National Aeronautics and Space Administration

NASA Astrobiology Institute 2016 Annual Science Report

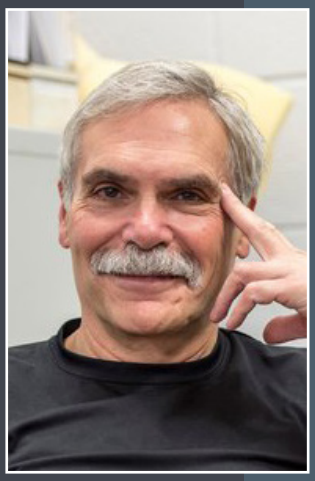
Team Report:

Reliving the Past:
Experimental Evolution of Major Transitions,

University of Montana/
Georgia Institute of Technology

Reliving the Past:
Experimental Evolution of Major Transitions

Lead Institution:
University of Montana /
Georgia Institute of Technology



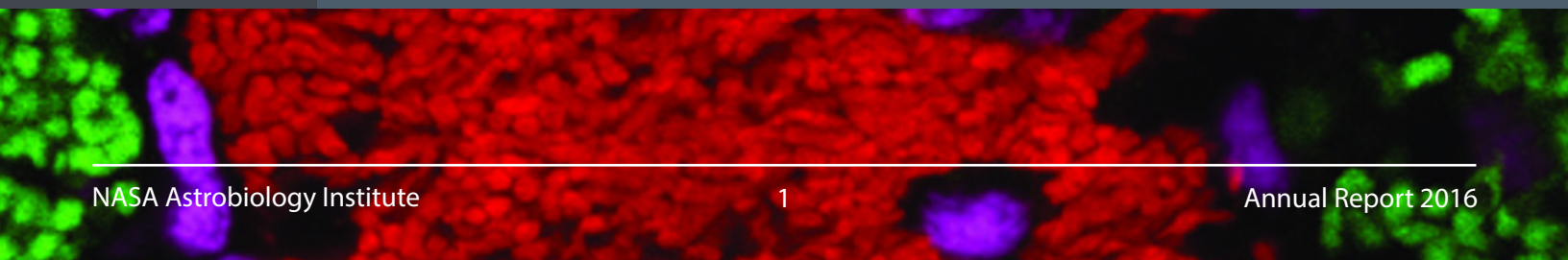
Principal Investigator:
Frank Rosenzweig

Team Overview

The Origin of Species concludes with a hymn to biocomplexity teeming on an English hillside. The hymn's most penetrating verse is "these elaborately constructed forms, so different from each other, and dependent upon each other, had all been produced by laws acting around us." To delve into these laws, to understand how differences are selected and how interdependence is enforced remains biology's grandest challenge. Our Team seeks to meet this challenge by Reliving the Past using experimental evolution, an approach that enables us to discern evolution's causes as well as its consequences, and to discover why evolution takes certain paths and not others. By tackling five questions we aim to illuminate what drove major transitions leading to the evolution of complex life:

- How do enzymes and metabolic networks evolve?
- How did the eukaryotic cell come to be?
- How do symbioses arise?
- How does multicellularity evolve?
- How do history, gene interactions and mutation constrain innovation?

We seek general principles likely to govern the emergence of complexity wherever life exists. Our enterprise falls squarely within Astrobiology, the study of the origins, evolution, distribution, and future of life in the universe, and addresses the fundamental question: How does life begin and evolve?



2016 Executive Summary

The Reliving the Past (RLP) Team seeks to understand evolutionary transitions where subunits coalesce to form autonomous, interdependent wholes. If life is a self-sustaining chemical system capable of Darwinian evolution, then the factors driving these quantum leaps in biocomplexity should be at work wherever life exists. The Team itself underwent a major transition in 2016: PI Rosenzweig, along with Co-Is Herron, Gerrish and Smith relocated to Georgia Tech, where they welcomed two new investigators Ratcliff (GaTech) and Kacar (Harvard/ELSI).

By 6 key performance indicators, Year-2 was a resounding success. In terms of scholarly output we published, or now have in press, 41 peer-reviewed manuscripts, including multiple reports in *Science*, *PNAS* and *Cell*. Co-I Smith co-authored the widely-praised "The Origin and Nature of Life on Earth" published by Cambridge Press (Fig. 1). Our work attracted media coverage: Co-I McCutcheon's ground-breaking work on lichen symbioses was featured not only in *Science* (cover) and *PNAS*, but also in the *NY Times*, *The Atlantic* and BBC news. *Popular Science* selected Ratcliff as one of its 2016 "Brilliant 10" most innovative young minds in science and engineering. Copley's work on how new enzymes are born was featured in *Science News*. Our CAN-7 is a wellspring of new ideas, generating 31 new grant proposals. By year's end 5 had been funded, worth >$3.2M, including a Packard Fellowship to Ratcliff. Reliving the Past investigators gave 50 invited seminars worldwide.

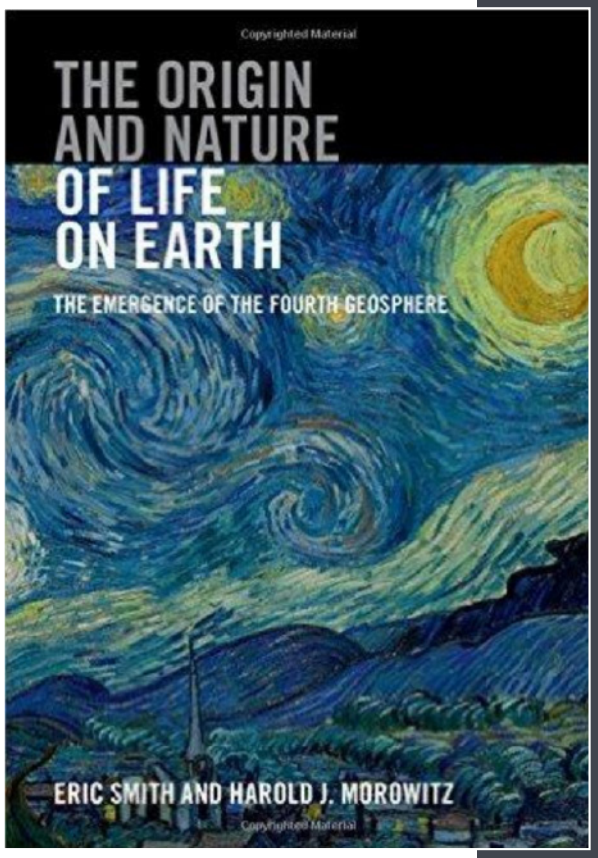


Fig. 1. The Origin and Nature of Life on Earth (Cambridge University Press) provides an integrated introduction to the main ideas needed to understand life on Earth as a planetary process. Smith and Morowitz draw from planetary science, organic and biochemistry, microbiology, fundamental physics, and information theory, providing a roadmap to engaging with the best ideas from all these domains at their highest level.

