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24. ORIGINS OF ORGANISMAL COMPLEXITY

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In the antecedent to this book, a case was made for the emergence of genome complexity as a passive response to mutational bias, the limited reach of natural selection, and lineage-specific differences in the power of genetic drift (Lynch 2007). This left open the question as to whether differences at the next highest level of organization – the cell – might also be a product of effectively neutral processes. One might anticipate that the higher the level of organization and the closer to outward phenotypic expression, the more likely it is that natural selection will be fully responsible for patterns of variation. Some would even argue that none of the internal molecular and cellular details matter, but the observations summarized on the preceding pages make clear that this is not the case. What follows is a synthesis of observations from comparative cell biology and evolutionary theory relevant to the matter of the emergence of cellular and organismal complexity.

A broad array of observations on the biology of unicellular prokaryotes and eukaryotes have been assembled on the preceding pages to help make sense of patterns of variation in light of basic evolutionary principles. Ideally, they will have also made clear that evolutionary biology is more than the century-old tradition of applying comparative analyses to phylogenetic trees and concocting adaptive hypotheses for the observed patterns. Understanding the mechanisms by which evolution proceeds requires an appreciation of the functional constraints on cells imposed by the chance historical roots of biology's invariants (e.g., the dependence on DNA-based genomes, RNA-based transcriptomes, and protein- and lipid-based infrastructures). Just as the design of a successful gourmet menu in the absence of an understanding of the potential ingredients is unlikely, a mechanistic understanding of evolution is inconceivable without information on its material basis.

Were life given the chance to start anew, genomes and external membranes would likely evolve. However, the underlying elemental features might be entirely different from the particular hand dealt to biology. The biochemical, bioenergetic, and biophysical properties of life's cellular components ultimately define the limited ways by which mutational processes can introduce variation into populations.

At the time of Wallace and Darwin, essentially nothing was known about genetics or the molecular constituents of cells, removing all barriers to thinking that natural selection is essentially all powerful. However, we now know that the need to expand evolutionary biology beyond a pan-adaptationist framework is deeply rooted in biological reality. Mutations are particulate in nature, with effects that are typically small and deleterious, and the transmission of alleles across generations is a stochastic process, with the role of chance in inheritance varying by several orders

of magnitude across phylogenetic lineages. Every genomic nucleotide site in every species is subject to mutational change and to the vagaries of genetic drift, but genomes are also finite in size, and these matters along with issues such as recombination rates and nonadditive gene interactions dictate the kinds of evolutionary pathways that are open to exploitation by natural selection.

Most notably, the capacity of natural selection to use genetic fuel to drive the expansion of biological diversity has significant bounds dictated by extrinsic factors such as population size. Not all mutations are available for adaptive evolutionary change, as the effective size of a population dictates the granularity of mutational effects that are visible to the eyes of natural selection. In small populations, adaptive change can only proceed by use of beneficial mutations of relatively large effects, and mildly deleterious mutations cannot be purged. In contrast, evolution in large populations is much more fine-tuned. This means that even in the ideal case in which mutation and selection pressures operate in identical ways, phylogenetic lineages are expected to vary in their response to selection, simply as a consequence of variation in the power of random genetic drift, which will not only alter modify the availability of existing variation but will, through history, have defined the genomic architectures from which mutational changes can be built.

Given various assumed selection coefficients, mutation and recombination rates, and aspects of population demography, evolutionary-genetics theory has been very good at defining how changes are expected to proceed in a generic way, usually without any explicit reference to phenotypes. The next step is to elucidate from first principles the connections between molecular/cell-level features and their selective consequences. The critical importance of cell biology to evolutionary biology resides in the exacting details by which the functional links between genotypes and phenotypic effects can be defined, an essential step in the development of a more mature science of evolutionary biology. This being said, although more than one physicist has claimed that all of biology is physics, the goal of this final chapter is to highlight why biophysics, biochemistry, and cell biology alone will never be sufficient to understand evolutionary processes.

Deconstructing the Great Chain of Being

For those who know the difference, it is commonly believed that eukaryotes are superior life forms to prokaryotes, that animals represent the pinnacle of the evolutionary process within eukaryotes, and that vertebrates occupy a higher rung on the ladder of superiority than invertebrates, with humans highest of all. For example, the implication one acquires from a reading of Lane and Martin (2010) is that prokaryotes are condemned to a pathetic and perpetual fate of simplicity in form and functions owing only to the absence of a mitochondrion.

This line of thinking might work well if there was justification for the medieval "Great Chain of Being" view of life, but there is no evidence that the ultimate goal of natural selection is to build bigger, bonier, and more complex organisms. Humans are better at being human than are bacteria, and bacteria are better at carrying out microbial functions than vertebrates, but one cannot directly compare the genetic fitnesses of reproductively isolated organisms, as evolutionary fitness is a function

of the relative transmission rate of genomic material within a cohesive population of compatible individuals. Nonetheless, where performances can be compared in an absolute sense, simpler organisms generally come out on top.

For example, heterotrophic bacteria, particularly large-celled species, are capable of assimilating biomass at substantially higher rates than any of the morphologically more complex unicellular eukaryotes of comparable size (Lynch et al. 2021; Chapter 8). The highest rate of cell division in the smallest unicellular eukaryotes is nearly an order of magnitude less than that in the largest bacteria, and the eukaryotic rate declines with increasing cell size. Notably, this ability of prokaryotes to produce daughter cells at higher rates than eukaryotes is accomplished without subsidizing a vast intracellular network of specialized organelles. It is also done with fewer and smaller proteins, which on average appear to have higher catalytic efficiencies and higher accuracy of substrate utilization in bacteria (Chapters 4 and 12). Thus, by all accounts, given identical metabolic tasks, prokaryotes are just as good if not better than their eukaryotic counterparts. Moreover, in multicellular eukaryotes, the maximum mass-specific growth rate (at any life stage) continues to decline with increasing size at maturity, such that the highest growth rates achievable by animals and land plants are one to two orders of magnitude below those for unicellular heterotrophs and phototrophs, respectively (Figure 24.1).

As in the case of reduced genome replication fidelity in organisms of increasing size (Chapter 4), this gradient in declining maximum growth rates may be a consequence of the accumulation of mildly deleterious growth-reducing mutations in small N_e species (Lynch et al. 2021). Particularly noteworthy in Figure 24.1 are the approximately -0.20 and -0.10 power-law scaling relationships for heterotrophs and autotrophs. As noted in Chapter 8, there has been an on-going debate as to whether mass-specific metabolic rate scales with the -0.25 or -0.33 power, but the observations for maximum growth rate are inconsistent with both hypotheses. One might imagine that biophysical constraints would set the baseline pattern, with deleterious-mutation accumulation then increasingly diminishing the capacity of larger organisms (with smaller N_e) relative to the biophysics barrier. However, this would lead to a steeper slope than the biophysical expectations, contrary to what is actually seen.

