Computational Evolution Experiments Reveal a Net Loss of Genetic Information Despite Selection

Chase W. Nelson[1,*] and John C. Sanford[2]

*[1]Rainbow Technologies, Inc., 877 Marshall Rd., Waterloo, NY 13165, USA. [2]Department of Horticulture, NYSAES, Cornell University, Geneva, NY 14456, USA. (*Corresponding author: cwnelson88@gmail.com)*

Abstract

Computational evolution experiments using the population genetics simulation Mendel's Accountant have suggested that deleterious mutation accumulation may pose a threat to the long-term survival of many biological species. By contrast, experiments using the program Avida have suggested that purifying selection is extremely effective and that novel genetic information can arise via selection for high-impact beneficial mutations. The present study shows that these approaches yield seemingly contradictory results only because of disparate parameter settings. Both agree when similar settings are used, and both reveal a net loss of genetic information under biologically relevant conditions. Further, both approaches establish the existence of three potentially prohibitive barriers to the evolution of novel genetic information: (1) the selection threshold and resulting genetic decay; (2) the waiting time to beneficial mutation; and (3) the pressure of reductive evolution, i.e., the selective pressure to shrink the genome and disable unused functions. The adequacy of mutation and natural selection for producing and sustaining novel genetic information cannot be properly assessed without a careful study of these issues.

Key words: Avida, digital organisms, experimental evolution, genetic entropy, irreducible complexity, Mendel's Accountant, reductive evolution, selection threshold, waiting time to beneficial mutation

Introduction

Mathematical models and numerical simulation have long suggested that the accumulation of slightly deleterious mutations may pose a threat to the long-term survival of many biological species, including humans [1–4]. Computational evolution experiments with the forward-time population genetics simulation Mendel's Accountant have predicted a substantial fitness decline in the human species under biologically relevant conditions [5]. Moreover, experiments with biological organisms have raised similar concerns, revealing that the majority of adaptive mutations cause a loss of functionality [6–10]. Lethal mutagenesis may also play a key role in pathogen attenuation [11–13]. Recently, however, experiments using

the digital genetics software Avida have suggested that purifying selection can be extremely effective and that novel genetic information can arise via selection for high-impact beneficial mutations [14]. Avida researchers have claimed a high degree of biological relevance for the program, using it to address numerous biological questions [15,16].

In this study, we investigate why Avida and Mendel's Accountant yield seemingly contradictory results. We find that most discrepancies are due to differences in default settings. Mendel's default settings implement values plausible for modeling the human species, while Avida's default settings have virtually no parallel in biological systems. Additionally, Avida introduces several un-biological mechanisms both for facilitating the development of novel genetic information and for preventing its loss. The most notable deviations from biological reality include the distribution of mutational fitness effects, the waiting time to high-impact beneficial mutation, and the selective neutrality of inert genomic material. When used with more realistic settings, Avida's results agree with other studies that reveal a net loss of genetic information under biologically realistic conditions. The results reported here suggest that three substantial barriers may prevent the evolution of genetic information by mutation and natural selection in biological organisms: (1) the selection threshold; (2) the waiting time to beneficial mutation; and (3) reductive evolution. Implications for theory and medicine are discussed.

Mendel's Accountant

Detailed descriptions of Mendel's Accountant (hereafter Mendel) are available elsewhere [17,18], and default settings are described in the Methods. Briefly, Mendel constitutes a numerical simulation that tracks mutations as they arise within the members of a model population. The user specifies parameters such as population size, genome size, mutation rate, and the proportion of beneficial mutations. Mutational fitness effects are represented by a Weibull distribution, with both deleterious and beneficial effects having lower and upper bounds. The largest deleterious fitness effect is –1.0 (lethal in most contexts), while the smallest effect is defined as the reciprocal of the functional genome size ($-1/G_e$), following the precedent of Kondrashov [2]. Beneficial mutations are limited by the same lower bound ($1/G_e$) and a user-defined upper bound (0.001 by default). Each mutation has its own fitness effect as well as its own location within an individual's genome, allowing the investigator to model linkage and recombination. To save computational resources, neutral mutations are not normally tracked. Instead, the mutation rate is scaled to exclude neutral mutations, such that the mutation rate defined by the user is the rate per effective genome, i.e., the rate of mutations affecting fitness.

The program periodically reports various statistics during an experiment, including the population's average fitness and the average number of deleterious and beneficial mutations per organism. The program is open source and is available online [19].

Avida

Avida differs from Mendel in that it represents genomes directly using machine code instructions, and generally requires more computer science knowledge for use and interpretation of results. Twenty-six genomic instructions are defined in the software, and each performs a specific computational task (e.g., adding two numbers). Individual genomes, called *digital organisms*, consist of about 100 instructions and undergo random mutation at a user-defined rate. Mutations may substitute, insert, or delete instructions at random. The Avidian organisms are themselves housed on a two dimensional grid. Replication is asexual, with daughter cells randomly replacing one of the eight surrounding neighbors. Because of this, replication rate determines fitness in Avida; any changes that allow an organism to copy its genome and replicate faster will allow it to replace other organisms, and its frequency in the population will increase.

Each organism in Avida has an associated *merit* value that determines its relative replication rate. This value reflects both genome size and the ability to perform one of nine computational functions (logic operations). Making merit proportional to genome size implements a scheme called *size neutrality* in which larger genomes are artificially given extra computational time. This removes the selective pressure to shrink genomes, making organisms with identical phenotypes but different genome sizes equivalent in fitness. Because of this, acquiring *merit bonuses* by performing any of the nine logic operations is the primary means by which organisms increase their replication rate in Avida. These functions arise when random mutations produce particular combinations of instructions that cause the functions to be executed. For example, the simplest logic operations, NAND and NOT, can occur when the instruction NAND arises in the correct combination with input-output and labeling instructions.

Considering its frequent application to biological questions, Avida's default range of beneficial mutational fitness effects is curiously high. The two simplest operations have a multiplicative merit bonus of 2, doubling an organism's fitness. Bonuses increase exponentially with the complexity of a function, and EQU (the most complex function in Avida) multiplies fitness by 32 (Table 1). For purposes of biological comparison, relative fitness may be defined as $w = 1 + s$, where s is the mutational fitness effect and w is the relative fitness of an organism expressing

Table 1. Default fitness bonuses for performing nine logic operations in Avida. Adapted from Lenski *et al*. [14].

Logic Operation	Computation	Number of NAND Operations Needed (n)	Default Multiplicative Bonus (2^n)	Default Fitness Effect ($w - 1$)
NOT	~A; ~B	1	2	1.0
NAND	~(A and B)	1	2	1.0
AND	A and B	2	4	3.0
ORNOT	(A or ~B); (~A or B)	2	4	3.0
OR	A or B	3	8	7.0
ANDNOT	(A and ~B); (~A and B)	3	8	7.0
NOR	~A and ~B	4	16	15.0
XOR	(A and ~B) or (~A and B)	4	16	15.0
EQU (XNOR)	(A and B) or (~A and ~B)	5	32	31.0

a particular function as compared to its function-free ancestor. Mutational fitness effects therefore range from 1.0 to 31.0 under Avida's default settings. The program is available online [20], and more detailed descriptions of the software are available elsewhere [21–23].

