Conclusion: The future of multicellularity research

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Abstract

In this chapter, we examine future directions for research on the evolution of multicellularity. Specifically, we review open questions about the evolution of multicellularity from a range of disciplinary perspectives, including philosophy, natural history, phylogenetics, biophysics and astrobiology. Further, we examine major outstanding questions about the evolution of multicellularity itself, including the origin and subsequent evolution of multicellular life cycles, the role of organismal size in the evolution of multicellular complexity, the origin of development, and the role of environmental drivers and niche construction over geological timescales. Finally, we highlight recent progress and the future potential of comparative, experimental, and theoretical approaches to studying multicellularity. There has never been a better time to study the evolution of multicellularity- recent developments in life science technologies have dramatically expanded experimental opportunities, yet the field remains small enough that breakthrough discoveries made by new members of the community are not just possible, but expected.

18.1 Introduction

In this final chapter, we examine some of the major outstanding questions and approaches for multicellularity research that we believe will define the intermediate-term future of the field. This is not meant to be an exhaustive list of potential future directions, but rather an overview of particularly compelling topics and questions that are well-poised to make major contributions to our understanding of the evolution of multicellularity, based on the current conceptual and technical state of the field. The list is inevitably affected by our backgrounds and biases, and we have no doubt that some important contributions will come from directions that we have not foreseen.

The chapter is organized around eight research areas that are not truly distinct—indeed, there are many interactions among these topics, and we expect insight will just as often come from the edges of these networks as the nodes.

18.2. Philosophical issues in multicellularity

The evolution of multicellularity has historically been a rich topic for philosophical inquiry. The evolution of a new type of individual requires us to confront questions for which we in biology have long relied on heuristic solutions, but which break down when examining this major transition. Understanding the transition from unicellular organisms to multicellular organisms challenges us to define what organisms and biological individuals are (Clarke, 2010; Godfrey-Smith, 2013; Pradeu, 2016; Queller and Strassmann, 2009), and what it means to be multicellular (Rose and Hammerschmidt, 2021). Once we have these definitions in hand, how do we know when the transition to multicellularity has occurred? Is multicellularity even a binary state, such that an organism is either multicellular or not, or is it a continuum? These are challenging philosophical questions, and addressing them has broad value for biology as a whole, not just the evolution of multicellularity.

In Chapter 2, Maureen O'Malley challenges the notion that multicellularity is even a coherent category. Specifically, she argues that multicellular organisms are so diverse that they do not naturally fit under a single label—and indeed, there may be many more differences than similarities between, say, a cellular slime mold such as *Dictylostelium* that spends most of its life in a unicellular state, only ephemerally inhabiting a multicellular state, and an animal, which does the opposite. This has relevance beyond categorization: the way we frame scientific questions depends on how we see the differences among organisms. For example, the question "Why have aggregative multicellular organisms remained relatively simple?" (Márquez-Zacarías et al., 2021) assumes that this is a reasonable question to ask (*i.e.*, that developmental mode is an evolutionarily-salient factor among the many other differences between these organisms).

In addition to these questions about the evolution of multicellularity *per se*, the philosophy of biology is concerned with understanding the approaches that are used to study it. In Chapter 4, Merlijn Staps, Jordi van Gestel, and Corina Tarnita classify these approaches into two broad categories: bottom-up approaches that address particular evolutionary trajectories, considering the roles of the nature of the unicellular ancestor and the environmental factors imposing natural selection, and top-down approaches that aim to identify general principles by comparing the features of existing multicellular organisms. Recognizing that both approaches have strengths and limitations, they advocate for integrating, for example, the top-down, multilevel selection approach of Rick Michod (Chapter 3) with a bottom-up, mechanistic model of the emergence of multicellular life cycles. The multilevel selection framework, including its application to the evolution of multicellularity, has itself been a frequent point of intersection between biologists and philosophers of biology (Bourrat, 2015; Godfrey-Smith, 2013; Michod, 2005; Okasha, 2006).

Philosophical research has significant potential to contextualize and generalize macroevolutionary trends. We believe that philosophy will continue to play a key role in describing how multicellular organisms become units of selection, how multicellular traits arise, become heritable, and affect fitness, and how parts of organisms emerge and become entrenched within a multicellular context.