If the drift-barrier hypothesis is the correct explanation for the patterns observed in Figure 24.1, the maintenance of a constant power-law relationship across a wide range of body sizes demands a particular distribution of growth-altering effects of mutations – for each proportional increase in organism size, there must be a constant proportional increase in the total fixed deleterious-mutation load. Because N_e declines with increasing organism size (Figure 4.3), this means that the magnitude of a deleterious mutational effect capable of avoiding selective elimination must also increase with organism size. Thus, to keep the increment in load constant, the number of genomic sites with a particular effect must be inversely proportional to the effect size, as the product of the two is the total load.

An unfortunate aspect of this effect-scaling expectation is that direct empirical quantification of very small mutational effects is currently beyond reach. However, observational limits are not grounds for dismissing the legitimacy of a hypothesis. Numerous areas of science, including particle physics, astrophysics, and even bioenergetics, have advanced significantly by building around theoretical constructs that

required decades of work by hundreds of investigators and enormous funding to achieve validation with direct observations.

Genome complexity and organismal complexity. Three decades of whole-genome sequencing have revealed clear phylogenetic patterns of genome structure and organization (Lynch 2007). Rarely exceeding 10 Mb in length, prokaryotic genomes typically contain 1000 to 8000 protein-coding genes, and these generally comprise $> 95\%$ of the entire genome, with slightly less than 1 kb of genomic space allocated per gene (Figure 24.3). Introns (intergenic DNAs that must be spliced out of transcripts to achieve productive messenger RNAs) are absent from prokaryotic genes, and gene regulation is often simplified to the point that multiple genes are coordinately expressed from the same operons. In contrast, eukaryotic genomes are rarely smaller than 8 Mb, with unicellular species commonly having genomes in the range of 20 to 100 Mb, and metazoan and land-plant genomes often expanding beyond 1 Gb.

This vast expansion of genome size from prokaryotes to complex multicellular eukaryotes is largely a consequence of the increase of intergenic DNA caused by the proliferation of multiple classes of mobile-genetic elements (essentially parasitic DNAs) and the colonization of protein-coding genes by introns. Less pronounced is the expansion of gene number in eukaryotes, which except in extreme cases of recent polyploidy, typically sum to $< 50,000$, and quite often to $< 20,000$. The smallest eukaryotic genomes are quite similar in nature to those in prokaryotes, being devoid of mobile elements and generally highly depauperate in intronic DNA. In contrast, in the enormously bloated genomes of animals and land plants, $< 2\%$ of DNA is associated with protein coding, and in the extreme cases, each gene is separated by ~ 100 kb.

In terms of their origin, these massive changes in gene and genome organization across the Tree of Life have little to do with adaptive evolutionary differentiation. For example, mobile-genetic elements are a burden to host cells for two reasons: 1) each such element typically spans a few hundred to a few thousand base-pairs, thereby imposing an energetic cost in terms of excess DNA synthesis; and 2) the random insertion of offspring elements into novel genomic sites often has added negative consequences for host-cell fitness as a consequence of gene disruption. Consistent with this view, despite having no innate immunity to mobile-element invasion, the genomes of microbial eukaryotes with relatively large effective population sizes are generally nearly devoid of such elements (as is the case in prokaryotes); such insertions are simply kept rare in these species by purifying natural selection. Likewise, introns, which can exceed 10 kb in length in multicellular species, are an energetic burden at both the DNA and RNA levels, and also impose upon their host genes an elevated rate of mutation to defective alleles owing to the need to maintain specific sequences at key nucleotide sites involved in splice-site recognition.

Thus, the overall message from comparative genomics is that the quantum leap in morphological complexity of eukaryotic cells is accompanied by an expansion of genome and gene-architectural complexity but only by a moderate increase in gene number. Notably, the smallest eukaryotic genomes contain fewer genes than bacterial genomes of comparable size (Figure 24.3), demonstrating that an expanded gene set is not a requirement for the development of a eukaryotic cell plan. This

upholds the the hypothesis that rather than being essential causal determinants of the emergence of cellular complexity, most of the changes in eukaryotic genome organization are simple by-products of the shift in the population-genetic environment permissive to the expansion of noncoding DNA. Recall from Chapter 17 that based on the energetic costs of DNA synthesis, species with small cells and large effective population sizes are able to purge insertions of nonfunctional DNA as small as a few base pairs by natural selection, whereas in large eukaryotic species, natural selection is essentially unaware of the relative energetic costs of insertions as large as several kb.

A final point of emphasis here is that the current function of a cellular feature and the forces of evolution operating on it may have little to do with its origin. For example, introns enable multicellular species to engage in tissue-specific alternative splicing of precursor messenger RNAs, thereby increasing proteome diversity without adding more genes. However, introns were present in large numbers in LECA, well before the emergence of multicellularity. Similar arguments can be made with respect to the increase of intergenic DNA as a substrate for the development of novel mechanisms of gene regulation (Lynch 2007). Thus, an expansion of genome size in the ancestral eukaryote was not promoted because of its immediate utility, and indeed resulted despite intrinsic disadvantages, but in the process set the stage for later adaptive exploitation by descent with modification. These observations again provide compelling support for the strong role of historical contingency in guiding the future paths of cellular evolution.

A shake-up of genomic organization in the ancestral eukaryote. Part of the expansion of eukaryotic gene number was a consequence of mitochondrial-to-nuclear genome transfer (Chapter 23), although other additions came from duplications within the nuclear genome itself (Lynch 2007). Incremental single-gene duplication is an on-going process in all genomes, with rates per gene often of the same magnitude as base-substitution mutation rates per nucleotide site, i.e., in the range of 10^{-9} to 10^{-8}/gene copy/generation. However, such processes are typically balanced in the long run by an approximately equal rate of gene loss, and there is no evidence of an inexorable climb in gene number in any domain of life. There are, nonetheless, occasional episodes of massive genome expansion with more permanent effects. For example, many whole-genome duplication events have been recorded in lineages of plants, animals, budding yeasts, ciliates, and many other eukaryotes, although not within prokaryotes.

Of special note here is a significant period of genome expansion on the road from FECA to LECA, leading to an addition of several thousand genes. Given that a substantial fraction of duplicate genes is not permanently retained (Chapter 6), the initial burst in gene number likely exceeded 10,000, with some eukaryotic lineages then losing more individual duplicates than others. One of the most pronounced lines of evidence for a basal expansion of eukaryotic gene number draws from the increased complexity of multimeric proteins in this lineage. Virtually every aspect of eukaryotic cell biology reveals the use of heteromeric protein complexes (with the component parts encoded by different genes) whose orthologs in prokaryotes are homomeric (with all subunits encoded by the same genetic locus). A large fraction of these changes occurred in the stem eukaryote, with the added subcomponents having

been derived by gene duplication. Already discussed in detail in prior chapters, just a few examples will be summarized here.