In terms of synergistic activities, RLP investigators served as peer reviewers for scores of manuscripts, served on editorial boards of *BMC Microbiology*, *J Biological Chemistry*, *Genome Biology Evolution* and *Biology Letters*, and evaluated proposals to NASA, NSF and NIH, both as panelists and ad hoc reviewers. Co-I Sherlock and PI Rosenzweig organized the 2nd ASM Conference on Experimental Microbial Evolution, which attracted >150 investigators from eighteen countries. We mentored 82 students (32 undergraduates/27 graduates/23 post-docs), including two NPP fellows (Weldon and Turner), one DDF Fellow (Lynch) and

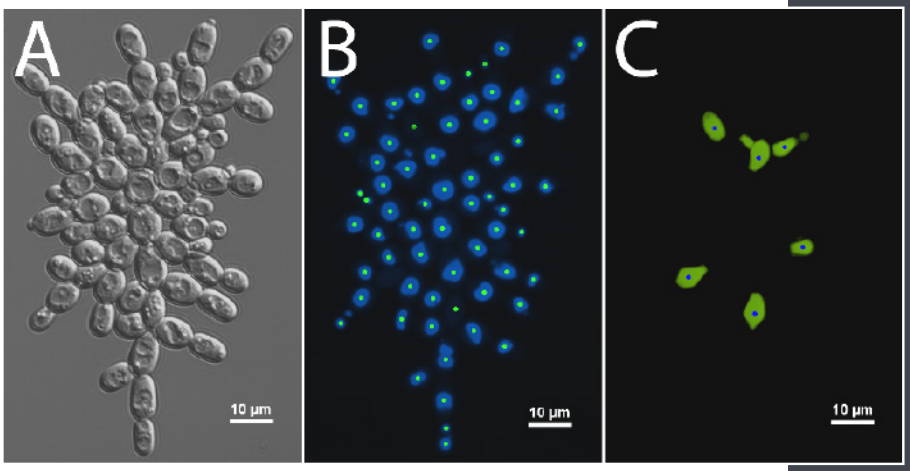


Fig. 2. Measuring apoptosis in multicellular "snowflake" yeast. Pictured is the same cluster imaged with (a) differential interference contrast microscopy, (b) the blue-fluorescent vacuole stain CellTracker Blue CMAC and (c) the green-fluorescent apoptosis stain dihydrorhodamine 123. We obtained counts of all cells by counting the number of vacuoles (white dots marking cells in b) and apoptotic cells (black dots marking cells in c) by using the spot-detection algorithm in NIS-elements v. 4.30.

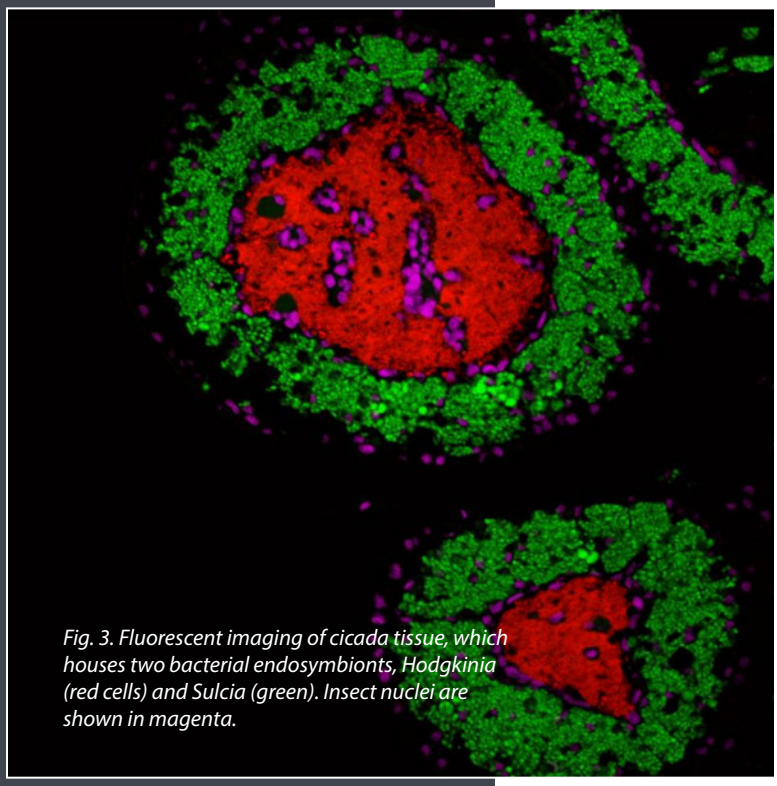


Fig. 3. Fluorescent imaging of cicada tissue, which houses two bacterial endosymbionts, Hodgkinia (red cells) and Sulcia (green). Insect nuclei are shown in magenta.

Fig. 4. Evolution of colony diversity within three populations of P. aeruginosa evolved under a daily biofilm life cycle for 90 days. (Cover, Journal Bacteriology special issue)

Fig. 5. Hypogymnia imshaugii, a macrolichen common in northwestern North America. Lichens were previously thought to contain two parts: an ascomycetous fungus and a photosynthetic alga. Spribille et al. show that a third element–a basidiomycete yeast–is part of the lichen cortex in the largest group of macrolichens. (Cover of Science 353(6298). Credit: Tim Wheeler)

one NSF Pre-doctoral fellow (Gulli). RLP's broader impacts were felt in E/PO activities that ranged from televised lectures to high school students to public, streamed lectures/interviews on the Origin of Life and on the Societal Implications of Astrobiology.

In September we hosted the NAI Executive Council. Team members reported out in a "Major Evolutionary Transitions" colloquium, joined by invited speakers Nicole King (UC-Berkeley), Loren Williams (GaTech), Doug Erwin (Smithsonian), Paula Welander (Stanford) and Neil Blackstone (N Illinois).