18.3. Natural history

One of the most important resources for the field of multicellularity is the only truly long-term natural experiments that we will ever have access to: lineages which have independently evolved multicellularity, in most cases hundreds of millions of years ago. Each such origin is a replicate natural experiment, and phylogenetic comparative methods make them useful for testing hypotheses. Most of what we know about multicellularity we've learned from these organisms, and yet we believe that we are only beginning to utilize this resource.

Future work will be critical for more accurately resolving the phylogenies of multicellular organisms. These will be essential for answering basic questions, for instance: How many times have both simple and complex multicellularity evolved? The most widely cited figure we have (informally) seen is 'at least 25 origins', which comes from an analysis by King (2004), performed on a phylogeny developed by Baldauf (2003) and widely popularized in a landmark review by Grosberg and Strathmann (2007). Recent work, however, suggests that the true number is likely far higher. For example, multicellularity appears to have evolved in the green algae alone at least 25 times (Umen and Herron, 2021), and complex multicellularity appears to have convergently evolved from simple multicellular ancestors in the fungi 8-11 times using the same suites of genes (Nagy et al., 2018). How and when has cellular differentiation evolved in different lineages (e.g., see Hammerschmidt et al. (2021) for an excellent discussion on the history of phylogenetic inference into the tempo and mode of multicellular evolution within the cyanobacteria)? Which phylum is sister to the remaining animals, sponges or ctenophores (Jékely and Budd, 2021)?

We still have much to learn about the natural diversity of less ecologically-dominant multicellular taxa. For example, recent work has shown that choanoflagellates, the closest known relative of animals, are capable of forming epithelia-like sheets of cells and can regulate phototactic swimming by manipulating the shape of their sheet-like bodies (Brunet et al., 2019). Another choanoflagellate species forms hollow spherical groups that contain a microbiome (Hake et al., 2021). Work with non-Metazoan Holozoans shows they are capable of expressing a remarkable diversity of cellular phenotypes (*i.e.*, flagellated cells, amoeboid cells, cysts and coenocyes) through time (Sebe-Pedros et al., 2017), lending support to the hypothesis that temporal cellular differentiation may have been co-opted for spatial cellular differentiation in animals (Brunet and King, 2017), as it likely was in the volvocine algae (Nedelcu and Michod, 2006). Within fungi, recent work has shown that large, complex multicellular structures do not necessarily require large genomes (Nguyen et al., 2017). It is clear that we are only beginning to appreciate the diversity of multicellular life on Earth, particularly among the 'minor' multicellular taxa (Herron et al., 2013).

Improved sampling of extant multicellular organisms will provide deeper insight into the manifold routes, mechanisms, and constraints on this major transition. In particular, it will be helpful to know how much the cell biology of the unicellular ancestor dictates the way in which multicellularity subsequently evolves. For example, we still don't have a widely-accepted explanation for why complex multicellularity has only evolved in eukaryotes (which is especially surprising given that bacterial multicellularity had a ~600 million to billion year head start (Bengtson et al., 2017; Gibson et al., 2018; Schirrmeister et al., 2015)), though hypotheses linking eukaryotic transcriptional regulation to complex multicellularity have been proposed (de Mendoza et al., 2013; Petroll et al., 2021; Tarver et al., 2015). Groundbreaking work has shown that genes put to extensive use in multicellular processes (*e.g.*, cellular adhesion, development, and cancer suppression) often predate this transition (King, 2004; Nedelcu and Michod, 2006;

Rokas, 2008; Ruiz-Trillo et al., 2008), suggesting that they were co-opted for novel multicellular use. Improved taxonomic sampling will refine our ability to infer how, when, and why genes have been co-opted for multicellular functionality, and how important this process is for the evolution of novel multicellular traits.

18.4. Life cycles: an organizing principle for multicellular origins

All multicellular organisms possess a life cycle, which characterizes their growth and reproduction. As Merljin Staps, Jordi van Gestel, and Corina Tarnita explain in Chapter 4, life cycles are a foundational concept for the origin of multicellularity, because they describe the manner in which groups of cells are generated and reproduce themselves (Bonner, 1965; Buss, 1987). Such group-level reproduction is an essential component in multicellular groups becoming Darwinian entities (Rainey and Kerr, 2010) and variation in the life cycle (*i.e.,*, how they partition genetic variation among multicellular groups) has profound implications for the subsequent evolution of multicellularity (Hammerschmidt et al., 2014; Ratcliff et al., 2017; Staps et al., 2019).