A previous study [24] has demonstrated that seven of the nine logic operations arise by mutation alone in Avida, without selection, reflecting their informational simplicity within the software environment. Under default settings lasting about 10,000 generations, an average of 8.6 (± 0.7) such functions successfully evolve (i.e., rise above a frequency of 50%), increasing fitness by an average of 20,000,000 fold. Increases of this magnitude are enabled by the large multiplicative fitness bonuses assigned to the logic operations ($2^2 \times 4^2 \times 8^2 \times 16^2 \times 32 =$ 33,554,432; Table 1). Fitness increases observed in biological evolution experiments are negligible by comparison; e.g., in experiments with *E. coli*, fitness increased by only 75% after 20,000 generations [6]. Interestingly, the Avidian logic functions are prevented from reaching fixation by the relatively high mutation rate (approximately 0.85 mutations per genome per generation). Fitness eventually levels off, as only nine functions are available.

Although Avida's default mutational fitness effects range from 1.0 to 31.0, the user may specify other values. Using alternative values ranging from 0 to 1.0, Nelson and Sanford [24] used an empirical approach to demonstrate that Avidian populations experience a *selection threshold*, or a critical fitness effect

below which drift dominates the behavior of a mutation. About half of the functions evolve (rise above a frequency of 50%) with fitness effects of approximately 0.2, the empirically determined threshold value. With fitness effects of ≤ 0.075, no new functions evolve, and those that have previously evolved break down.

Selection threshold and genetic entropy

Muller [25] was one of the first to allude to a selection threshold, writing in 1964 that "There comes a level of advantage… that is too small to be effectively seized upon by selection." Population size is the most studied factor affecting the selection threshold [26], and its role is expressed in Kimura's [27] inequality, $|s| < 1/(2N_e)$. This states that a mutation's fate will be dominated by random genetic drift if the absolute value of its fitness effect (s) is less than the reciprocal of twice the effective population size (N_e). However, many other factors influence the efficacy of selection, including developmental canalization and environmental effects. Any factor that influences reproduction in a way that is independent of the genotype will raise the threshold, causing more mutations to behave as if they are neutral. The point is well summarized by Eyre-Walker and Keightley:

> … it seems unlikely that any mutation is truly neutral in the sense that it has no effect on fitness. All mutations must have some effect, even if that effect is vanishingly small. However, there is a class of mutations that we can term effectively neutral… As such, the definition of neutrality is operational rather than functional; it depends on whether natural selection is effective on the mutation in the population or the genomic context in which it segregates, not solely on the effect of the mutation on fitness [28].

Nei [29] has pointed out that natural selection operates as the result of the production of different genotypes in a population, and is therefore not the fundamental cause of evolution. Selection can only alter the survival of variation that has already arisen in nature. As a result, net fitness can decrease even when natural selection is successful. ReMine [30] makes this point clear by using the analogy of soldiers marching uphill on a descending conveyor belt. The conveyor belt represents the load of deleterious mutations that consistently decreases fitness. The soldiers near the bottom are less fit, and tend to be eliminated as they fall off the lower edge (representing natural selection). Those that survive may replicate at a certain rate, and take a step upward each time a beneficial mutation occurs. This interplay is known as the *mutation-selection balance* [31]. If the

rate of beneficial mutations (rare steps upward) is insufficient to counteract the load of deleterious mutations (common steps downward), natural selection may work very effectively but concurrently be unable to prevent net information loss and eventual extinction. In such a situation, the entire population eventually slides off the conveyor belt, experiencing *error catastrophe* or *mutational meltdown*.

It is obvious that the potential lethality of deleterious mutational load is magnified when selection is less effective. Because the majority of mutations are deleterious [28], random genetic drift imposes a high degree of directionality on evolution by favoring the fixation of mutations that decrease fitness [32]. These issues have caused concern about the long-term survival of numerous species, including humans [4], inspiring titles like "Contamination of the genome by very slightly deleterious mutations: why have we not died 100 times over?" [2]. No compelling solutions to this paradox have yet emerged, though many possibilities have been proposed [2,3] (see Discussion).

These considerations lead to the realization that, especially in species with large genomes, it is possible that mutation rates are so high and deleterious mutations so common that genetic information cannot be maintained. Sanford [33] has introduced the term *genetic entropy* to describe the deterministic deterioration of genetic information resulting from ineffective purifying selection. The aforementioned experiments with Avida have demonstrated genetic entropy, providing empirical evidence that selection thresholds exist, and showing that ineffective selection may pose a substantial barrier to the evolutionary origin and maintenance of complexity [24]. Experiments using Mendel have provided further evidence of a selection threshold, and have explored the evolutionary fate of both beneficial and deleterious mutations [5,34–36].

The present study explores potential barriers to the progressive evolution of novel genetic information by pursuing several lines of experimentation with Mendel and Avida. First, Mendel is used to replicate results obtained under Avida's default settings. This demonstrates Mendel's versatility and reveals the parameters that are necessary to obtain results typical of an Avida experiment. Two additional sets of Mendel experiments are performed, one using default settings, and another using settings more conducive to the occurrence of high-impact beneficial mutations. Next, Avida is used to pursue two additional questions. First, functional precursors of the EQU operation are assigned neutral fitness effects in order to explore the evolutionary origin of complexity when beneficial mutations are not readily available. Second, various mechanisms preventing reductive evolution (adaptive loss of genetic material and functionality) are disabled and the evolutionary consequences observed.

Methods

Experiments using Mendel's Accountant

All Mendel experiments used version 1.8.5. Random number seeds were chosen as integer values from 1 to 1,000. Experiments were performed using settings that: (1) approximate Avida's default settings, (2) employ Mendel's default settings, and (3) use Mendel settings more conducive to the occurrence of high-impact beneficial mutations. A full list of experimental settings appears in Table 2.

First, ten Mendel experiments were performed to approximate Avida's default results. The most notable changes to Mendel's default settings were a reduced genomic mutation rate of 0.01 (reflecting the size and selective neutrality of much of the ancestral Avidian genome), a proportion of 0.000023 mutations being beneficial, and uniform multiplicative beneficial fitness effects of 5.5. (Mendel does not lend itself to studying the large discrete fitness effects implemented in Avida, so uniform fitness effects were used.)

Next, ten experiments were performed under Mendel's default settings. Following this, twenty experiments were performed under settings more conducive to the occurrence and selection of high-impact beneficial mutations. The fraction of beneficial mutations was increased to 0.001, the maximum beneficial fitness effect increased to 0.5, heritability increased to 0.5, and experiment length increased to 1,000 generations.