Finally, our Team made significant research advances towards understanding how biological systems increase in complexity. To give but three examples: Will Ratcliff has significantly advanced our understanding of how multicellular groups can form (both genetically and geometrically), as well as how these simple groups establish new multicellular life cycles that incorporate programmed cell death (apoptosis) (Fig. 2). Vaughn Cooper has shown how bacterial biofilms evolve into synergistic communities via regulatory mutations that coordinate attachment and dispersal and/or the shift from anaerobic to anaerobic metabolism. Vaughn's work was recently featured on the cover of a special issue of *J. Bacteriology* (Fig. 3). John McCutcheon continues to unravel the mysteries of insect-bacterial symbioses (see Fig. 4) and to reveal more parallels between these symbioses and the 'classic' organelles, mitochondria and plastids. John's team has shed new light on the lichen symbiosis (Fig. 5), one of the most spectacular examples of macro-scale complexity built exclusively from microbial 'parts.'

Year-2 was therefore a great success for the Reliving The Past Team. We expect to achieve even more in Year-3 by strengthening collaborations within our Team and across the NAI, and by promoting experimental evolution as a way to evaluate the ecological and genetic factors that increase complexity in living systems.

Project Reports

How Do Enzymes and Metabolic Networks Evolve?

The earliest life on earth, known as the LUCA (last universal common ancestor) had only a few hundred genes. Extant organisms have thousands. The massive expansion of the genetic repertoire in the last 3.8 billion years has been fueled in part by gene duplication followed by divergence of one copy to encode a protein with a new function (Fig. 6).

This project, led by Shelley Copley, addresses the process of enzyme evolution by gene amplification and divergence using a model system in which a gene that encodes an essential enzyme (ArgC) is deleted. Another enzyme (ProA) present in the bacterium has a promiscuous secondary activity that corresponds to that of the missing enzyme, but it is too inefficient to rescue growth. However, a single mutation allows this enzyme to serve both functions, albeit poorly. (This mutant enzyme is referred to as ProA*.) This system sets the stage for her studies of the genetic changes that improve fitness when an inefficient enzyme serves two essential functions.

This year, Shelley's team published a paper in the *Journal of Bacteriology* describing genetic changes that improve fitness in *E. coli* when ProA* must serve the functions of both ProA and ArgC. Mutations in the promoter of the operon encoding ProA* (Fig. 7a, b) increased expression of the bifunctional enzyme. A synonymous mutation in the upstream gene (*proB*) created a new promoter for the gene encoding ProA* (Fig. 7c). Each of these mutations increases growth rate by 3-5-fold. Finally, massive segmental amplifications – the requisite first step for divergence toward two specialist enzymes – were observed (Fig. 8).

Surprisingly, segmental amplification does not occur in the same experimental

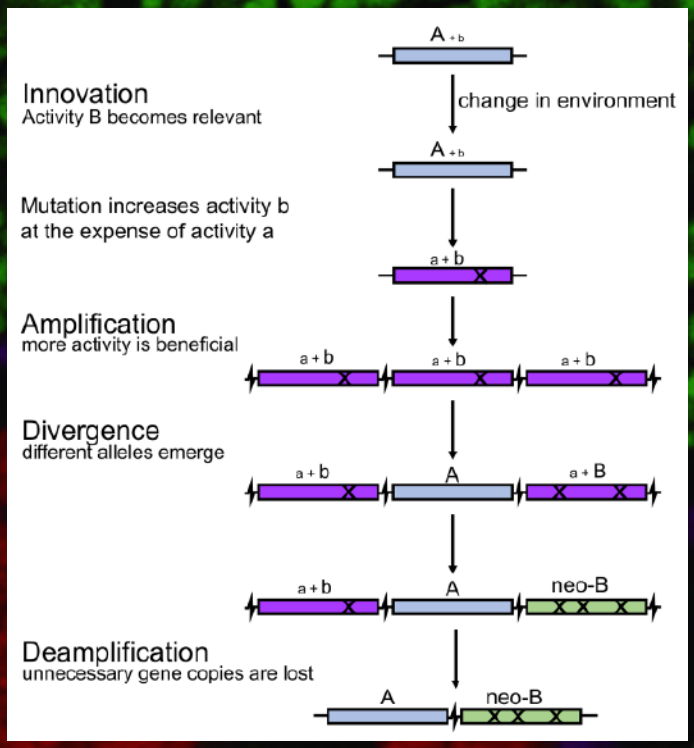


Fig. 6. The Innovation-Amplification-Divergence model for evolution of a new enzyme (neo-B) starting from a gene encoding an enzyme with a primary activity A and a weak secondary activity b that becomes useful after a change in environment.

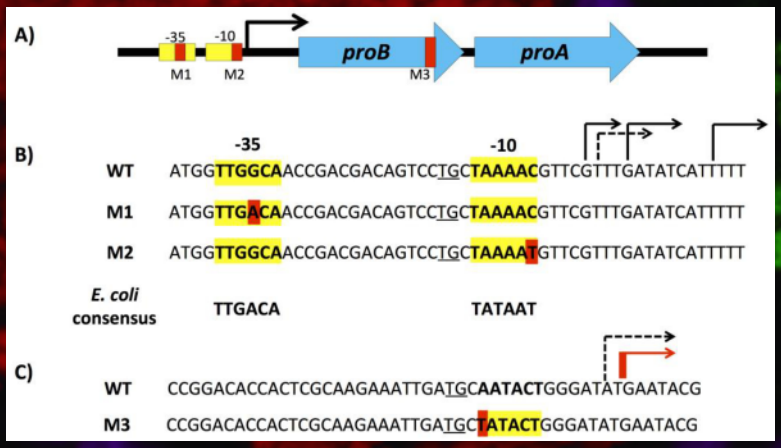
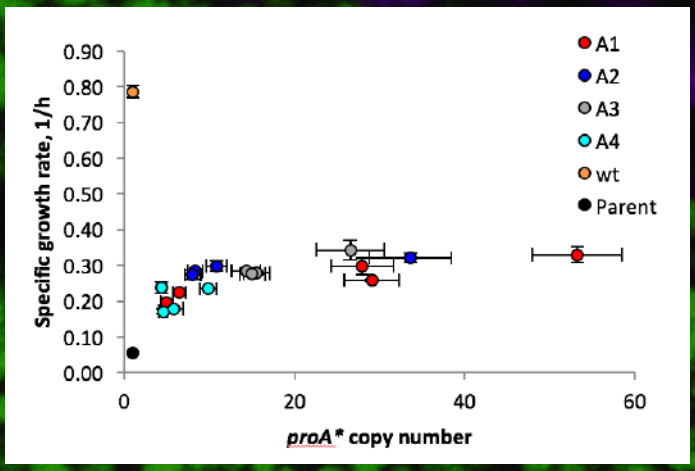