First, as outlined in Chapter 10, many of the homomeric proteins involved in prokaryotic maintenance of chromosome integrity and replication have eukaryotic orthologs involved in mitosis and meiosis that assemble into heteromers from subunits encoded by duplicated genes. The existence of parallel and/or ancestral duplications in archaeal orthologs implies that the ancestral eukaryote (likely a member of the archaea) may have been endowed with such features from the outset (Makarova and Koonin 2013). Second, numerous proteins involved in RNA processing exhibit a similar syndrome. Consider, for example, the Sm family of proteins, which are involved in the processing of single-stranded RNAs, including forming the core of the eukaryotic spliceosome that removes introns from transcripts. Whereas bacterial Sm proteins form 6- or 7-subunit homomeric rings, they are fully heteromeric in eukaryotes, with each subunit encoded by a different genetic locus (Scofield and Lynch 2008). Third, many of the key molecular machines involved in protein surveillance and processing, including chaperones, the proteasome, and the exosome, obtained their heteromeric structures in the pre-LECA era (Chapter 14). Fourth, the guardian of the nuclear environment, the nuclear-pore complex, consists of a layered series of duplications, and this is also true of nearly every aspect of the eukaryotic vesicle transport system (Chapter 15). Finally, the α and β subunits of structural tubulin filaments emerged prior to LECA, as did the δ and ϵ subunits deployed in the eukaryotic flagellum (Chapter 16).

Although many more examples could be given, these kinds of observations suffice to reveal that there was a substantial amount of gene duplication in the stem eukaryote, with an especially significant amount of duplicate preservation associated with the structural features of protein complexes. Given the frequency with which whole-genome duplications occur in modern-day lineages of eukaryotes, the possibility that one or more of such events precipitated this expansion in genome size in the ancestral eukaryote cannot be ruled out (Makarova et al. 2005; Zhou et al. 2010). However, a formal evaluation of the matter is made difficult by secondary chromosome rearrangements and removals of large numbers of duplicates over the vast reach of time since the origin of eukaryotes.

Whatever the mechanism – one or two whole-genome duplications or cumulative duplications of smaller chromosomal regions, Marakova et al. (2005) suggest that the basal increase in eukaryotic gene number may have been precipitated by a cataclysmic event inducing a sudden and prolonged reduction in population size. Part of the rationale for this argument is the fact that the preservation of duplicate genes by subfunctionalization (as opposed to neofunctionalization) is facilitated in populations of small size. Recall from Chapter 6 that subfunctionalization is a process by which duplicate genes are preserved by the complementary loss of key subfunctions. Unless there is a resolution of an adaptive conflict, no fitness gain results from such a process. Indeed, there are weak bioenergetic and mutational costs of relying on duplicated genes relative to single-copy genes with the same functions, which are too small to be affected by purifying selection in sufficiently small populations.

Was this expansion of the complexity of key molecular machines of any relevance to the establishment of the altered eukaryotic cell plan or to the subsequent diversification of eukaryotes into morphologically diverse descendent lineages? Evidence

that increases in multimeric complexity endow organisms with superior molecular performance or with a dramatic shift in function or diversity of functions has been elusive (Chapter 13). On the other hand, as outlined in Chapter 6, the differential loss of duplicate genes in parallel lineages can passively lead to genetic map changes that manifest as post-reproductive isolating between sister taxa, thereby establishing novel and independent lineages. This again illustrates the point that cellular modifications arising entirely by nonadaptive mechanisms may have had a foundational role in channeling downstream evolutionary pathways.

Multicellularity

Under the common belief that organismal complexity represents adaptive progress, the evolution of multicellularity is often viewed as the ultimate goal of natural selection. In this view, single-celled lineages (comprising the vast majority of the Tree of Life) are condemned to a perpetual state of simplicity by the lack of one or more critical ingredients, such as the mitochondrion, rather than by a lack of selective incentive. For this reason, the evolution of multicellularity is often touted as a "major transition" in the history of life (Maynard Smith and Szathmáry 1995), the iconic forms being metazoans and land plants, made not just of multiple cells but multiple cell types. It should be remembered, however, that complex multicellularity with multiple cell types has arisen many times across the eukaryotic Tree of Life (Sebé-Pedrós et al. 2017), including on multiple occasions in fungi, red and green algae, slime molds, ciliates, and as noted immediately below, even in bacteria. Moreover, this broad phylogenetic distribution still understates the ease with which multicellularity can evolve.

Under appropriate laboratory settings on short time scales, it is not difficult to coax multicellularity out of simple cultures of unicellular organisms (Hammerschmidt et al. 2014). This is most dramatically illustrated by an experiment that self-selected for aggregations of *S. cerevisiae* cells, so-called snowflake colonies that rapidly settle in test-tube environments (Ratcliff and Travisano 2014; Ratcliff et al. 2015). In parallel experiments, the initial transition to snowflake form resulted from a mutation in a single gene preventing mother-daughter cell separation. As discussed below, a persistent challenge in the establishment of multicellularity is the emergence of cheater genotypes that harvest the benefits of group living without paying a price incurred by other group members (e.g., the release of group-beneficial products). In this particular system, however, genetic conflict is thwarted, as selection favors large colonies produced by fragmentation, eliminating the opportunities for transmission of cheaters that fail to engage in aggregation.

In situations like this, as long as the ecological conditions selecting for multicellularity remain, secondary genomic changes that refine such a state will be further enforced, even if contrary to the interests of the individual, although as noted below, it helps if the group members are close relatives. In this process, the colony (rather than the individual cell) becomes the unit of selection, and even with snowflake yeast, a crude form of cellular differentiation emerges, including apoptosis (suicide) of central cells, which although disadvantageous to the individual cell is essential to offspring colony production. In effect, the colony becomes an individual.

Engineering a more contrived system, Wahl and Murray (2016) developed another form of multicellular yeast with somatic differentiation. In this case, a construct was engineered to enable the production of two cell types, one from the other. Upon chemical induction, a faster-growing "germ cell" gave rise to a slower growing "somatic" cell type that secreted into the medium invertase. This enzyme then hydrolyzed nonutilizable sucrose into fructose and glucose molecules essential for the growth of the germ cells. Multicellularity was maintained in this system because cheating cells rapidly segregate into cheating-only colonies, with reduced reproductive rates.

These two examples clearly show that the key issue with respect to the evolution of complex multicellularity is not an intrinsic barrier to initial emergence. Rather, multicellularity is typically held back by the limited ecological opportunities that encourage such body plans, by the investment costs imposed by larger somas, and by the presence of internal threats that can thwart persistence after initial establishment. Before addressing these issues in more detail for eukaryotes, a brief consideration of bacteria will make clear that multicellularity is by no means restricted to the eukaryotic domain.

