individual cells (diamonds), and the settling rate of the cluster per unit size (horizontal lines; one size unit is Ln[cluster area in µm^2]). In phase 1 (7–28 days), snowflake yeast evolved faster settling only through an increase in the number of cells per cluster. In phase 2 (28–65 days), snowflake yeast begin modifying the nature of individual cells, increasing their mass by more than twofold, which similarly increases cluster mass. In phase 3 (65–227 days), snowflake yeast evolves to settle more efficiently, forming faster settling, more hydrodynamic clusters. Arrows are scaled only to other arrows of the same type (e.g., solid to solid).**

In our experiment we see an important shift in the nature of adaptation. In periods 1 and 2, cluster size, and hence

settling rate, is a direct function of the number and size of cellular units. In period 3, however, faster settling evolves through changes in the overall shape of the cluster, a novel adaptation that is not a direct function of the cluster's cellular units (this distinction is similar to the difference between types I and II multilevel selection; Damuth and Heisler 1988). Across taxa, there is a broad correlation between organism size and complexity (Bell and Mooers 1997; Bonner 2004). A nice example of this comes from the Volvocine algae: the only species that have evolved cellular division of labor form colonies with > 32 cells (Koufopanou 1994). Our results suggest the intriguing possibility that early evolutionary innovations, leading to larger size, promote subsequent increases in complexity. The first snowflake yeast to evolve were small and slow settling, but settling rate rapidly increased as they evolved larger cluster size. Larger clusters, however, grow slower than smaller clusters, probably because of reduced resource diffusion to internal cells (Ratcliff et al. 2012). Continued evolution of faster settling through increased cluster size would impose greater growth rate costs, placing a premium on traits that increase settling rate without causing an increase in cluster size, such as the evolution of a more hydrodynamic shape. An additional adaptation to large cluster size is apoptosis. We previously found that after 60 transfers with settling selection, large cluster-forming snowflake yeast began using apoptotic cells as "breakpoints" within the cluster, producing proportionally smaller, faster growing propagules as a result (Ratcliff et al. 2012).

It therefore seems likely that costs associated with the first adaptations to faster settling, larger cluster size, impose secondary selection for novel, more complex adaptations. We might expect this result to be general in the evolution of multicellularity, as the geometric implications (lower surface area : volume ratio) of larger biological aggregates are universal (West et al. 1999), potentially reducing the growth rate of both heterotrophic (Wentland et al. 1996) and autotrophic microbes (Niklas and Spatz 2012). Indeed, cluster formation in nonmotile algae increases settling speed (Lürling and Van Dank 2000), but reduces growth rates (Becks et al. 2010; Yokota and Sterner 2011).

More generally, our results are consistent with observations of trait hierarchy: emergent functional properties arise from underlying physical structures (Alfaro et al. 2005). Because natural selection acts on a trait's functional properties, rather than directly on the underlying basis, there is potential for multiple mechanisms to affect the same emergent trait (Marks and Lechowicz 2006). The evolutionary consequences of such divergent mechanisms are thought to be profound, as they may provide avenues for divergent modes of adaptation, as observed in cichlid fish (Schaefer and Lauder 1996; Hulsey et al. 2006). Prior microbial selection experiments have supported these observations, with largely phenotypic parallelism in response to uniform selective conditions (Lenski and Travisano 1994; Woods et al. 2006) masking more divergent genetic responses (Travisano and Lenski 1996; Barrick et al. 2009), which have persistent evolutionary consequences (Travisano et al. 1995; Blount et al. 2008; Meyer et al. 2012). Our current results on multicellularity indicate multiple adaptive mechanisms readily evolve within a lineage, providing avenues for further adaptation and diversification.

In summary, we show that continuous selection on yeast for fast settling through liquid media can result in rapid multicellular-level adaptation. Faster settling occurred through multiple, complementary routes involving both quantitative and qualitative structural modifications to the snowflake yeast body plan. The timing and nature of adaptation in our experiment suggests that the reduced growth rate of larger clusters favors novel routes to faster settling, ultimately driving an increase in organismal complexity. Selection to mitigate the costs incurred by larger body size may be a fundamental driver of increased multicellular complexity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Movie S1. The settling speed of multicellular yeast was measured with a custom built microscope.

Figure S1. Selection scheme used in this experiment.

Figure S2. Despite a small decrease in cell density, larger more massive cells should increase settling speed.

Figure S3. 2D outlines of 49 randomly chosen snowflake yeast clusters from the day-65 strain.

Figure S4. 2D outlines of 49 randomly chosen snowflake yeast clusters from the 227-day strain. 227 day yeast were significantly more round than 65-day yeast (Figure 4B,C).

Figure S5. The faster settling of day-227 yeast is not an artifact of their larger average size: day-227 yeast clusters were more round across the size distribution.