Fig. 7. Point mutations that enhance growth of an E. coli strain in which a weak-link enzyme (Glu383Ala ProA) serves as a poor catalyst for two essential reactions (its own reaction in proline synthesis and the reaction normally catalyzed by ArgC). A) Locations of point mutations in the proBA operon; B) Mutations M1 and M2 in the promoter of the proBA* operon increase transcription of the operon and increase growth rate by 4.7- and 3.2-fold, respectively. The dashed and black lines indicate the transcription start sites previously determined by other laboratories. C) Mutation M3, a synonymous mutation in the 3'-end of proB, creates a new promoter for proA* and increases growth-rate by 5.1-fold. The red line indicates the new transcription start we identified. The dashed line indicates a weak transcription start site previously identified by another laboratory.*



system in a different bacterium, *Salmonella enterica*. Instead, mutations just upstream and in the coding region of the gene substantially increase growth rate, suggesting not all bacteria evolve a new enzyme via gene amplification and divergence when exposed to the same selective pressure.

Fig. 8. Amplification of the region surrounding proA of up to 55 copies in four independent lineages increases growth rate relative to the parental strain by up to 6-fold.*

How Did the Eukaryotic Cell Come to Be, Specifically the Cell that Contained a mitochondrion? How Do Symbioses Arise?

The highlight of 2016 in the McCutcheon lab was probably the *Science* paper showing that the most diverse and abundant lichens are actually composed of two fungal partners, not one (Fig. 9). The Team showed that the outer layer—called the cortex—of these fungi is composed of a mix of the known ascomycete filamentous fungus and a heretofore unknown basidiomycete fungus that exists in yeast form. This is in some ways a simple result, but it has implications far beyond lichen biology and symbiosis. Lichens are almost unique in symbiosis: they are macroorganisms that are composed of microorganism components that do not form visible structures when grown alone.

Our paper for the first time reveals the complete partner list for the most diverse and species-rich group of macrolichens, and finally allows research into the fascinating developmental biology problem of how eukaryotic microorganisms come together to make macro structures.

We also made progress on our mealybug system in our *PNAS* paper, where we showed that symbiont replacement is possible (indeed, relatively common) in even the most intricate and interdependent endosymbioses (Fig. 10). This symbiosis exists with a highly unusual bacteria-within-bacteria structure (Fig. 11), which is important because of the likely prokaryote-within-prokaryote origin

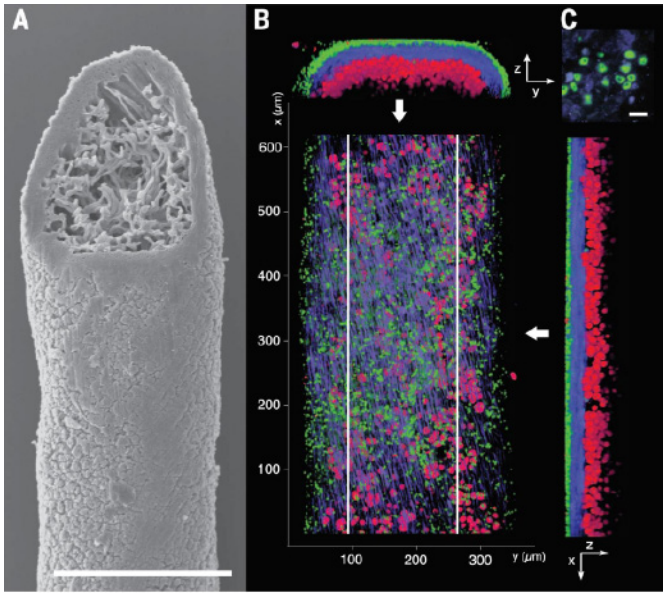


Fig. 9. From Spribille, Science. Fluorescent cell imaging of dual fungal elements in lichen thalli (known fungus, blue; newly described fungus, green).

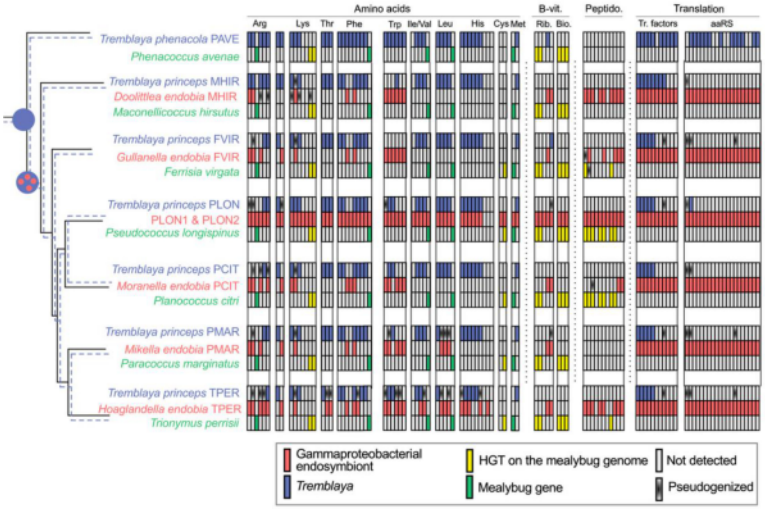


Fig. 10. From Husnik, PNAS. A complex history of gene retention, loss, and acquisition in the mealybug symbiosis.

of the mitochondrion. We showed that the innermost bacterium in this symbiosis (called *Moranella* in some species) has been replaced several times over the course of mealybug evolution by bacteria from a related group of widely distributed insect associates. Remarkably, the outermost bacterium, called *Tremblaya*, has a very small and degenerate genome encoding few genes but remains stable in the face of dramatic changes to the identity of the bacterium living in its cytoplasm. This result has implications for our thinking on how other endosymbioses such as mitochondria might have come to be: taken together, we argue that these results support "gradualist" hypotheses for organelle evolution.