Multicellularity and cooperativity in bacteria. Although they have not gone to the extremes of metazoans and land plants, prokaryotes are by no means immune to evolving physical or behavioral consortia of mutually beneficial cells with specialized functions (Claessen et al. 2014). In effect, the individual cells of such collectives are similar to those in asexually propagating, multicellular eukaryotes, in that cell fitness is a function of the emergent properties of the group. Cell-cell communication is typically involved, and in some cases, subsets of cells terminally differentiate to the point of relinquishing the capacity to reproduce, reminiscent of the altruistic behaviors observed in social animals, where the sacrifice of individual fitness is advantageous provided sufficient benefits are accrued by close relatives (Hamilton 1964a,b; Diggle et al. 2007). The one major difference between multicellularity in eukaryotes and bacteria is that in the former case cells are usually aggregated from the outset (slime molds being exceptions), whereas in bacteria individual cells come together to form a unit (cyanobacteria being exceptions).

Just a sampling of the deep list of examples will be given here. Numerous species of filamentous cyanobacteria that are capable of producing specialized cells with anaerobic interiors for nitrogen fixation (heterocysts) as well as specialized spore cells (akinetes). The soil bacterium *Bacillus subtilis* undergoes periodic switching between motile unicellular and sessile filamentous states, with one transition being essentially random and the other involving a timer (Norman et al. 2013, 2015). Multicellular magnetotactic bacteria have no known unicellular stage, and are linked together via structural filaments contained within a central acellular chamber (Keim et al. 2004; Shapiro et al. 2011). Mycobacteria harbor a family of excretion-system loci that coordinate a form of sexual reproduction known as conjugation (Gray et al. 2016).

Particularly striking are the widespread quorum-sensing systems that bacteria use for inducing intraspecific neighbors to cooperate in the production of biofilms, planktonic aggregates, or swarming motility (Ng and Bassler 2009). Such systems generally involve simple positive-feedback loops, wherein above a certain cell-density

threshold, cells begin to release a chemical pheromone (a low molecular-weight chemical called an auto-inducer). Pheromones are costly, and of little use at low population densities, but when the external autoinducer concentration exceeds a threshold level, cognate receptors bind them, triggering a signal-transduction cascade that coordinately turns on a group of response genes, including the gene that produces the pheromone.

In some systems, the pheromone molecules are small enough to simply diffuse through the the cell membrane and are picked up by cytoplasmic factors (e.g., lactones in *Pseudomonas,* Ng and Bassler 2009), whereas in other cases larger message molecules (e.g., modified peptides in Gram-positive bacteria) are bound by transporters located on the cell surface, which then transfer them to kinases used to phosphorylate response regulator proteins (Chapter 22). Some species have multiple quorum-sensing systems, providing a basis for complex environmental sensing mechanisms and combinatorial communication (Cornforth et al. 2014; Feng et al. 2015; Jemielita et al. 2018), behavioral attributes that are typically assumed to be restricted to metazoans.

Bacteria even have memories, in the sense that prior exposure to an environmental challenge can enhance the response at a later point in time, even extending into the next generation. This occurs when the lifespans of key proteins exceeds the time needed for cell division (Mitchell et al. 2009; Lambert and Kussell 2014; Mathis and Ackermann 2016). The benefits of collective behavior are not always clear, but include presumed advantages associated with biofilm formation, including the aggregative behavior of food-degrading and/or tissue-invading enzymes and antibiotic production.

Given that signaling proteins are bioenergetically expensive, such systems are vulnerable to invasion by mutant cheaters that relinquish the costs of signaling but gain the benefits from the public goods produced by the remaining group members, a classical weak-link in social systems. A powerful defense against such invasions is made possible by kin recognition, wherein individuals can discriminate close from distant relatives, thereby providing opportunities for dispensing benefits only to individuals likely harboring similar genetic constitutions, including the underlying genes for helping (Diggle et al. 2007).

Such a recognition system is known to operate in a striking example of multicellularity in bacteria, the social soil bacterium *Myxococcus xanthus.* In this species, individual cells aggregate into swarming masses that assemble into fruiting bodies, within which only a fraction of cells actually produce spores. Such aggregations are genetically highly homogeneous, with kin detection and restricted cell adhesion being governed by a simple two-locus system sensitive to even single amino-acid changes (Cao and Wall 2017). Dozens to hundreds of mutually exclusive recognition groups, each operating in effect as a unit of selection, can coexist in single soil samples (Vos and Velicer 2009).

These kinds of kin-recognition groups extend beyond *Myxococcus.* For example, when grown on agar substrates, the soil bacterium *B. subtilis* forms distinct boundaries between incompatibility groups (Stefanic et al. 2015). Eukaryotic slime molds (*Dictyostelium*) are social amoebae that form stalks and fruiting bodies after solitary cells aggregate into kin-recognition groups with a simple genetic basis (Benabentos et al. 2009; Hirose et al. 2011). With collective benefits being dispensed only to

close kin, these kinds of systems are organized in ways that are not much different than multicellular eukaryotes where all cells within individuals are clone-mates.

How does a single bacterial species come to consist of multiple kin-recognition groups, as opposed to operating as a single species-wide unit? One possibility is that the coexistence of incompatibility groups is facilitated by a form of frequency-dependent selection, as demonstrated by experiments with *B. subtilis* (Pollak et al. 2016). In a mixed population, rare strains with one particular sensing mechanism are unable to activate their own quorum-sensing system because the concentration of their messenger molecule is too low, but such strains can still gain advantages elicited by common strains with activated quorum-sensing systems. However, as initially rare strains increase in abundance, and their own systems become active, they then become subject to exploitation by other rare strains. In principle, the entire system facilitates coexistence, with the fitnesses of strains increasing as their relative frequencies decrease, thereby bounding them away from extinction.

Finally, it is worth noting that just as the individual cells of multicellular eukaryotes exhibit division of labor, there are many examples of bacterial consortia in which different members provide complementary resources for metabolic cross-feeding. For example, Rosenthal et al. (2018) found that genetically uniform laboratory populations of *Bacillus subtilis* can subdivide into two subpopulations of metabolically differentiated cells, one producing harmful acetate, and another converting the latter into a benign storage molecule. Likewise, simple test-tube populations of *E. coli* often develop spatial structure and cross-feeding in ways that promote coexistence of different evolved clades (Good et al. 2017; Behringer et al. 2018). In an engineered example, Pande et al. (2014) constructed two strains of *E. coli* with complementary amino-acid requirements – each lacked the ability to synthesize one amino acid but released the other into the environment. Over a short time period, the strains evolved a division of metabolic labor that enhanced the productivity of the overall system, as the cost of producing excess amino acid for the partner was less than the advantage gained from being provisioned for the other.