Despite the centrality of life cycles for this major evolutionary transition, we know relatively little about how they arise. Experimental evolution with yeast (Koschwanez et al., 2013; Ratcliff et al., 2012) and green algae (Herron et al., 2019; Ratcliff et al., 2013) shows that life cycles can arise through growth followed by physical fracture. In both of these examples, the emergent life cycles can include unicellular genetic bottlenecks, efficiently partitioning genetic variation between groups. Ecology itself can act as a 'scaffold' (Black et al., 2020)–creating an environment that favors a multicellular phase (*i.e.*, a biofilm) followed by a unicellular phase (Hammerschmidt et al., 2014). Over time, the cycling between unicellular and multicellular states could be brought under developmental control (Black et al., 2020). Alternatively, life cycles may arise due to environment-specific cues (*e.g.*, expression of aggregative proteins during starvation) (Dworkin, 1963; Mahadeo and Parent, 2006). Despite the plausibility of these different routes, we do not yet have a general theory for how life cycles arise, nor have we resolved the ancestral life cycle state of all extant clades of multicellular organisms. Further, we know little about how, when and why developmental processes modify multicellular life cycles, taking them from simple, stochastic beginnings to the robust and highly-regulated processes that characterize most extant multicellular organisms.

The above framework raises an intriguing, and largely unexplored hypothesis: the ecology of early multicellular life cycles may constrain the types of multicellularity that ultimately evolve (Pichugin and Traulsen, 2020). Some early multicellular life cycles provide a clear ecological advantage—for example, single cells aggregating to form groups when stressed. Such a behavior can provide group survival benefits (Smukalla et al., 2008), and may limit the costs associated with multicellularity (*i.e.*, resource diffusion slowing growth (Pentz et al., 2020), or limited dispersal (Queller and Strassmann, 2014)). Indeed, simple environmentally-dependent aggregation has evolved many times among otherwise unicellular organisms, leading to the ubiquity of microbial biofilms (Flemming and Wuertz, 2019), though in some cases this simple life cycle has served as the basis for more significant multicellular innovation (see Chapters 5-8). Might we be missing modes of multicellularity simply because those early life cycles were not ecologically advantageous enough to persist? Future work will be necessary to disentangle this effect from the downstream evolutionary consequences of how these life cycles affect the evolution of multicellularity itself.

18.5. The critical role of organismal size

As John Tyler Bonner made clear (Bonner, 2006), size is a universally-important trait for multicellular organisms. The benefits of multicellularity stem in part from advantages of size derived from group formation, and many multicellular lineages have undergone selection to form larger, more mechanically-robust multicellular bodies at some point in their evolutionary history. A number of key questions remain unanswered regarding the evolution of larger size in early multicellular organisms, however.

Biophysically, how do larger organisms evolve? This is not a trivial question: multicellular organisms face biophysical stresses that act over evolutionarily novel length scales, and we have no reason to believe that early multicellular bodies are particularly robust. Cells growing within groups face a challenge–as cells divide, they exert strain on neighboring cells, which accumulates until it causes the group to fracture (Jacobeen et al., 2018). In order avoid fragmenting, organisms growing in groups must either stop dividing, reduce the accumulation of cell-cell strain, or evolve to tolerate this strain by becoming tougher. Extant organisms do all three. For example, aggregative organisms like *Dictylostelium discoideum* typically do not divide in the multicellular phase, which is induced by starvation (Jang and Gomer, 2011). In some clonal multicellular organisms, such as plants, growth is often developmentally regulated to limit strain accumulation by producing new cells in parallel sheets (Jackson et al., 2017), or incorporating information about packing-induced strain into cellular division planes (Dupuy et al., 2010). Other mechanisms for increasing multicellular toughness include cell-cell adhesion (Abedin and King, 2010; El-Kirat-Chatel et al., 2015) and the entanglement of filamentous biological materials (Zou et al., 2009). We know relatively little about how biophysical toughness evolves prior to the evolution of developmental systems that coordinate strain reduction and increased toughness. Further work should examine the biophysical organizing principles for multicellularity and assess how this constrains the types of multicellular organisms that can evolve.