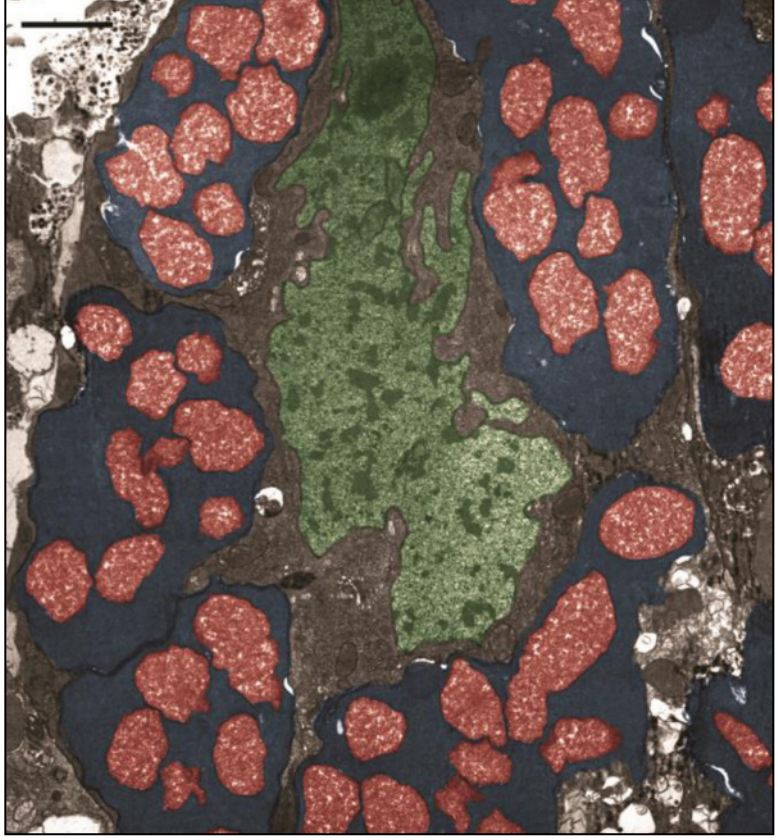


Fig. 11. The bacteria-within-bacteria structure shown by TEM (Tremblaya cells are blue, Moranella cells are red; insect nucleus is green).

How Do Symbioses and Synergistic Interactions Arise?

Most bacteria are found growing on surfaces in biofilms; inevitably these must disperse to recolonize new surfaces when resources become limiting. When such populations repeatedly undergo a life cycle of surface attachment, biofilm assembly, dispersal, and reattachment, they often evolve into different forms by acquiring adaptive mutations specific to particular aspects of this cycle.

Using a laboratory model of this cycle, Vaughn Cooper's team has learned this diversity often produces a whole that is greater than the sum of its parts. The overall objective of this project is to define the ecological mechanisms that produce these synergistic interactions. A related goal is to define the identity and trajectories of the adaptive mutations that produce diversity using high-throughput population-genomic sequencing.

Recently, Cooper learned that populations of *Pseudomonas aeruginosa* evolving under this cycle repeatedly differentiate into early-attaching mutants that colonize the plastic surface and

Fig.12. Evolution of colony diversity within three populations of P. aeruginosa evolved under a daily biofilm life cycle for 90 days.

late-attaching variants that attach to established biofilm. These populations also become dominated by mutator genotypes defective in DNA mismatch repair that rapidly differentiate (Fig. 12).

His team also discovered that major adaptations were produced by mutations in genes that coordinate attachment and dispersal and in other genes that govern the shift from anaerobic to anaerobic growth. Mutants that first colonize the surface enjoy brief dominance but must adapt to low-oxygen conditions when late colonists attach and bury them. Both types later disperse and continue the cycle, ultimately producing greater biomass together than alone (Fig. 13). Cooper is working to define the ecological and genetic boundary conditions that enable mutator genotypes to invade as well as the first altered functions that set the stage for these synergistic, multicellular communities. His research is relevant both to understanding how multicellular organisms might have evolved, and why bacterial biofilms are intrinsically resistant to a wide range of antimicrobials.