Experiments using Avida

All Avida experiments used version 2.8.1. Random number seeds were chosen randomly as an integer value from 1 to 1,000,000,000. Two sets of experiments were performed, one in which various precursor functions were assigned neutral fitness bonuses, and one in which mechanisms preventing genome shrinkage were disabled.

For experiments in which functions were assigned neutral fitness bonuses, the number of neutral functions varied from zero to nine, with zero corresponding to Avida's default settings and nine corresponding to all functions (including EQU) having no fitness effect. Two sets of 20 replicates were performed, one in which functions were made neutral from simple-to-complex (beginning with NOT), and one in which functions were made neutral from complex-to-simple (beginning with XOR). Each replicate therefore consisted of 10 experiments, one for each combination of neutral functions. In all instances, EQU was the last function made neutral (all nine neutral functions). Default fitness bonuses were maintained for advantageous functions, and functions were made neutral by defining multiplicative

Table 2. Parameter settings used in experiments with Mendel's Accountant. A dash (–) indicates the use of default values.

Parameter Category	Parameter	Mendel Default Values (Expt 1)	Avida Approximation Values (Expt 2)	Altered Mutation Values (Expt 3)
Basic	New mutations per offspring	10	0.01	-
	Fraction of mutations beneficial	1.0×10^{-5}	2.3×10^{-5}	1.0×10^{-3}
	Offspring per female	6	4	-
	Population size (per tribe)	1000	3600	-
	Generations	500	10000	1000
Mutation	Functional genome size	3.0×10^{9}	100	-
	Fraction of mutations having a large effect	0.001	Not applicable	-
	Minimum deleterious mutation effect considered large	0.1	Not applicable	-
	Maximum beneficial effect per mutation	0.001	Not applicable	0.5
	Number of initial beneficial loci	0	-	-
	Fraction recessive	0	-	-
	Combine mutations in multiplicative manner	No	Yes	-
	Fraction multiplicative effect	Not applicable	1	-
	Consider all mutations equal	No	Yes	-
	Equal effect for each deleterious mutation	Not applicable	0.001	-
	Equal effect for each beneficial mutation	Not applicable	5.5	-
	Synergistic epistasis	No	-	-
	Allow back mutations	No	-	-
Selection	Random death	0	0.1	-
	Heritability	0.2	1	0.5
	Non-scaling noise	0		-
	Fertility declining with fitness	Yes	No	-
	Selection scheme	Unrestricted probability selection	-	-

(*Continued*)

Table 2. (*Continued*)

Population	Clonal reproduction	No	Yes	-
	Haploid	No	Yes	-
	Fraction self fertilization	0	-	-
	Initial heterozygous alleles	No	-	-
	Dynamic linkage	Yes	-	-
	Number of chromosome pairs	23	-	-
	Number of linkage subunits	989	-	-
	Dynamic population size	No	-	-
	Population substructure	No	-	-
	Bottleneck	No	-	-
Computation	Tracking threshold	1.0×10^{-5}	-	-
	Parallel processing	No	-	-
	Queuing system	PBS	-	-
	Simulation engine	Fortran	-	-

bonuses of 1.0 (fitness effect of 0) in the environment.cfg file (type=mult, value=1.0).

To examine the role of genome shrinkage in evolution, two sets of 30 replicates were performed, one each for genome sizes of 50 and 100. The default genome contained in default-classic.org was used for size 100, and genomes of size 50 were constructed by removing 50 of the unnecessary NOP-C instructions from the default genome. For each replicate, three alternative scenarios were compared: (1) size neutrality on (default; SNON); (2) size neutrality off (SNOFF); and (3) size neutrality off with mutations to the H-COPY instruction disabled (SNOFF NHC). To disable size neutrality, the avida.cfg file was altered to make base merit constant (BASE_MERIT_METHOD 0). To disable mutations to H-COPY, the instset-classic.cfg file was altered (h-copy 0). Mutations substituting the H-COPY instruction into the Avidian replication loop allow a doubling of the replication rate, and it was found that this process can circumvent the pressure to reduce genome size.

Results

Experiments using Mendel's Accountant

Under Mendel's default settings (Table 2), end-of-experiment fitness declined to an average of 0.76 (± 0.01) after 500 generations. Populations contained an

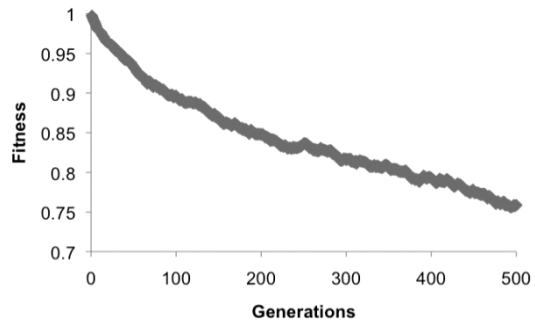
average of 4,906.1 (± 34.3) deleterious mutations and 0.03 (± 0.04) beneficial mutations per genome. Figure 1(A) displays the fitness trajectory of a case study population under these conditions.

Under settings designed to approximate results obtained under Avida's default settings lasting 10,000 generations, fitness increased to an average of 35,730,000 (ranging up to 126,900,000) relative to the ancestral population. These results matched Avida very well, which produces an average fitness increase of approximately 19,749,130. Populations contained an average of 62.7 (± 5.2) deleterious mutations and 8.8 (± 0.9) beneficial mutations per genome. Figure 1(B) displays the fitness trajectory of a case study population under these conditions.

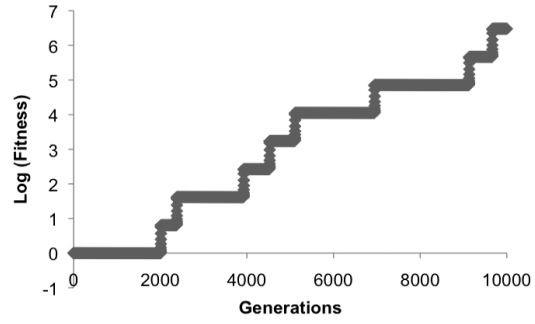
To explore evolution under conditions similar to the default settings but more favorable to beneficial mutation, the proportion of beneficial mutations was increased to 0.001, with a maximum effect of 0.5, heritability was increased to 0.5, and experiment length was increased to 1,000 generations. Under these conditions, end-of-experiment fitness decreased to an average of 0.8 (± 0.1), with an average of 9,739.3 (± 50.2) deleterious mutations and 14.8 (± 3.2) beneficial mutations per genome. Although no end-of-experiment fitnesses were above the ancestral fitness of 1.0, fitness did rise above 1.0 during the course of three (15%) of these experiments, with a maximum of 1.01. One of these cases is shown in Figure 1(C). Here, a high-impact beneficial mutation (fitness effect of approximately 0.2) occurred around generation 270 and rapidly moved to fixation. No other mutations (beneficial or deleterious) reached fixation over the 1,000 generations of this experiment. End-of-experiment fitness was 0.85.