All of these observations on bacteria highlight the importance of a key issue in evolutionary biology – the unit of selection. In principle, selection can operate at any level, discriminating among individuals within populations, among groups of individuals based on their emergent properties, or even among species on very long time scales. Moreover, a trait that is beneficial at one level need not be beneficial at another, altruistic behavior being a prime case in point, as in this case the individual incurs a fitness cost while other members of the population experience a fitness gain. Generally, when conflicts like this arise, we expect selection at the individual level to prevail for the simple reason that the turnover rate of individuals exceeds that of groups, but the distinction between individual- and group-selection becomes blurred when the interacting members of the population are close relatives.

What is an individual from an evolutionary perspective? In almost all organisms, individuality is clearly demarcated in a physical sense, e.g., by cell membranes in the case of unicellular species, and distinct soma in the case of multicellulars. However, things are not so clear at the genetic level, as some individuals are more closely related than others, the extreme cases being monozygotic twins or members of an asexually propagating lineage. This matters because behavior that is harmful to an individual but sufficiently beneficial to close relatives can be promoted by

kin selection. For example, a suicidal behavior that sufficiently elevates the fitness of clone-mates will be advanced by selection. Given the clonal nature of microbial aggregates, it is then clear that the unit of selection at the genetic level extends beyond individual cells to the local kin group, not greatly different than the situation in multicellular eukaryotes in which the soma is derived by clonal expansion. In this sense, many microbes are multicellular in an evolutionary sense even in the absence of structural connections between cells. The key point is that eukaryogenesis was not a pre-requisite for admission into the multicellular world.

The costs of multicellularity. Certain forms of multicellularity can open up access to novel resource pools not physically possible for single cells, e.g., the ability of animals to overwhelm smaller prey items and land plants to experience the full spectrum of ambient sunlight. Multicellularity can also provide added benefits in terms of survival, e.g., avoiding predation or providing protection against physical environmental challenges, although increased size may invite the attention of still larger consumers. However, to be promoted by natural selection, transitions to novel ecological niches require that any benefits accrued outweigh the costs, at least in the early stages of establishment. In particular, a mutation that expands dietary breadth needs to do so in a way that magnifies the net rate of acquisition of resources beyond what was possible in the ancestral state, and needs to do so for a long enough time period to move to fixation (with the number of required generations being on the order of the effective population size).

With their structural support systems, animals and land plants were predisposed to proliferate across uncolonized land masses, provided the advantages (in terms of resource acquisition or predator avoidance) outweighed the costs of cellular cooperation. However, once enough multicellular lineages had evolved diverse and refined mechanisms for occupying most niches afforded by large size, there would be no continuing evolutionary incentive for the mass movement of microbes towards multicellularity, any more than established multicellular species would be expected to undergo regressive evolution to unicellularity.

Taken in the broader picture of evolution across the Tree of Life, the often-stated beauty and wondrous nature of land plants and animals may have artistic appeal, but belies the underlying negative aspects of multicellularity. Yes, such organisms have undergone adaptive diversification in ways that enable them to successfully forage and find mates in sometimes unexpected ways, but the same is true for every microbial organism. What bears emphasis is that, in the long run, the transition to multicellularity comes with a price. What is often viewed as an indication of superiority from one perspective is quite the opposite from another.

First, as discussed above, whereas large complex organisms may have access to novel resources, they also ultimately succumb to a reduction in bioenergetic capacity (Figure 24.1). In particular, maximum growth rates decline with organism size. That is, even with an unlimited food supply, large organisms are unable to convert resources into biomass at the rates of smaller organisms. Thus, the long-term benefits of multicellularity do not reside in a greater capacity for assimilating biomass essential for growth and reproduction, at least not at high resource levels. Are there energetic advantages at low resource levels? Given the increased maintenance costs of larger organisms per unit time and their longer generation times (Chapter 8), this

too seems unlikely.

Second, the idea that selection relentlessly promotes increased complexity to confer robustness in the face of environmental and mutational changes also comes up short, as it ignores both the physical costs of complexity and the constraints on evolutionary processes. As noted above, more complex gene structures magnify both the bioenergetic costs and the mutational vulnerability of alleles. There is no evidence that genes endowed with introns, proteins of greater length, or expansions of mobile-element families were promoted by advantages conferred on their host genomes. Quite the contrary, their initial establishment appears to have made possible only by the reduction in the efficiency of negative selection.

Third, as outlined in Chapter 20, while added layers of intracellular surveillance, such DNA polymerase proof-readers, constitute an increase in complexity by any definition of the term and superficially lead to the impression of an elevation in robustness, this is an illusion. Although the initial establishment of a new layer of protection may be promoted by positive selection, in the long run, the overall advantage is expected to dissipate as a consequence of the further accumulation of mildly deleterious mutations to the multiple-component system. In the long-term, this leads to a more complex system with no better capacity than the originally simpler system, but now with added costs of energetic investment and mutational vulnerability.

Fourth, multicellularity imposes the necessity of costly mechanisms for the suppression of renegade cells. In particular, the constant threat from emerging cheater cells is arguably one of the greatest challenges to the maintenance of multicellularity (Frank 1995; Michod 1999). A recurrent problem for species with differentiated somatic tissues is the emergence of cells that proliferate at the expense of the overall organism, cancer being one of the most obvious manifestations of this kind of problem. Indeed, because somatic mutations are not inherited across generations, selection for genomic repair mechanisms appears to be substantially reduced relative to the situation in the germline, with mutation rates in somatic cells typically being elevated at least ten-fold (Lynch 2010; Blokzijl et al. 2016; Abascal et al. 2021). As a consequence, as intrasomatic selection progressively selects for clonal variants that expand at the expense of their neighboring cells, somatic mutations inevitably lead to senescence (Nelson and Masel 2017).

The claim here is not that multicellularity emerged in the face of short-term disadvantages, but rather that once set in motion, the transition to such body plans creates unavoidable downstream consequences. Short-term opportunities guided particular lineages down trajectories leading to larger body plans with structural support only possible with multicellularity. However, once established, such body plans not only result in lineages with reduced bioenergetic capacity but alter the population-genetic environment in ways that encourage the establishment of genomic and subcellular features with added expenses. In particular, multicellularity sets up a scenario by which effectively neutral (but absolutely deleterious) changes can accumulate through time by mutation pressure, perhaps in some cases even being driven by positive selection for mechanisms that enhance competitive ability while reducing individual productivity (e.g., dominance of larger over smaller individuals). Combined with the partitioning of the full repertoire of functions essential in unicellular organisms to specialized cell types (discussed in the following

section), complex multicellularity then becomes an evolutionary trap in the sense that reversion to unicellularity becomes impossible.

Finally, additional considerations remain to be explored in this area from the standpoint of population-genetic mechanisms. In particular, in the initial stages of a transition, mutations to multicellularity would normally need to proliferate in a background population of unicellularity. Assuming a life cycle with a sexual phase, this leaves many questions unanswered with respect to genetic compatibility of unicellular and multicellular variants. This is a key issue given that, owing to initial rarity in the critical phase of establishment, all mutations face the challenge of the fitness consequences of mating with foreign types and diluting their phenotypic effects. Thus, long-term phases of clonal propagation, haploidy, and local inbreeding would seem to facilitate the emergence of multicellularity.