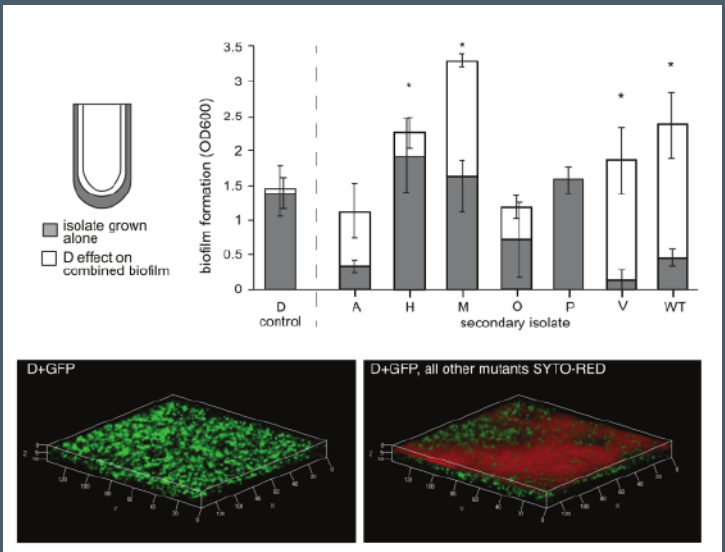
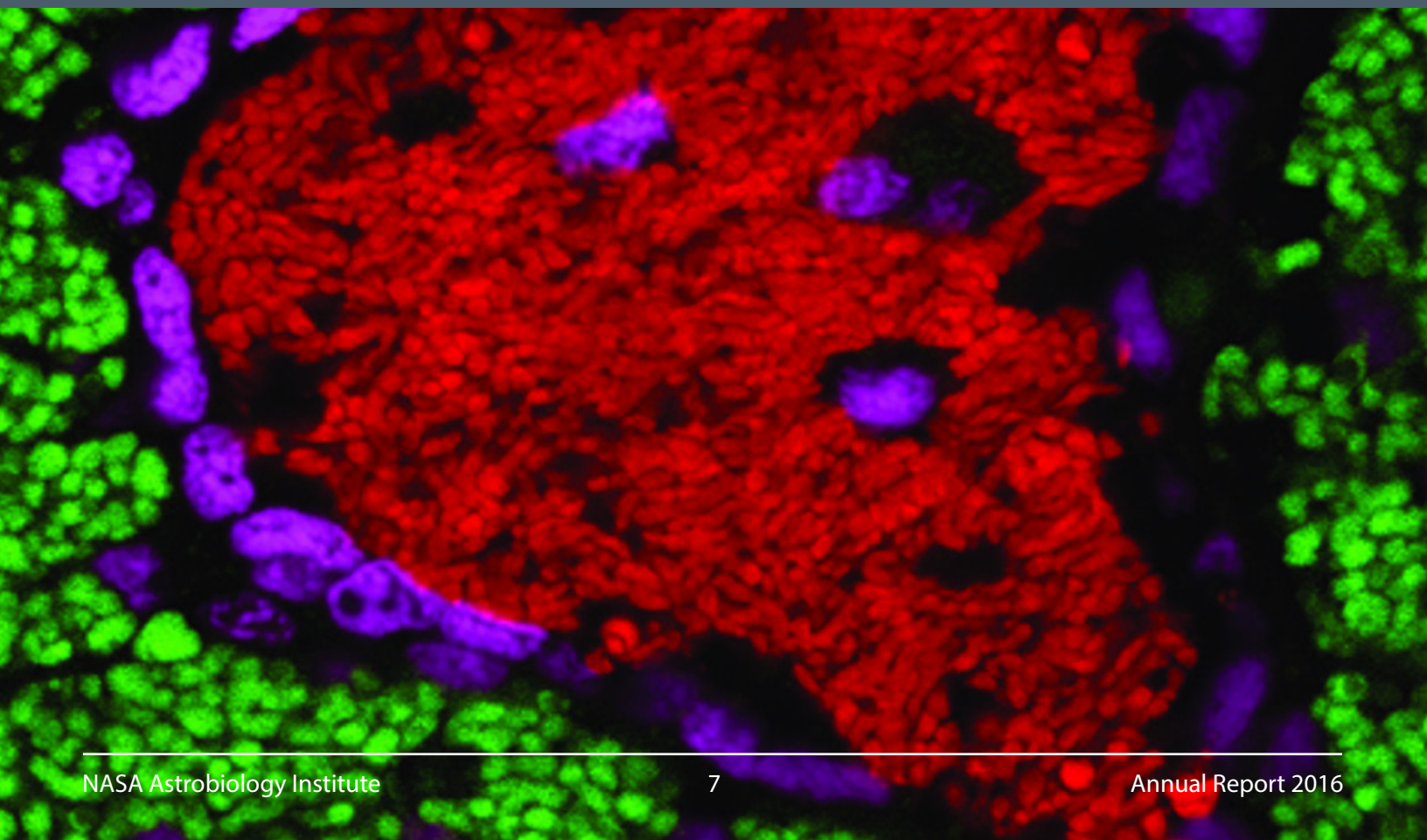


Fig. 13. The early colonizing D type facilitates the attachment of other types and enhances biofilm output. (TOP) Pairwise biofilm assays were conducted in which the D type was allowed to colonize a polystyrene surface for 4 h before adding a secondary isolate. Biofilm formation of individual members growing alone (gray) and the increase in total biofilm following D colonization (dark gray) are shown. Asterisks signify combinations that are significantly more productive than both the member grown alone and D with more D added to control for greater cell density. (BOTTOM) Confocal microscopy of the constructed biofilm community inoculated simultaneously with D labeled with GFP. The remainder of cells was imaged after staining with red Syto62 dye. Left, only GFP-expressing cells are shown. Right, combined image of GFP and all red-stained cells attaching to the D type.



How Does Multicellularity Evolve?

As one of the major transitions in life's history, multicellularity enabled unprecedented increases in complexity, including cellular differentiation and complex developmental programs. Predation has long been hypothesized as a cause for why multicellularity evolved, as most predators can only consume prey of a certain size.

Led by Matt Herron, this project has determined that simple multicellularity can evolve in the unicellular green alga *Chlamydomonas reinhardtii* in response to predation by the ciliate *Paramecium tetraurelia* (Fig. 14). Eight isoclonal lines were isolated from each of two populations where multicellularity had evolved plus one control population that evolved in the absence of predators. The 24 isolates belong to four genetic groups with high variability among and low variability within groups. Following on from prior work, where multicellularity evolved in *C. reinhardtii* in response to settling selection, Herron's team has analyzed gene expression over the course of this novel organism's life cycle; they find that genes differentially expressed between unicellular and multicellular isolates are enriched for volvocine-specific and *C. reinhardtii*-specific genes (Fig. 15). Differentially expressed genes are also enriched for gene ontologies related to regulation of multicellular development.

Finally, Matt has modeled relationships between particle-level heritability and collective-level heritability during major transitions and shown that, given certain assumptions, heritability of collective-level traits is higher than that of the corresponding particle-level trait under most conditions. In Year 3 his team will complete life-cycle analyses of evolved isolates from the predation experiment, and identify the genetic bases of multicellularity in two experimental populations. They will also initiate forward genetic analyses, and begin analyzing cell-specific patterns of gene expression. The multicellular structures that have evolved in these experiments represent the very origins of multicellular development. We now have a unique opportunity to observe the evolution of development from the ground up, in real time, in a species that never had a multicellular ancestor.