Experiments using Avida

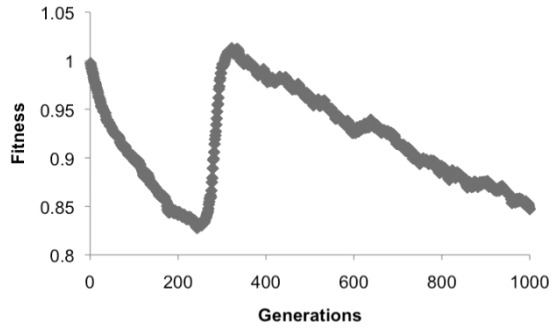
Experiments were conducted to determine how many functional precursors must be rewarded to enable the evolution of EQU in Avida. Results are summarized in Figure 2. EQU never evolved when seven or more precursor functions were neutral. It also never evolved with six neutral precursors under the complex-to-simple scenario, and evolved only once with six neutral functions under the simple-to-complex scenario. These findings expand the results of other studies, in which EQU never evolved when all simpler functions were neutral [14] and certain combinations of neutral functions involving NOR and XOR were found to hinder the evolution of EQU [37]. The evolution of XOR and EQU therefore requires selection for functional precursors, and at least two precursors must be rewarded for EQU to evolve. EQU is more likely to evolve when relatively complex operations are rewarded, because complex operations are less likely to arise without a selective advantage. Hitchhiking of neutral functions to high frequencies (> 50%) was common in these experiments.



(A)



(B)



(C)

Fig. 1. Fitness trajectories of case study populations in Mendel's Accountant. Note that the axes differ. (A) Under Mendel's default conditions, fitness decayed to an end-of-experiment value of 0.76 as a result of the accumulation of approximately 4,897.2 deleterious mutations per individual. (B) Under conditions approximating Avida's default settings, fitness leaped in stages to an end-of-experiment value of 3,014,000 as a result of the spread of eight beneficial mutations with fitness effects of 5.5. Roughly 55.8 deleterious mutations were present per individual. Note that the y-axis is log base 10. (C) Under altered Mendel settings, fitness declined sharply, then leaped to 1.01 following the introduction of a high-impact beneficial mutation (fitness effect of approximately 0.2) around generation 270. This offset the adverse effects of approximately 2,643.4 deleterious mutations that had accumulated in the individual in which it occurred. End-of-experiment fitness was 0.85.

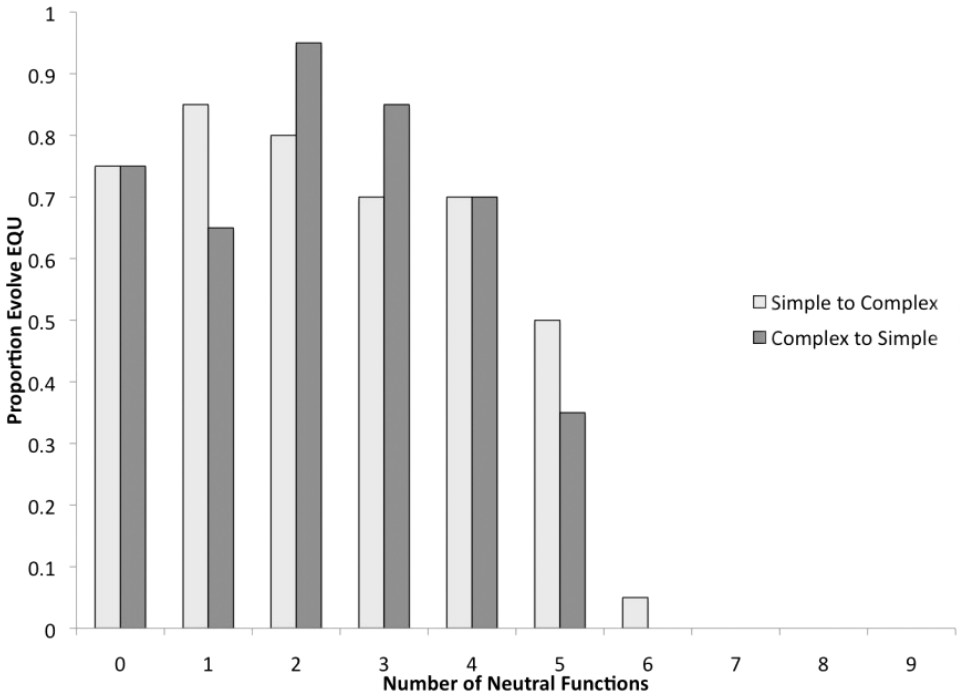


Fig. 2. The effects of selectively neutral precursors on the evolution of EQU. EQU never evolved when seven or more functions were assigned neutral fitness bonuses.

Avida experiments were also performed to examine the evolutionary consequences of selection acting on genome size (results not shown). Fewer functions evolved when size neutrality mechanisms were disabled, and this difference was more pronounced for organisms with smaller genomes. EQU evolved less often, and end-of-experiment fitnesses were lower for both genome sizes of 50 and 100. Though genome size tended to increase somewhat under default settings, this pattern was reversed when size neutrality was not enforced. Therefore, size neutrality artificially facilitates the evolution of complexity in Avida, presumably by maintaining inert genomic code that can be used as raw material for evolutionary innovation.

Discussion

Selection threshold and genetic entropy

A previous study [24] demonstrated that a fitness effect *selection threshold* exists in Avida. The selection threshold is defined as the mutational fitness effect at

which natural selection and random genetic drift contribute equally to the fate of a mutation in the population. Practically, this is the fitness effect for which positive selection successfully captures half of the beneficial mutations that arise. In Avida, this occurs at a beneficial fitness effect of approximately 0.2 (of course, this is a lower estimate of the threshold value, as multiple mutations produce the same logic operations in each run). Moreover, zero new functions evolve when fitness effects are ≤ 0.075, and those that have previously evolved break down. Likewise, experiments with Mendel have estimated a selection threshold of approximately 10^{-4} to 10^{-3} under conditions typical of mammalian populations [5,34].

The selection threshold can act as a barrier preventing the evolutionary origin and maintenance of novel adaptive genetic information. Unless selection is able to "see" the fitness effects of mutations, they will drift in the population as if neutral. Because the majority of mutations are deleterious, the suspension of selection for low-impact mutations strongly favors the fixation of mutations that decrease fitness [32]. The net result is a phenomenon that Sanford [33] has termed *genetic entropy*. When this occurs, purifying selection is unable to counteract the accumulation of low-impact deleterious mutations. Even when rare beneficial mutations cause a selective sweep, they are linked to numerous deleterious mutations across many loci, such that the total number of functional loci decreases. Experiments with Mendel have confirmed that deleterious mutations accumulate in a linear fashion despite selection [5], consistent with biological studies (e.g., with *E. coli* [38]). It is worth emphasizing that the gradual fitness declines shown in Figures 1(A) and 1(C) occur despite the concurrent action of reasonably strong selection; in these cases, selection is simply unable to counteract the net adverse effects of new mutations.