Is selection for larger size itself a driver of increased complexity? Size is costly, as it reduces access to extracellular resources (*i.e.*, food and oxygen), providing a selective incentive to evolve traits that overcome these limitations (Bonner, 2006; Knoll, 2011). Examples of such traits include circulatory systems, low surface-area to volume morphologies, and even oxygen-binding proteins like myoglobin. Many of these require the evolution of increased developmental regulation and multicellular integration. Despite the conceptual strength of this hypothesis, however, it has yet to be directly tested.

18.6. Origins of multicellular development

Few topics are more important for the evolution of complex multicellular life than the origin of multicellular development. Developmental regulation of cells within a multicellular group (defined as the genetic, bioelectric, and biophysical mechanisms that allow cells to perform spatially and temporally-explicit behaviors) is required for the expression of any reasonably sophisticated multicellular structure, yet we still know little about how development evolves *de novo.*

One school of thought argues that development is an outcome of multicellular adaptation. Whether this occurs via the rewiring of phenotypic variation that was expressed through time in the unicellular ancestor (*e.g.,* flagellar swimming, amoebal crawling, etc.) to be expressed in a spatially-dependent manner (Brunet and King, 2017; Mikhailov et al., 2009; Sebe-Pedros et al.,

2017), or the *de novo* evolution of cellular differentiation (Arendt, 2008). Alternatively, Stuart Newman and Ramray Bhat have proposed that the initial steps of development may have been non adaptive. Specifically, they hypothesize that dynamical patterning modules may initially have arisen through the interactions of cellular behaviors and biophysical mechanisms that would have had novel developmental consequences once in a multicellular context. These could then have been refined by selection for improved multicellular functionality (Newman and Bhat, 2009).

Future work resolving how development has evolved in disparate lineages, and how it can evolve from scratch in initially undifferentiated groups of cells, will be critical for developing a comprehensive understanding of this process. Major questions remain: how critical is the evolution of the life cycle to the origin and ultimate evolution of development (Fortezza et al., 2021)? How and when will cell-cell communication be important during development? What is the relative importance of co-opting ancestral plasticity in cellular phenotype vs. evolving plasticity *de novo*? This work will require understanding not just the selective advantages of development, but also the mechanisms through which it arises. This research area will benefit greatly from interdisciplinary collaboration among theorists, natural historians, and groups leveraging emerging tools in synthetic biology / experimental evolution to directly test hypotheses.

18.7. Environmental drivers and niche construction

One of the most fundamental lines of inquiry in the field of multicellularity concerns when and why multicellularity evolved on Earth (Knoll, 2011). This is critically important for developing a robust understanding of the environmental drivers of simple multicellularity and the subsequent evolution of more complex lineages. This is a challenging research topic, because these transitions occurred in the deep past, and early multicellular lineages tend not to be well preserved in the fossil record. Even with exceptional fossils (like those from the Doushantuo Formation, where even cellular organelles remain visible (Sun et al., 2020)) it can be difficult to place the fossils into phylogenetic context (Chen et al., 2014). Future work will be crucial for increasing not just the sampling resolution of ancient multicellular lineages, but resolving their broader Earth context, their local environment, and their biotic interactions. The latter may be especially important for resolving how organismal interactions (*e.g.*, arms races, co-evolution) underlie the evolution of increased multicellular complexity (Sperling et al., 2013), and subsequently impact biogeochemical processes (Butterfield, 2018).

It is clear that multicellularity has fundamentally transformed Earth's surface and biogeochemical processes–for instance, plants alone account for approximately 80% of the biomass of all life on Earth by one estimate (Bar-On et al., 2018). Despite recent progress, we do not fully understand how multicellular organisms have affected the evolution of Earth's biogeochemical cycles and climate, or how multicellular niche construction has affected the subsequent evolution of multicellularity. For example, we often define our biomes by the dominant multicellular taxa in that environment (*e.g.,* forests, grasslands, coral reefs, kelp forests), and nearly all of the organisms in these environments are there because of the niches created, directly or indirectly, by the dominant multicellular taxa. Plants give rise to herbivores and pollinators, which in turn give rise to predators, predators of the predators, parasites of all of these, hyperparasites, predators of the hyperparasites, and so on. Clearly, the evolution of multicellularity depends on the prior evolution of multicellularity- but how, why, and are these principles general or lineage-specific?