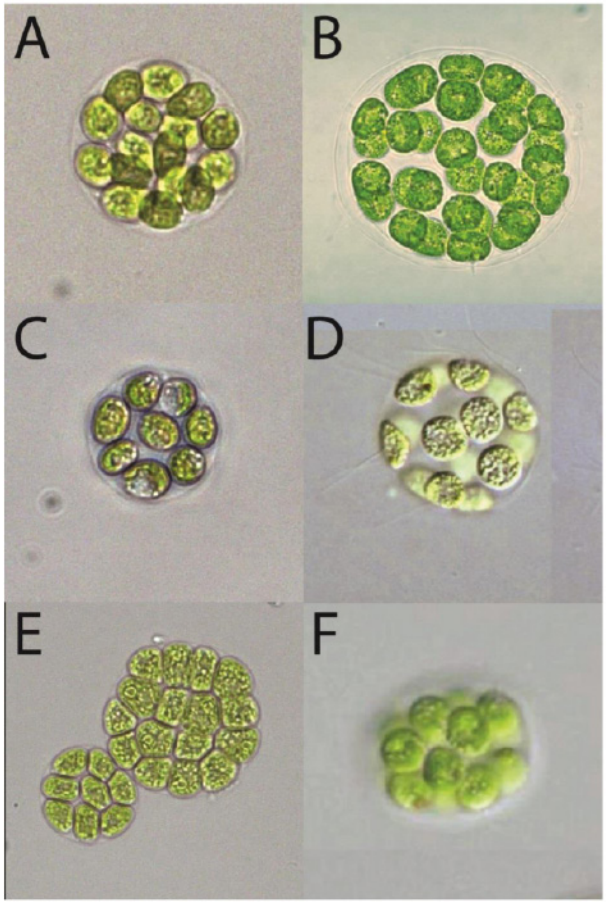


Fig. 14. Naturally occurring and experimentally evolved volvocine algae. A, C, E: multicellular structures from the Paramecium predation experiment. B: Eudorina elegans. D: Volvulina steinii. F: Yamagishiella unicocca.

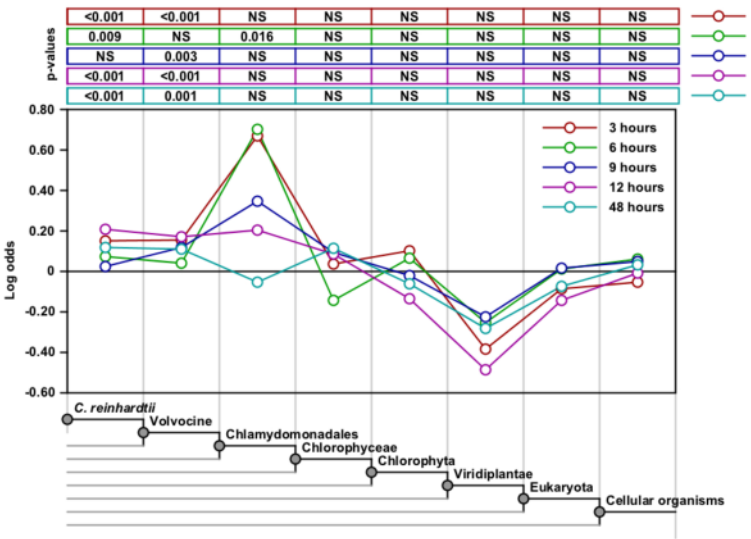


Fig. 15. Results of phylostratigraphy analysis. The y-axis represents the log odds of the observed degree of over/underrepresentation relative to genome-wide frequencies. Bonferroni-corrected p-values result from a hypergeometric test (alpha = 0.0025, equivalent to a false discovery rate of 1%) performed in GeneMerge v.1.4.3 "NS" = not significant.

How do Pleiotropy, Epistasis and Mutation Rate Constrain the Evolution of Novel Traits?

Gavin Sherlock's lab at Stanford is investigating the effect of historical contingency, specifically looking at how existing mutations constrain and/or affect future evolution. This is related to Stephen Jay Gould's idea of "replaying the tape of life", but Gavin and his team are replaying it from slightly different starting points, to see whether the paths taken ever cross or converge, or whether they remain forever separate. This work is key to understanding how tumors evolve, or how antibiotic resistance arises in infections. Team members previously selected 3 adaptive lineages (Levy et al, 2015, *Nature* 519, 181-186), each of which presumably had one gain-of-function, or one loss-of-function beneficial mutation, affecting either Ras/Protein Kinase A signaling, or the Tor signaling (Fig. 16 and 17). To track their further evolution, team members re-barcoded them: Adaptive lineages were backcrossed to the ancestral strain containing a "landing pad", and then sporulated, to isolate individuals having both a beneficial mutation and a blank landing pad. They then performed pairwise fitness competitions between independent segregants of each lineage against a fluorescently marked ancestor strain, and unequivocally proved that, the new mutations do provide a fitness benefit.

Gavin's team have now rebarcoded each of these strains, with ~500,000 lineage tags, which will allow them to track the emergence of new beneficial mutations as each mutant is independently experimentally evolved. Already they have evolved, in duplicate, each of these three barcoded genotypes for 168 generations, under the original evolutionary conditions of Levy et al., and are now sequencing the lineage tags throughout the evolutions, to determine the rates at which they are evolving, and their distribution of fitness effects. They will then isolate hundreds of clones from each evolution, remeasure their fitness, and select hundreds of adaptive clones for whole genome sequencing.

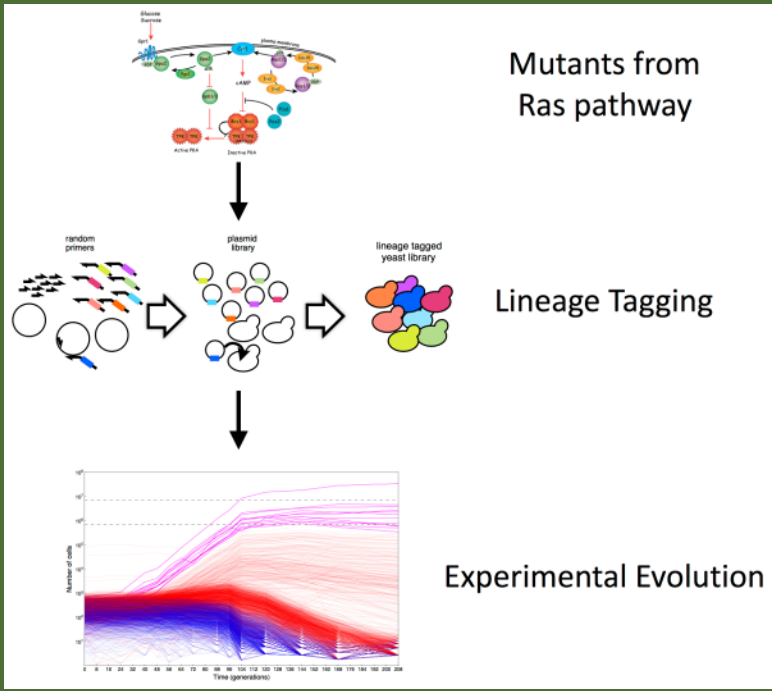


Fig. 16. Barcode lineage tagging in yeast. An experimental population is founded by millions of yeast cells that represent a single clone, except for a molecular barcode by which they can be distinguished by DNA sequencing. This procedure enables one to track at high resolution those lineages that acquire beneficial mutations as well as the fitness of those mutations, which is related to how quickly they increase in relative frequency.