Genetic entropy is not merely a theoretical concern. Numerous analyses have confirmed that the accumulation of slightly deleterious mutations can cause gradual fitness loss leading to extinction in asexual species [12,25,39–42], and similar processes are relevant to sexual species [1,43], including humans [2–4, 44–47]. Lethal mutagenesis of pathogens, due to elevated mutation rates and periodic bottlenecking upon infection, may also be applicable in novel medical approaches [11–13]. Novel means of genetic intervention to reduce mutation rates may be necessary to prevent the extinction of numerous species, though it is unclear whether this would be feasible.

High-impact beneficial mutations

The Mendel case study displayed in Figure 1(C) is an informative example of the effects of high-impact beneficial mutations. A single high-impact beneficial

mutation (fitness effect of approximately 0.2) occurred around generation 270, offsetting the effects of approximately 2,643 deleterious mutations in the individual in which it arose. Beneficial mutations with large effects have certainly occurred in nature. For example, in the presence of an antibiotic, the fitness effect of any mutation conferring drug resistance is so large as to be mathematically undefined, as the ancestral fitness is rendered zero in that environment. Other examples of high-impact beneficial mutations have been reported in viruses in the presence of heat [48]. However, even though these mutations are beneficial in their respective environments, they work by damaging or eliminating genetic information [8], not producing it (see below).

This phenomenon highlights one disadvantage of Mendel's Accountant, namely, that it treats evolution merely as an accounting problem, in keeping with traditional population genetics. Evolution is seen as an exercise in fitness addition and subtraction, without any reference to the underlying genomic mechanisms or architecture. This favors progressive evolution, as it allows single beneficial mutations of large effect to compensate for large numbers of deleterious mutations. This phenomenon is made possible by the infinite allele model, and is precisely the process that Kimura invoked to explain the problem of very slightly deleterious mutation accumulation [27]. However, even though this model is clearly more conducive to progressive evolution, there are several reasons why it is not biologically realistic. Scenarios in which large numbers of deleterious mutations are regularly offset by relatively few high-impact beneficial mutations lead inevitably to shrinkage of the functional genome. If such beneficial mutations are the sole source of progressive evolution, the functional genome must shrink each time evolution takes a step forward (i.e., each selective sweep). This type of change is not sustainable and cannot constitute the sole source of progressive evolution. (For this reason, deleterious and beneficial mutations have heretofore been studied separately with Mendel, with high-impact beneficial mutations being studied as a special case [34].) Instead, plausible scenarios of progressive adaptive evolution must allow the deterministic elimination of most deleterious mutations through purifying selection. Additionally, the gradual accumulation of beneficial mutations through natural selection must have the potential to build every complex biological feature requiring explanation. This process requires qualities of linkage and functional integration that cannot be adequately represented with numerical simulation.

Distribution of mutational fitness effects

The mutational fitness effects implemented under Avida's default settings (1.0 – 31.0) are extremely rare or nonexistent in the biological realm (but see Bull

et al. [48] on high-impact mutations in viruses). This renders published Avida results irrelevant to the great majority of biological mutations. Some readers may object that, while Avida's fitness effects are too large, those implemented in Mendel are too small. On the contrary, it is well established that (1) most mutations are deleterious, and (2) most mutations have very slight effects [28]. For example, a recent study of nonessential ribosomal genes in *Salmonella typhimurium* [49] examined a total of 126 single bp substitutions, revealing that 120 were weakly deleterious and 6 were neutral or nearly-neutral. Average deleterious selection coefficients were 0.0096 and 0.0131 for synonymous and nonsynonymous mutations, respectively. No significantly advantageous mutations were found, and no mutations caused a complete loss of function. In humans, most nonsynonymous mutations in protein coding regions have effects in the range of 10^{-3} to 10^{-1} [28]. Moreover, mutations in functional regions of the genome that are nonprotein-coding are likely to have even smaller effects. Viruses are somewhat exceptional for their high mutational sensitivity. Approximately 20 to 41% of viral mutations are lethal, while viable mutations have an average deleterious fitness effect of 0.10 to 0.13, and many mutations appear neutral [50,51]. However, viable mutations of small effect in viruses are still more abundant than those of large effect, and, as Lind *et al.* [49] have noted, it is possible that such experiments report large numbers of neutral mutations because of assays that lack sufficient sensitivity to detect low-impact mutations.

Junk DNA

A final concern is the existence of inert or "junk" DNA, i.e., genomic material for which mutation does not affect functionality. It does seem possible that many genomic sites play functional roles that are (at least partially) independent of sequence. Avida accounts for this by specifying *no-operation* instructions for 85% of the ancestral genome. Mendel also corrects for this possibility in two ways. First, Mendel models only the effective (functional) genome size, G_e, with 10% as the default. Second, to account for truly neutral mutations ($s = 0$), only the genomic rate of mutations affecting fitness, not the total rate, is used in default settings. Neutral mutations are thus excluded from the mutation rate. Mendel therefore uses a human mutation rate of 10 per genome per generation, rather than the actual mutation rate of approximately 50 – 100 [2,4,52–54], and a genome size of 3.0×10^8 (rather than 3.0×10^9). This genome size limits the magnitude of fitness effects to $1 / (3.0 \times 10^8) = 3.33 \times 10^{-9}$ and larger, allowing selection to act more effectively on mutations affecting fitness. These steps serve to account for neutral mutations and inert genomic material, to minimize the

required computational resources, and to focus the use of Mendel on the effective (functional) genome (though the ability to track neutral mutations is currently being implemented).

The above considerations grant the common assumption that approximately 90% of the genome is indeed "junk." However, this has been subject to challenge for some time [55]. Importantly, the term "junk DNA" was first introduced by Ohno not as a result of experimentation, but rather as a theoretical necessity to avoid the evolutionary barrier of genetic entropy:

> … there seems to be a strict upper limit for the number of gene loci which we can afford to keep in our genome. Consequently, only a fraction of our DNA appears to function as genes. … the moment we acquire 10^5 gene loci, the overall deleterious mutation rate per generation becomes 1.0 which appears to represent an unbearably heavy genetic load. … Even if allowance is made for the existence in multiplicates of certain genes, it is still concluded that, at the most, only 6% of our DNA base sequences is utilized as genes. … More than 90% degeneracy contained within our genome should be kept in mind when we consider evolutional changes in genome sizes. … it is not likely that these sequences came into being as a result of positive selection. Our view is that they are the remains of nature's experiments which failed [56].