The emergence of cell-type specialization. As noted above, although the average land-plant and animal genome harbors two- to three-fold more genes than in their unicellular relatives, little of this increase seems to be related to necessities associated with multicellularity, as opposed to being indirect by-products of alterations in the population-genetic environment. Consider that in the early days of genome sequencing, the observation that numerous genes in mammalian genomes were absent from *Drosophila* and nematode genomes led to the idea that such genes play key roles in the development of vertebrates. However, as a broader phylogenetic survey of genome contents began to emerge, it became clear that these apparent "vertebrate-specific" genes were actually cases of gene loss in select invertebrate lineages.

Similarly, many of the genes originally thought to have been unique to metazoans have since been found in basal unicellular lineages (e.g., choanoflagellates and ichthyosporeans), and in some cases even more deeply. For example, the integrin proteins used for cell-cell adhesion in metazoans are present in the apusozoan lineage (basal to both animals and fungi) but absent from fungi and choanoflagellates (Sebé-Pedrós et al. 2010). Likewise, many proteins initially thought to be uniquely involved in cell signaling, immune response, and animal development were subsequently found to be present in choanoflagellates (King et al. 2008; Richter et al. 2018). While there are many gene gains on the branch subtending the metazoan lineage, the number of gene losses (among these the genes for the biosynthesis of nine amino acids) appears to be just as great (Richter et al. 2018). Thus, the evolution of complex multicellularity did not involve a major influx of new genes. Rather, many of the genes deployed in the unique features of complex multicellular organisms are modified descendants of those used in related functions in unicellular ancestors.

The substantial increase in cell-type number in the face of only a moderate increase in gene number is informative with respect to the mode of origin of multicellularity in eukaryotes. Although metazoans and land plants exhibit dozens to hundreds of cell types, the increase in complexity of cellular functions is relatively small. Rather than evolving entirely new sets of tasks, most cell types in multicellular species have simply lost a range of features found in ancestral unicellular species. Whereas, the latter must be capable of multitasking with respect to nutrient acquisition, avoidance of predators, dealing with unfavorable environments, etc., multicellularity offers the possibility of division of labor among cell types. Thus, un-

der the assumption that such partitioning leads to the whole being more than the sum of its parts, it has been suggested that such partitioning is critical to the evolution of complex multicellularity (Maynard Smith and Szathmáry 1995; Michod 1999). A key point, however, is that the emergence of multicellularity is not heavily dependent on neofunctionalization. Rather, following a pattern similar to that seen when genes duplicate, subfunctionalization at the level of cell-type specialization has played an enormous role in the evolution of multicellular organisms. Indeed, drawing from such patterns, some have suggested that cell types can be classified based on shared and divergent patterns of gene expression (Arendt 2008; Arendt et al. 2016).

The most common way in which cell specialization emerges from a single-celled mode of life appears to be the conversion of a prior temporal pattern of cell life-cycle differentiation into a developmentally regulated spatial pattern (Mikhailov et al. 2009; Sebé-Pedrós et al. 2017). The expected scenario here is one in which cell differentiation based on external environmental cues is progressively eliminated and replaced by internal mechanisms such as cell-cell communication and developmental regulation. This partitioning up of pre-existing gene functions combined with regulatory rewiring clarifies why the evolution of multicellularity does not require a massive investment in new genomic real estate.

Although division of labor among cell types is a natural evolved outcome of multicellularity, once established, it also plays a key role in ensuring the stability of such body plans, just as the loss of complementary functions by subfunctionalization leads to the preservation of duplicate genes. That is, the establishment of mechanisms that contribute to group fitness but come at an expense to free-living cells (e.g., functional partitioning) will reduce the likelihood of reversion to unicellularity even if this were to be beneficial. Apoptosis (developmentally controlled cell death) is a prime example of a trait that can be of no benefit to a single-celled organism. In this sense, the division of labor in multicellular organisms, whether intrinsically beneficial or not, has a ratchet-like effect on the stability of multicellularity – as cell types become more and more specialized, the likelihood of reversion of any single cell type to a form containing all ancestral-cell features necessary for independent living becomes less and less likely (Libby and Ratcliff 2014; Cooper and West 2018).

Separation of germ and somatic cells represents an extreme form of division of labor, as the former become increasingly specialized for the single function of propagation, releasing the latter from any special requirements associated with cell fusion, meiosis, and offspring production. However, to see that such an extreme form of partitioning does not depend on the prior establishment of multicellularity, one need only look to the ciliated protozoa (Chapter 10). These highly diverse and globally distributed protists are binucleate, with the diploid micronucleus serving as a transcriptionally silent germline, recombining during sexual reproduction, and a highly polyploid and edited version of it, the macronucleus, serving as the locale of somatic gene expression, The latter is transmitted without recombination during asexual cell division, but disposed of and then replaced following sexual reproduction.

Studies on the Volvocales, an order of green algae that includes the unicellular *Chlamydomonas,* provide some empirical insight into many of the above points. The order includes species with 2-, 4-, 8-celled body plans, etc., extending up to the large spherical, multicellular forms known as *Volvox,* which contain thousands of

cells. Notably, the increase in colony size is accompanied by a substantial expansion in noncoding DNA in both the mitochondrial and plastid genomes, consistent with a shift in the population-genetic environment as these organelles do not harbor the genes associated with cell development (Smith et el. 2013). The advantages of multicellularity vs. unicellularity in the group remain to be worked out. Although it has been argued that collective aggregates are more efficient at responding to weak chemotactic signals (Colizzi et al. 2020), and that flagellar stirring of boundary layers can magnify advective transport beyond the limits of diffusion (Solari et al. 2006), this leaves unexplained the predominance of the simplest body plans.

The first steps in the transition to multicellularity in this group involve the evolution of colonies of undifferentiated cells, with division of labor arising secondarily (Kirk 2005; Herron et al. 2009; Herron 2016), and accomplished in the absence of gene-number expansion (Featherston et al. 2018; Matt and Umen 2018). Notably, the genus *Volvox* is polyphyletic, having evolved on several independent occasions, the most extreme form being *Volvox carteri,* which harbors 16 large nonmobile germ cells embedded in an extracellular matrix surrounded by a single-layered sphere of $\sim$ 2000 terminally differentiated flagellated cells. Notably, the germ cells exhibit the greatest breadth of gene expression, relative to the more specialized transcriptomes of the somatic cells, a pattern similar to that seen in pluripotent stem cells in metazoans (Matt and Umen 2018).