Finally, it's unclear to what extent Earth's environment has constrained the evolution of multicellularity. For example, how has environmental oxygen affected the evolution of multicellular size (Bozdag et al., 2021; Cole et al., 2020)? Prior to the rise of near modern oxygen levels in the Phanerozoic (Lyons et al., 2014), did low ocean/atmospheric oxygen constrain the evolution of tissues more than a few cells thick? How might environmental constraints interact with the origin of life cycles, the environment in which multicellular organisms could persist (Turner, 2021), the topology of multicellular groups (Yanni et al., 2020), and the subsequent evolution of multicellular development?

18.8. Astrobiology: the ultimate generalization

A comprehensive understanding of how, when, and why multicellularity has evolved on Earth should provide insight into a fundamental question in astrobiology: assuming cellular life has independently evolved elsewhere in the Universe, what is the probability that it would evolve to be multicellular? This is not quite the f_i of the Drake equation ("the fraction of planets with life that actually go on to develop intelligent life (civilizations)"), but it is probably a factor contributing to f_i, unless we are imagining intelligent unicells (as some science fiction authors have done).

What biotic and abiotic factors are critical for the initial transition to multicellularity and the subsequent evolution of complex multicellularity, and how might these apply to other planetary contexts? Is this process necessarily slow, or can it occur quickly given the right conditions? Understanding the evolution of multicellularity on Earth, and in particular developing robust theory from evolutionary first principles, should allow us to make strong astrobiological hypotheses. In addition to its importance for the field of astrobiology, this has deep significance for humanity, helping address a fundamental existential question: are we alone, or is sentient multicellular life cosmologically common?

18.9. Key methods

Understanding the evolution of multicellularity will take a plurality of methods and disciplines. Here we highlight some of the key approaches that will be crucial for research progress. These approaches can be roughly classified in three broad categories: comparative, experimental, and theoretical. Although there is some overlap, and although other classifications are certainly possible, we think this is a reasonable first pass at making sense of the diversity of methods.

Comparative methods often involve describing the differences between multicellular organisms and their closest unicellular relatives. Differences between the two must have evolved since they diverged from a (presumably unicellular) ancestor, and some of these differences will be causes and consequences of the transition to multicellularity. We are employing a broad definition of comparative methods, to include comparisons of morphology, physiology, biochemistry, genetics, and so on.

Of course, such comparisons are only meaningful when we understand the evolutionary relationships among the organisms we're comparing, so accurate phylogenies are crucial. There is simply no substitute for knowing how and when multicellularity evolved in extant lineages. As we describe in section 18.3, improved phylogenetic inferences have often led to new insights into the evolution of multicellularity.

Paleontology and Earth science play critical roles in ground-truthing comparative methods. Fossils are indispensable in calibrating divergence time estimates in phylogenies, and they

provide a unique snapshot into the phenotype of early multicellular life. Earth system science (i.e., isotope geochemistry) will allow for more precise inference of the environmental and planetary contexts of early multicellular evolution.

Although comparative methods are powerful tools for understanding the evolution of multicellularity, they do have some important limitations. In most cases, the closest unicellular relatives of extant multicellular organisms diverged from them hundreds of millions of years ago, and subsequent, uncharacterized changes in the unicellular lineage limit their utility as stand-ins for the unicellular ancestor. Experimental / directed evolution offers the unique opportunity to examine the origin of multicellularity directly and has been recently applied to fungi (Ratcliff et al., 2012), bacteria (Hammerschmidt et al., 2014), algae (Boraas et al., 1998; Herron et al., 2019; Ratcliff et al., 2013), and ichthyosporea (Dudin et al., 2021). In addition to examining how simple multicellularity can evolve in various lineages, this method can provide insight into how increasingly complex multicellularity evolves, and allows one to directly test hypotheses that are otherwise experimentally intractable (*e.g.*, how early life cycles or environmental conditions affect the evolution of multicellularity (Bozdag et al., 2021)). The trade-off for this approach is that it sacrifices relevance to particular historical origins of multicellularity for tractability and (possibly) generality. It can, in other words, tell us about how multicellularity can evolve, but not about how it did evolve.