This reasoning is common. For example, upon reporting a human mutation rate of 64 mutations per generation, Drake *et al.* [52] note that:

> It is hard to image [*sic*] that so many new deleterious mutations each generation is compatible with life, even with an efficient mechanism for mutation removal. Thus, the great majority of mutations in the noncoding DNA must be neutral.

Following the introduction of the junk DNA concept, many biologists quickly adopted the *selfish DNA* mechanism [57–59] to explain repetitive DNA [60], suggesting that "The search for other explanations may prove, if not intellectually sterile, ultimately futile" [58]. Others resisted this line of reasoning and suggested that repetitive DNA may function in gene regulation [61,62].

A full discussion of the functionality of nonprotein-coding DNA is beyond the scope of this study. However, it is worth noting that junk DNA assumptions have proven to be largely incorrect, while hypotheses suggesting functionality are being increasingly vindicated. Mattick has remarked that the junk DNA dogma may "be a classic story of orthodoxy derailing objective analysis of the facts, in this case for a quarter of a century… [it] may well go down as one of the biggest mistakes in the history of molecular biology" (quoted in reference [63]). A wide range of

evidence now exists which suggests that nonprotein-coding DNA is indeed functional. Nonprotein-coding DNA is often strongly conserved, and over 90% of the human genome is transcribed [28,64,65]. This pervasive transcription includes repetitive elements, which are generally expressed in a tissue-specific manner and perform regulatory roles [66]. Studies that dismiss these results [67] exclude nonprotein-coding RNAs as simply "transcriptional noise." However, it is increasingly clear that such RNAs constitute the majority of the transcriptome and arise abundantly from intergenic regions [68]. Moreover, it has been shown that even mutations at "silent" (synonymous) sites in protein-coding regions can affect fitness and lead to disease [69,70]. Other evidence presented in this volume, such as that for genome-wide sequence patterns [71] and overlapping genomic codes [72], suggests functionality for a large fraction of the genome. If a large portion of nonprotein-coding DNA is indeed functional and sequence specificity is necessary for that functionality, then a very large class of mutations must exist in eukaryotes with very slight effects, smaller than the $10^{-3} - 10^{-1}$ range. These findings revive the concerns of Ohno [56] that humans may experience an "unbearably heavy genetic load" (i.e., genetic entropy), and suggest that human fitness may decline substantially in coming generations [4,45].

Several other mechanisms have been proposed to solve the paradox of how genomes could have survived extinction by genetic entropy [2,3]. These include recombination, back mutation, mutation rate heterogeneity, and synergistic epistasis between deleterious mutations. Such explanations are unlikely. Though theoretically possible, the perpetual back mutation or chance recombination of deleterious mutations into a single genotype represent sequences of events too rare to be plausible. As such, these mechanisms constitute appeals to rare chance events, not in keeping with the law-like operation of natural selection. For example, though uniform fitness effects and high heritability allow selection for mutation count under certain conditions [42], this effect disappears if there is a spectrum of fitness effects, and synergistic epistasis makes genomic decay more severe [35]. One other possibility is that the mutation rate has become elevated in the recent past, though this has not been studied in detail. Further work will be necessary before firm conclusions can be made about these issues and the severity of an impending fitness decline in the human species.

Irreducible complexity and the waiting time to beneficial mutation

All nine logic operations in Avida require the coordination of multiple instructions. Yet it has been shown that seven of these operations (NOT, NAND, AND, ORNOT, OR, ANDNOT, and NOR) arise even without a selective advantage [24], indicating

that they are relatively simple in the Avida environment. By contrast, XOR and EQU require selection for functional precursors. At least two precursors must be rewarded for EQU to evolve. EQU is more likely to evolve when more complex operations are rewarded, because complex operations occur at lower frequencies without a selective advantage. These results are relevant to a central issue in the study of progressive evolution, namely, the waiting time to beneficial mutation. This parameter determines the speed at which adaptation based on novel genetic information can progress. Indeed, billions of mutations have occurred in long-term evolution experiments with *E. coli*, greatly exceeding the number of possible point mutations in its genome of ~4.6 million bp, suggesting that all beneficial one-step mutations have likely been tested [73]. Many adaptive steps therefore seem to require multiple changes, yet the waiting time increases exponentially with each additional genomic site required to change [74]. If the waiting time becomes too great, a particular adaptive step can prevent an adaptive scenario. The Avida results reported here demonstrate that this evolutionary barrier can indeed be prohibitive.

Whether adaptive steps are generally difficult to achieve (i.e., whether they involve multiple genomic sites) is an empirical question that must be addressed by biological studies. On one hand, it has become clear from protein studies that the proportion of amino acid sequences that can be translated into functional proteins is very small. For proteins about 100 amino acids long, there are $20^{100} = 10^{130}$ possible sequences, yet only about 1 in 10^{74} [75] to 1 in 10^{63} [76] are capable of forming functional structures, and most enzymes in an organism such as *E. coli* are over 300 amino acids long [77]. By comparison, it has been estimated that only 10^{120} to 10^{140} quantum particle interactions can have occurred in the entire universe since the Big Bang [78,79], and the probabilistic resources relevant to chemical reactions on Earth allow only about 10^{70} events [80]. As only a minute fraction of these events were amino acid interactions exploring protein space, it is clear that Earth has insufficient probabilistic resources for generating even one functional protein sequence by chance [77].

However, evolution need only wait for single adaptive steps, not entire proteins. Nevertheless, adaptive steps may require mutations at multiple genomic positions. The results reported in this study show that, given the probabilistic resources available in roughly 10,000 generations of an Avida experiment (testing an average of 10.8 billion instructions [37]), the waiting time to beneficial mutation is prohibitive to the evolution of the EQU function when intermediate states are neutral. This is in agreement with results reported elsewhere [14,37]. Turning to biological organisms, we may ask if there are any complex features we should expect not to arise in Earth's history because too many intermediates are neutral or maladaptive. Certainly, many complex biological features seem to require numerous steps (e.g., hundreds of nucleotides).

Though it has been suggested that, counterintuitively, the waiting time to beneficial mutation does not increase exponentially with the number of necessary sites involved [81], the results reported here suggest otherwise. Further, Axe [74] has provided a detailed mathematical treatment of this evolutionary barrier by modeling a bacterial population of 10^9 individuals experiencing 1000 generations each year for all of Earth's history. Under these conditions, if intermediate states are neutral, adaptations involving at most six genomic sites can be expected to arise over the course of history; if intermediate states are maladaptive, adaptations involving at most two sites can be expected. (This hypothetical population strongly favors progressive evolution.) It follows that there is not enough time in Earth's history for mutation to generate any adaptive step involving > 6 genomic sites in any species. Several studies have alluded to these limitations. Orr has noted that "natural selection is essentially constrained to surveying those [sequences] that differ from wild-type by single-point mutations… Double mutations are too rare to be of much evolutionary significance" [82]. Similarly, the eventual stasis observed in long-term evolution experiments with *E. coli* has been explained thus: "Either further major improvements (with fitness increments of more than a few percent in this environment) do not exist or else they are evolutionarily inaccessible (e.g., adaptations requiring multiple genetic changes in which the intermediate states are unfit)" [83].