Finally, many have argued that the establishment of mitochondria were central to the emergence of multicellularity (e.g., Bendich 2010; Lane and Martin 2010; Medini et al. 2020). The reasoning behind this speculation is diverse, ranging from the supposed bioenergetic advantages of mitochondria (which, in preceding chapters, have been repeatedly shown to be overstated), to the sequestration of germ cells from mutagenic by-products of organelle respiration, to the involvement of mitochondria in various aspects of developmental control. All of these arguments ignore the existence of multicellular prokaryotes and fail to make the distinction between mechanisms of origin of organismal features and their secondary, downstream modifications. As just one example, as noted above, cellular apoptosis is often viewed as a unique innovation that emerged after the evolution of multicellularity. The process is triggered by events associated with the mitochondrion, and can be essential to development and cancer suppression, leading to the superficial view of a causal link between mitochondria and the evolution of multicellularity. However, the apoptotic machinery appears to predate not only the evolution of complex multicellularity but even the origin of eukaryotes, as it is present in multiple lineages of unicellular eukaryotes as well as in bacteria (Koonin and Aravind 2002; Klim et al. 2018).

Closing Comments

We get excited by things we see. Up to now, the majority of research in evolutionary biology has focused on things like butterflies, zebras, and flowering plants. From this work, we have learned a lot about agents of natural and sexual selection, at least for multicellular species. What remains to be understood are the molecular/cellular mechanisms underlying phenotypic divergence and the degree to which these vary among phylogenetic lineages. Although these issues are common to all organisms,

unicellular organisms, which comprise the vast majority of life, provide a logical starting point for such investigation. The root of the Tree of Life is unicellular, and each cell in all of today's organisms is a product of a unique but continuous cell-to-cell lineage tracing back to the beginning of biological time.

We also like things to be simple. However, although it is relatively easy to understand the superficial features of the process of natural selection and to concoct adaptive stories as to how biodiversity arose, evolution is not a simple matter of natural selection pushing around mean phenotypes. Rather, the paths open to exploitation by selection are governed by the nonadaptive processes associated with population-genetic environment. Although mutation, recombination, and random genetic drift are universal genetic forces, and have been so since the origin of life, their relative strengths vary by orders of magnitude among today's phylogenetic lineages, often in ways that scale with organism size. Such variation has significant consequences for the emergence and utilization of the genetic material upon which natural selection acts.

Thus, whereas the technical field of population genetics is often conveniently viewed as being marginal with respect to questions concerning deep phylogenetic divergence, it is actually front and center. Likewise, while the details of molecular and cellular biology are often viewed as largely irrelevant to the ways in which populations respond to natural selection, it is precisely here that modifications must be made to yield new phenotypes, so these details also matter.

Evolutionary processes are inherently stochastic, with the potential paths open to evolutionary exploitation depending on historical contingencies and the granularity of mutational effects relative to the power of random genetic drift. This makes the development of evolutionary theory more challenging than desirable for those confined to a Darwinian mode of thinking. If this view that selection operates in an effectively deterministic manner, with the underlying details being immaterial, could be shown formally to be correct, it would be a great achievement for evolutionary biology, effectively confining the future of the field to the monotonous cataloging of selective forces operating on different organisms.

The preceding pages challenge this view. Although no scientist any longer argues against the central role played by natural selection in evolutionary change, it is now clear that organisms exposed to identical selection pressures will respond in qualitatively different ways depending on their population and cellular environments, in some cases being completely impervious to selection and largely driven by mutation pressure. Once the subject of a long debate confined to molecular evolution, it is also clear that, since the origin of life, effectively neutral processes have played a key role in the evolution of genome architecture, cellular features, and by extension, whole-organism biology. If this view is correct, it offers a unifying and mechanistic approach to thinking about the history and dynamics of evolutionary change across the Tree of Life, liberating us from the century-old tradition of assuming that all of biodiversity reflects an optimization process dictated by a supreme designer called natural selection.

Summary

- Contrary to popular belief, evolution is not on an inexorable path to build more complex organisms. Not only is there no evidence for an intrinsic advantage of complexity at the molecular, cellular, or organismal levels, but empirical observations show that simple bacteria often carry out the most basic functions of life, such as growth and DNA replication fidelity, more efficiently than do the more complex cells of eukaryotes.

- The emergence of eukaryotes from prokaryotic ancestors involved a substantial increase in genome size, particularly in multicellular lineages. However, the vast majority of this expansion is a consequence of the colonization of noncoding DNA, with an increase in gene number being secondary. Rather than being driven by positive selection, such changes appear to have arisen as passive by-products of a reduction in effective population size, which diminishes the ability of natural selection to oppose insertions of excess DNA.

- There is no evidence that an increase in gene number played a primary role in the establishment of the eukaryotic body plan. Instead, it appears that the reorganization of the ancestral eukaryotic genome, possibly spawned in part by one or two whole-genome duplication events, followed by gene loss and subfunctionalization, led to the emergence of a new form of genomic architecture, opening up new paths for evolutionary change. One of the most striking sets of such changes involved the transition of homomeric molecular complexes in prokaryotes to heteromeric forms in eukaryotes.

- Although complex multicellularity, as embodied in land plants and metazoans, is restricted to eukaryotes, with just two instances of such evolution across the Tree of Life, there is no statistical basis for arguing that such evolution was dependent on the prior emergence of the eukaryotic cell plan.

- Although not as extensive as land plants and animals, bacterial species exhibit a wide range of features that enable collectives of individuals to operate in ways that are more than the sum of their parts, including division of labor among different cell types. Such traits include morphological differentiation at the cellular level, quorum-sensing mechanisms that promote coordinated behavioral changes in response to changes in cell density, and cross-feeding of complementary resources. Notably, many of these mutual benefits are specifically dispensed to close relatives (kin-recognition) groups, an essential behavior for thwarting the emergence of cheater cells.

- Ecological opportunity played a central role in the emergence of land plants and animals, but such establishment induced downstream costs, some of which were inevitable consequences of a reduction in long-term effective population sizes. These include a reduced ability to assimilate biomass, a substantial increase in

the energetic cost and mutational vulnerability of genomes, gratuitous investment in overly complex cellular features, and the constant threat of mutant cheater cells.

- The emergence of multicellularity relies much less on the evolution of novel gene functions than on the partitioning and/or loss of ancestral cell functions, which leads to the division of labor among cell types. As multicellular forms become more complex, this leads to a situation in which reversion to unicellularity becomes impossible. As a consequence, multicellular lineages become terminal branches in the Tree of Life, whereas unicellular lineages retain the capacity to become multicellular should the ecological opportunity emerge.

- Many arguments about the causes and consequences of multicellularity may profit from a broader consideration of the unicellular branches on the Tree of Life. For example, although multicellular organisms often have sequestered germlines, multicellularity is not a prerequisite for this condition, as all ciliates carry out such partitioning by use of separate germline and somatic nuclei. Likewise, although mitochondria are involved in apoptotic (targeted cell death) in multicellular species, and the process is often viewed as a unique feature of multicellular organisms, the antecedents of apoptotic pathways can be found in unicellular lineages.