Other experimental approaches lie at different points along this continuum. Some, for example detailed studies of genetic and developmental mechanisms, synergize well with comparative methods by fleshing out the differences between unicellular and multicellular organisms. Others, for example biophysics, may derive general principles that should be relevant to many or all origins of multicellularity.

Another promising experimental approach is synthetic biology. Despite its potential as a sandbox allowing virtually unlimited experimental possibilities, relatively little work has leveraged synthetic approaches to study multicellularity (Basu et al., 2005; Solé et al., 2018; Toda et al., 2018). We expect that this will change as methods for gene editing become increasingly routine in non-model organisms (Booth and King, 2020), and as techniques for engineering organoids allow for increasingly precise control on *in vitro* morphogenesis (Hofer and Lutolf, 2021).

Theory plays a key role in multicellularity research, complementing natural history (which tells us what has happened in this one run of Earth's timeline) and experiments with organisms (which let us test various hypotheses about when, why and how these things happened). Theory thus provides a formal method for summarizing our knowledge about evolutionary processes, allowing hypotheses to be clearly defined and subsequently tested. By the same token, theory and computation allow us to explore the evolution of multicellularity in a way that is unencumbered by the historical contingencies that are otherwise an unimpeachable constraint of real organisms living in a specific environment. Computational and theoretical approaches are thus an invaluable tool for exploration, hypothesis generation, and the identification and formulation of general principles.

One theoretical approach that has been crucial for understanding the evolution of multicellularity is that of multilevel selection. In Chapter 3, Rick Michod reviews the development of multilevel selection theory, especially as it applies to the evolution of multicellularity and of cellular differentiation, including his own considerable contributions to this application. Among the most influential of these is a collection of population genetic models based on life-history tradeoffs

that address the evolution of a division of labor among cells, or cellular differentiation. In Chapter 12, Guilhem Doulcier, Katrin Hammerschmidt, and Pierrick Bourrat analyze the life history model and its underlying assumptions, limitations, and interpretation as a critical step in the transformation of cell groups into multicellular organisms.

Just as the origins of multicellularity in plants, animals, and other taxa are particular cases of a broader category (but see Chapter 2), the evolution of multicellularity writ large is a particular case of the broader category of "major transitions" in evolution (Maynard Smith and Szathmáry, 1995) or evolutionary transitions in individuality (Michod and Roze, 1997). Much of the theory that has been developed for the evolution of multicellularity can be applied more broadly to the larger category, though just how much of the theory can be so applied, and to which transitions, are very much open questions (Calcott and Sterelny, 2011; Herron, 2021; McShea and Simpson, 2011; O'Malley and Powell, 2016).

Comparative, experimental, and theoretical approaches can all benefit from the development of new model systems. Model systems of extant multicellular organisms, such as *Dictylostelium discoideum, Myxococcus xanthus, Neurospora crassa, Volvox carteri*, and *Salpingoeca rosetta* have been crucial for generating and testing hypotheses about the evolution of multicellularity. Given how contingent each of these model systems is (each species is nested within a lineage that has independently evolved multicellularity in largely idiosyncratic ways), new model systems will significantly expand our suite of experimental possibilities.

18.10. Conclusion

There has never been a better time to work on the evolution of multicellularity. Rapid advances in life sciences technology have opened new avenues for research that would have been unimaginable a generation ago, and will no doubt continue to revolutionize progress. And, despite the deep history of the field, it has remained a small enough niche that major conceptual breakthroughs are not only possible, but are a regular occurrence.

David Kirk (1998), quoting Jerome Gross, advised that the best path to a happy and productive scientific career is to find "a quiet backwater where there are lots of big fish to be caught, but not many people fishing." The evolution of multicellularity is much less of a backwater today than it was when Kirk wrote that, but it is still a relatively small pond, and we are convinced that there are indeed still big fish to be caught. We hope that this book has been useful to you, and we invite readers to reach out to any of the authors if you have questions about the evolution of multicellularity or are interested in wetting a line.

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