These concerns are usually discussed in terms of the waiting time to beneficial mutation, and generate spirited discussion in the literature [74,81,84–88]. However, although such calculations are usually interpreted to support the Darwinian mechanism of evolution, they are often incompatible with current theory. For example, Durrett and Schmidt [88] have calculated that the waiting time for a beneficial step involving only two sites, assuming a neutral intermediate, is roughly 100 million years in humans—yet humans are thought to have diverged from chimpanzees within the past 10 million years. Moreover, the challenge of generating the necessary adaptive mutations is complemented by the subsequent challenge of their fixation. This issue, classically known as the cost of substitution, is discussed elsewhere by ReMine [30,89].

The waiting time to beneficial mutation may alternatively be framed in terms of *irreducible complexity* [87,90]. The concept of irreducible complexity has had a great impact on the biological community, with numerous studies attempting to dismiss its importance. Avida has been used for this purpose [14–16,91]. Ironically, the program confirms that the problem is a reality by introducing what Dembski and Marks [92] have called *stair step active information* in order to evolve the EQU function, i.e., it provides information about the target (EQU) by rewarding the necessary building blocks, each of which can be feasibly constructed by mutation alone. This provides an easily scalable fitness landscape, in which successive

steps are advantageous (Figure 3). Thus the EQU function can be built gradually from precursors of lower complexity, each of which is easy to generate through random mutation.

To justify the fitness scheme implemented in Avida, Lenski *et al.* have noted merely that this "is precisely what evolutionary theory requires" [14]. However, evidence suggests that paths to adaptive functions in biological organisms involve many genomic sites, with many of the intermediate states being maladaptive. For example, experiments with TEM-1 β-lactamase have shown that, for homologues of <~66% identity, intermediate protein sequences are typically non-functional when hybridized by random composite. This is the case even when only a fraction

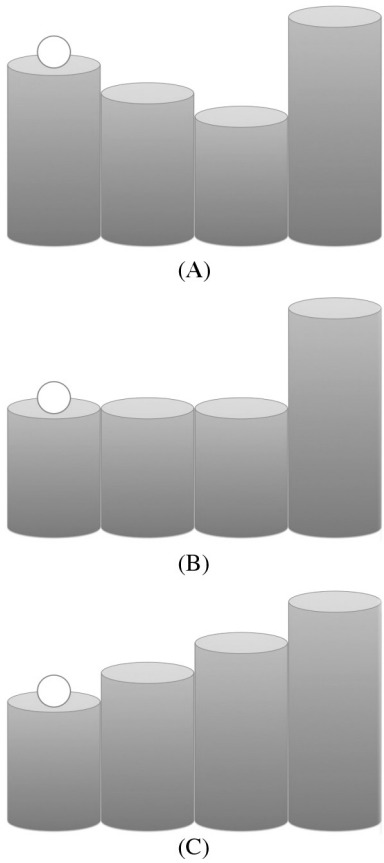


Fig. 3. Simple two-dimensional adaptive landscapes that become increasingly conducive to progressive evolution. The initial state is represented by the white ball. Natural selection can only promote intermediate states that increase fitness (steps "upward"). Shown are landscapes in which: (A) intermediate states are maladaptive; (B) intermediates states are neutral; and (C) intermediate states are beneficial.

of the total protein length is hybridized and sequences exhibit ~90% identity to wild type proteins [93]. These data suggest that contiguous stretches of co-optimized residues exist in biological proteins, and many intermediates between similar proteins may be nonfunctional. Moreover, most readily available adaptive changes are loss-of-function mutations [6,8,9]. These paths will be preferred by selection, as longer adaptive paths confer no advantage until distant targets are reached.

Avida demonstrates that the waiting time to beneficial mutation increases with the number of neutral intermediates, and that certain features cannot be expected to evolve unless simpler precursors are highly beneficial. While the problem of excessive waiting times does not make adaptive evolution formally impossible, it does render certain evolutionary scenarios implausible. Irreducible complexity means *complexity that is not reducible to parts that have a selective advantage on their own*, such that multiple coordinated changes are required without the help of selection. In other words, adaptations requiring multiple mutations are simply less likely, and the waiting time for their occurrence is greater. As Avida shows, this barrier can be prohibitive to progressive evolution. Unfortunately, computer simulations cannot provide a thorough understanding of the waiting times to adaptive steps in biology. As more is learned about the distribution of mutational fitness effects [28,94] and the genetic basis of adaptive change [8], the answers to these problems will become clearer.

Reductive evolution

Reductive evolution can entail an advantageous reduction in either genomic material or gene expression [9,95–97]. In both instances, organisms benefit from eliminating superfluous energy expenditure. The pressure to eliminate excess genomic material has been termed “compression selection” [97] and has been demonstrated in several biological systems. For example, in a classic serial transfer experiment with Qβ bacteriophage, replication rate increased by a factor of 15 and genome size decreased by 83%, with biological competency lost by the fifth transfer [95]. Some reductions in genome size have also been observed in evolution experiments with *E. coli* (e.g., reduced by 1.2% [38]). Although compression selection may not be strong in organisms for which the cost of maintaining and replicating DNA is a small fraction of the cell’s total energy budget [26], it is clearly operational in some smaller systems.

More frequently, reductive evolution proceeds via the elimination of unnecessary gene expression. Gauger *et al.* [9] have shown that, because these types of mutations are relatively common [96], reductive evolutionary paths are usually

taken even when short progressive paths are available. Long-term evolution experiments with *E. coli* have provided numerous examples of this process. One mutation that reduced *glmUS* expression by 10% was highly (~5%) beneficial [98], as was another mutation that reduced *spoT* expression [96]. Moreover, Cooper & Lenski [6] have reported that unused catabolic functions decayed as fitness increased in 12 experimental populations of *E. coli*, reducing diet breadth. One mutation, loss of the ability to use D-ribose, occurred in all 12 populations in the first 2,000 generations as a result of highly advantageous deletion mutations, increasing fitness by ~1.4% [7].

These studies indicate that the reduction of biological information can be highly advantageous. Recent reviews [8,10,99] have reported that the majority of studied adaptations involve the loss of traits and the reduction of genetic information. Whether a mutation is beneficial may depend critically upon the environment in which it arises (e.g., whether nutrients are available or antibiotics are present), meaning that the effect of a mutation on genetic information cannot be inferred from relative growth rates alone. Reductive changes are often (though not always) associated with fitness loss in other environments [100]. For example, the ability to transport (and therefore metabolize) citrate in oxic conditions evolved in one *E. coli* population after about 31,000 generations of experimental evolution [73]. However, the mutant is inferior on glucose, likely because it involves the alteration of a citrate transporter that normally operates only in anoxic conditions. Other decreases in channel constriction have also conferred advantages [100]. Similarly, Bull *et al.* [48] have reported high-impact beneficial mutations in the virus ϕX174 that increase fitness in an inhibitory, hot environment, but all of which reduce fitness at normal temperatures.