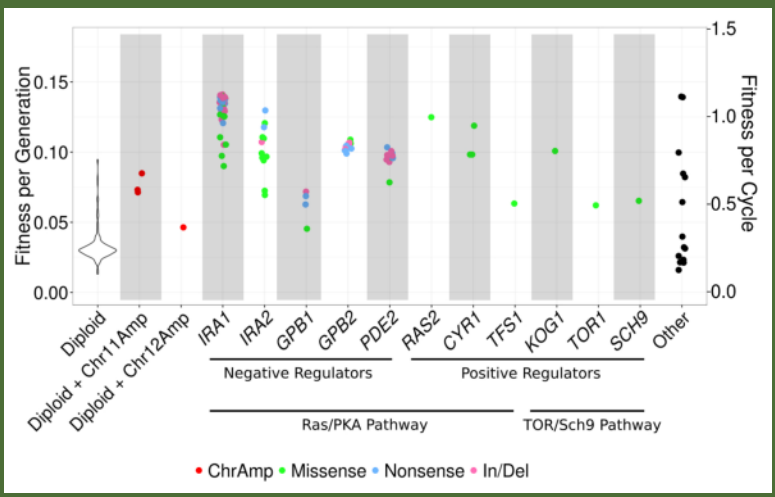


Fig. 17. The fitness spectrum (genotype-to-fitness map) of evolved clones with different adaptive mutations. The inverse variance weighted fitness averaged across all batches and replicates is plotted. Mutations are colored by their molecular basis (i.e. chromosomal amplification, insertion/deletion, nonsense or missense). The "other" class includes the 14 adaptive haploid clones for which we did not identify a nutrient response pathway mutation. Within-batch standard deviations (not shown for clarity) are ≤ 1% for > 90% of clones with nutrient response pathway mutations, while between-batch standard deviations are ~2% for all clones. To highlight the effect of single mutations on fitness, the six diploid clones with nutrient response pathway mutations are not shown. We show per-cycle fitness (8 generations per cycle) as a secondary y-axis (right side), as the fitness benefit of these mutations may not exclusively be due to changes in per-generation fermentative growth rate, but due to changes in other parts of the growth cycle such as growth lag, diauxic shift, aerobic growth, or increased viability after stationary phase.

Theoretical Integration: The Emergence and Stability of Biocomplexity

To understand both the information-carrying behavior of ecosystems with co-evolving member species, and the robustness of their ordered states, requires an analysis of the fluctuation statistics of these systems. Work by Co-I Eric Smith during 2016 has focused on two aspects of stochastic dynamics on either chemical or evolutionary networks constrained by stoichiometric relations: A) the status of ecosystems as fundamental information-carrying entities within the formal structure of population genetics, and B) the stochastic-process methods needed to analyze fluctuation dynamics on the kinds of concurrent-action networks that characterize both chemical and ecological stoichiometry. One project in ecological stoichiometry uses computational reaction-network theory in chemistry to construct models of co-evolutionary dynamics on configuration spaces constrained by stoichiometry. Such networks are models of the symbiosis studied by Co-I McCutcheon, or among members within syntrophic communities being investigated by Rosenzweig and Kinnersley (Fig. 18).

Ecosystem co-evolutionary dynamics must solve these computationally hard problems of resource balance if ecosystems are to settle into asymptotically robust steady states. Current work concerns evolutionary analysis using generalizations of the Price equation, to characterize the patterns that are transmitted at the ecosystem level rather than merely the hereditary types of the member species.

A second project builds on classical results in the stochastic process theory of chemical reaction networks, to provide new classes of solutions comparable to those that have existed for computationally simpler diffusive systems. We have shown how to decompose the statistics of complex networks on such a way that all-orders of fluctuation moments can be solved systematically with a method of matched asymptotic expansions. We have also proved the existence of a class of duality relations between the stochastic dynamics of such systems, and the propagation of information in statistical problems of inference about their dynamics and structure.

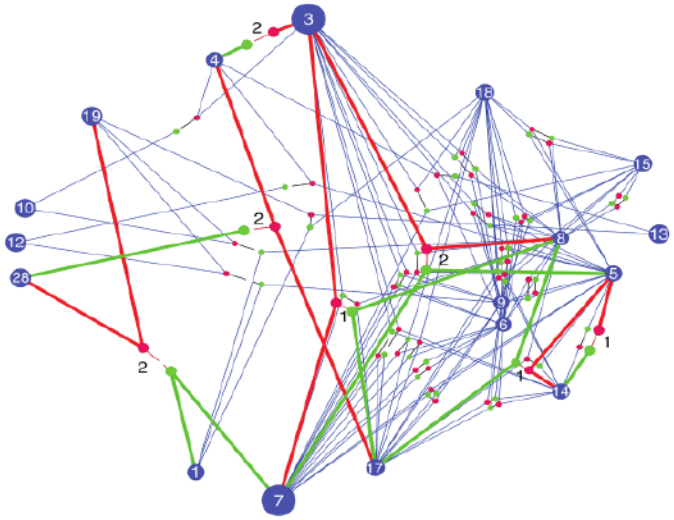


Fig. 18. A population-level stoichiometric optimization problem with an unbounded number of solutions. The fine network shows all directed reactions needed to implement the first 200 of an unbounded number of solutions to the problem of converting five units of resource 3 into three units of resource 7, with no waste. Solid circles are metabolites, and dashed lines are conversions performed by each population member, going from red to green. Highlighted sub-network is one particular solution, reflecting a stable ecosystem configuration maintained by coevolution.

Team Members

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John Carlo Kristofich
Reid Longley
Kennda Lynch
Ankur Makani
Chris Marshall
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Gavin Sherlock
Emily Sileo
Tanya Singh
Eric Smith
Paul Sniegowski
Toby Spribille
Caroline Turner
Jillian Walke
Stephanie Weldon

Reliving the Past: 2016 Publications

Dillon, M.M., and Cooper, V.S. (2016). The fitness effects of spontaneous mutations nearly unseen by selection in a bacterium with multiple chromosomes. *Genetics*, 204(3), 1225-1238. DOI: 10.1534/genetics.116.193060

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