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Figure 24.1. Scaling of maximum interval-specific growth rates with size at maturity. For unicellular species, the growth rates are equivalent to cell-division rates, whereas for multicellular species, they are the maximum rates among all age classes during individual development. All data are standardized to 20°C. From Lynch et al. (2021).

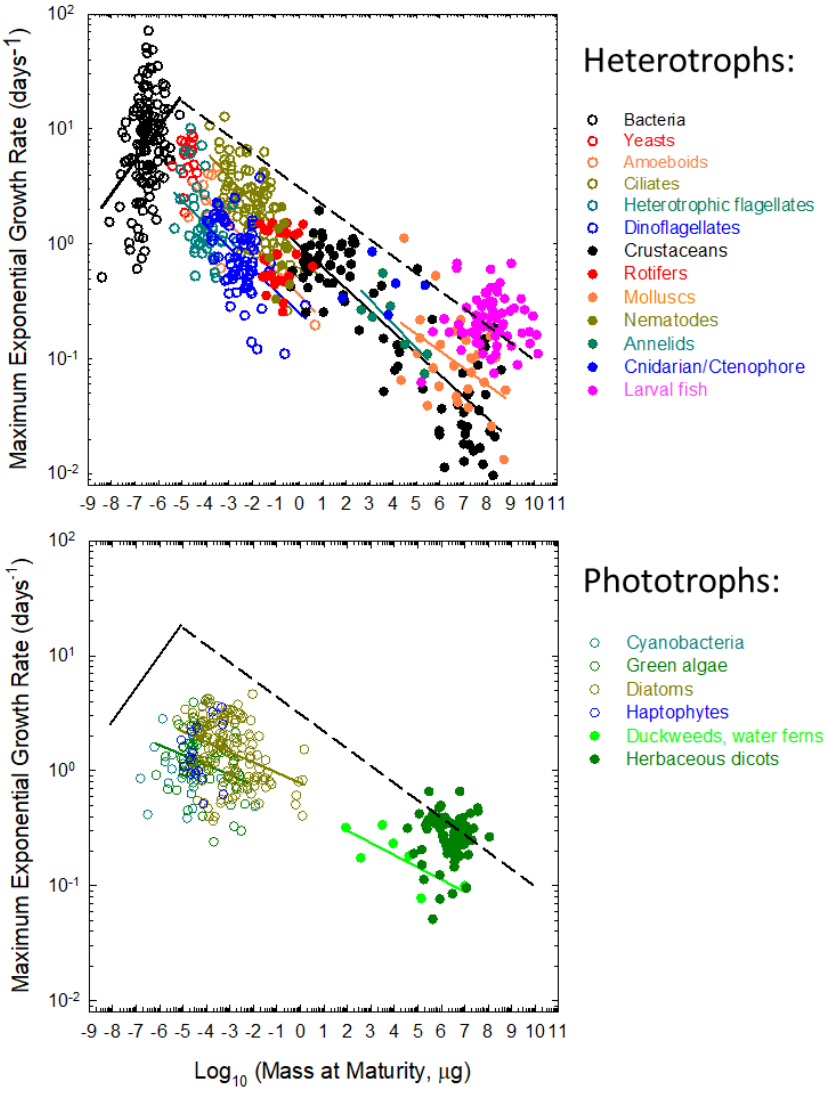

Figure 24.2. The approximate form of the density profile for deleterious mutations of effect s^* necessary to explain the power-law relationships in Figure 24.1, where s^* is defined as the pivot point between the domain of effective neutrality and efficient selection defined by the requirement that the product of the selection coefficient s and the effective population size N_e must exceed 1.0 for efficient selection. The upper axis label translates the lower-axis scale to size at maturity, B_a, by use of the pattern in Figure 4.3, which gives the relationship of the former to N_e.. Areas under the shaded triangles denote the summed loads of growth-reducing mutations for two different 1000-fold increases in B_a – as organisms increase in size, they become vulnerable to the fixation of mutations of larger effect, but the number of mutations is inversely proportional to the individual effects, keeping the sum of added effects constant as B_a increases, which yields a slope for the distribution of effects equal to -1 on a log scale. The elevation of the line is lower for phototrophs owing to the reduced power-law scaling in this group relative to heterotrophs.

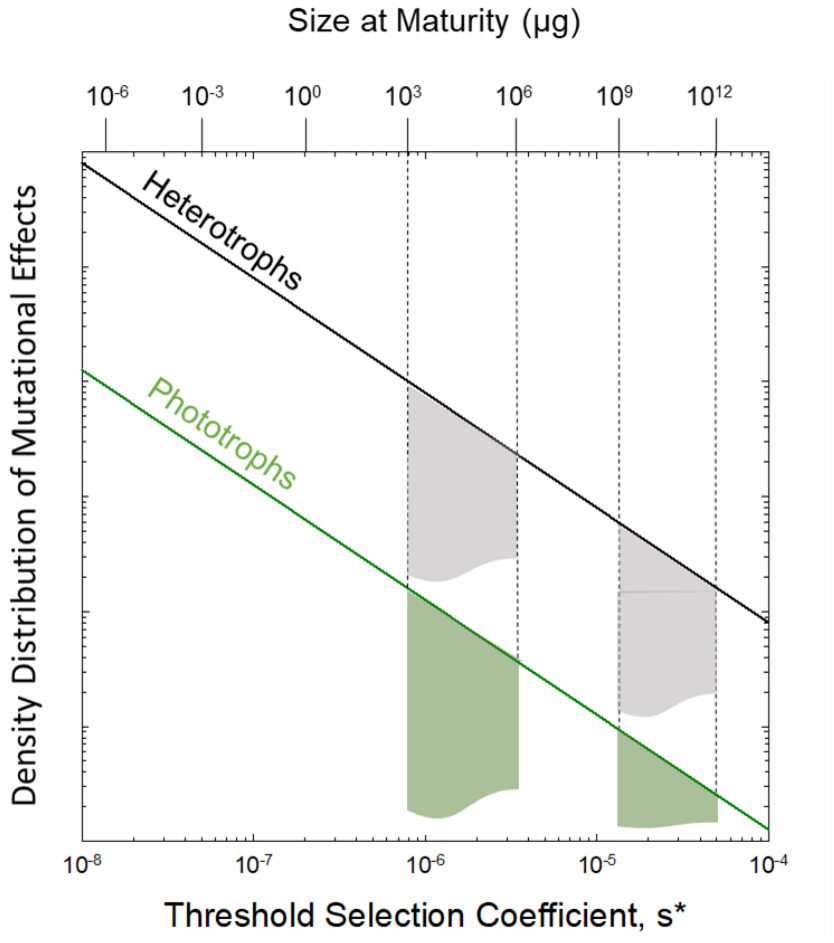

Figure 24.3. The scaling of the number of number of protein-coding genes per genome vs. total genome size (in millions of bases) across the Tree of Life. Data obtained from the NCBI genome summaries.