The Mendel software uses the classic infinite allele model, and so is not conducive to a straightforward study of the evolution of genome size. On the other hand, Avida is very tractable for this purpose. Importantly, the biological examples of adaptation discussed above involve reductive evolution, in contrast with adaptation under Avida's default settings, where novel functions arise and provide extreme advantages. It is somewhat surprising that Avidian populations achieve in only 10,000 generations what *E. coli* populations fail to glimpse in 50,000 generations. This occurs partly because artificial size neutrality mechanisms were introduced into the Avida software as a means of preventing the pressure of reductive evolution:

> The advantage gained by shrinking the code is so dramatic, however, that cells might even choose to shed sections of code that trigger moderate bonuses. Such a method certainly provides for very efficient optimization while discouraging the evolution of complex code by magnifying the barrier to neighboring local minima

> in the fitness landscape. ... Another possibility is to distribute CPU time in a manner proportional to the length of the code. This is the *size-neutral* scheme also used in tierra. The resulting fitness landscape is intuitively much smoother; strings that behave in the same way but differ in length of code are degenerate as far as their replication rate is concerned and far-lying regions in genotype-space can be accessed easily. Clearly this mechanism is much more conducive to the evolution of complexity… Note that enforcing size neutrality is strictly speaking un-biological, as it is known that self-replicating strings will shed all unnecessary instructions if given the opportunity. In avida, size neutrality is necessary in order to jump start the evolution of complexity [21].

Although the results reported here suggest that size neutrality is not strictly necessary for the evolution of complexity in Avida, it certainly improves success. Therefore Avida confirms that reductive evolution is also a potential barrier to the evolution of novel genetic information. Moreover, this barrier will be more prohibitive if new functions confer more realistic fitness bonuses.

The barrier that compression selection poses for progressive evolution is most extreme for small genomes. These results demonstrate that, when size neutrality is disabled, larger genomes evolve more logic operations than smaller genomes. This occurs because large genomes contain more superfluous material that may be used as raw material for evolutionary tinkering. If highly beneficial adaptations arise before prohibitive genome shrinkage occurs, the pressure to maintain highly beneficial functions can prevent further shrinking, which is only slightly adaptive. The large default rewards implemented in Avida dwarf the advantages gained by shrinking the genome, so evolved functions are retained once a minimal genome size is reached. This appears to be another case in which the waiting time to beneficial mutation is an important consideration, as innovations that require too much time may not arise before the extraneous genomic raw material is removed by selection.

Conclusions

This study used the evolutionary simulations Avida and Mendel's Accountant to examine three barriers to the production of genetic information by the neo-Darwinian mechanism of mutation and natural selection: (1) the selection threshold and resultant genetic entropy; (2) the waiting time to beneficial mutation, i.e., irreducible complexity; and (3) the pressure of reductive evolution, i.e., the pressure to shrink genomes and to disable unnecessary functions. The apparent disparity between the two programs results primarily from differences in default

settings. When used with similar settings that reflect biological systems, both confirm that all three of the aforementioned barriers can prevent the progressive evolution of novel genetic information. Though neutral or even maladaptive changes (e.g., gene duplication) are often considered "complex features" [16,26,101,102], it is important to note that this is not synonymous with genetic information. Even adaptive changes typically eliminate genetic information within a genome [8,10].

The evolutionary barriers discussed in this report are not merely of theoretical importance. As Lynch [4] and others [2,3,44–47] have shown, the human species faces a potentially lethal threat from the accumulation of very slightly deleterious mutations. Additionally, the lethal mutagenesis of pathogen populations may be applicable in novel medical approaches to cure infection and thwart pandemics [11–13]. It may be the case that novel means of genetic intervention to reduce mutation will be necessary to prevent the extinction of numerous species, including our own.

While both Avida and Mendel demonstrate that neo-Darwinian evolution may be a theoretical possibility under certain conditions, both programs also suggest that it is not a plausible explanation of most biological information. Such computational approaches can provide informative predictions of the values that key parameters (e.g., the distribution of mutational fitness effects) must assume if neo-Darwinian theory is viable. However, biological studies will be necessary to determine the values that these parameters actually assume in nature.

Digital genetics pioneer Thomas Ray made the following point about computational evolutionary studies:

> To understand the biology of digital organisms requires a knowledge of the properties of machine instructions and machine language algorithms. ... there exists a complementary relationship between biological theory and the synthesis of life. Theory suggests how the synthesis can be achieved, while application of the theory in the synthesis is a test of the theory. If theory suggests that a certain factor will contribute to increasing diversity, then synthetic systems can be run with and without that factor. The process of synthesis becomes a test of the theory [103].

It would seem, then, that the "unbiological" [21] parameters required to make the neo-Darwinian mechanism succeed in computational experiments should call the biological theory into question. As science commentator David Berlinski has remarked, "Computer simulations of Darwinian evolution fail when they are honest and succeed only when they are not" [104]. As more is learned about the genetic basis of adaptive change and the distribution of mutational fitness effects, the severity of these concerns for theory and medicine will become clearer.

Addendum

Because of a delay in this work's publication, several new relevant studies are not discussed therein. First, the authors have expanded upon the topic of numerical genetic simulation in another paper (Sanford, J.C., Nelson, C.W.: The next step in understanding population dynamics: comprehensive numerical simulation. In: Fusté, M.C. (ed.), Studies in Population Genetics, InTech, pp. 117–136). This paper reviews population genetic simulations, comments on Avida, and discusses general population genetic principles revealed by Mendel's Accountant, especially as concerns fixation. The selection threshold concept is also further developed, as first discussed by Nelson and Sanford in reference 24. Two papers utilizing the Avida platform have been released. The first (Adami, C., Qian, J., Rupp, M., Hintze, A.: Information content of colored motifs in complex networks. Artif Life 17, 375–390) traces network evolution in Avidian organisms, implementing typical parameter values. Fitness increases 100,000-fold over 90,000 updates (approximately 9,000 generations), reflecting the program's high-impact beneficial mutations. Another study (Clune, J., Pennock, R.T., Ofria, C., Lenski, R.E.: Ontogeny tends to recapitulate phylogeny in digital organisms. Am Nat 180, E54–63) also used default fitness effects. To our knowledge, biologically meaningful fitness effects have not been used, and direct mutational paths to complex instruction combinations are implemented. Thus, Avida researchers have not yet addressed concerns (e.g., those first raised in reference 24) regarding the relevance of Avida to biological organisms.

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