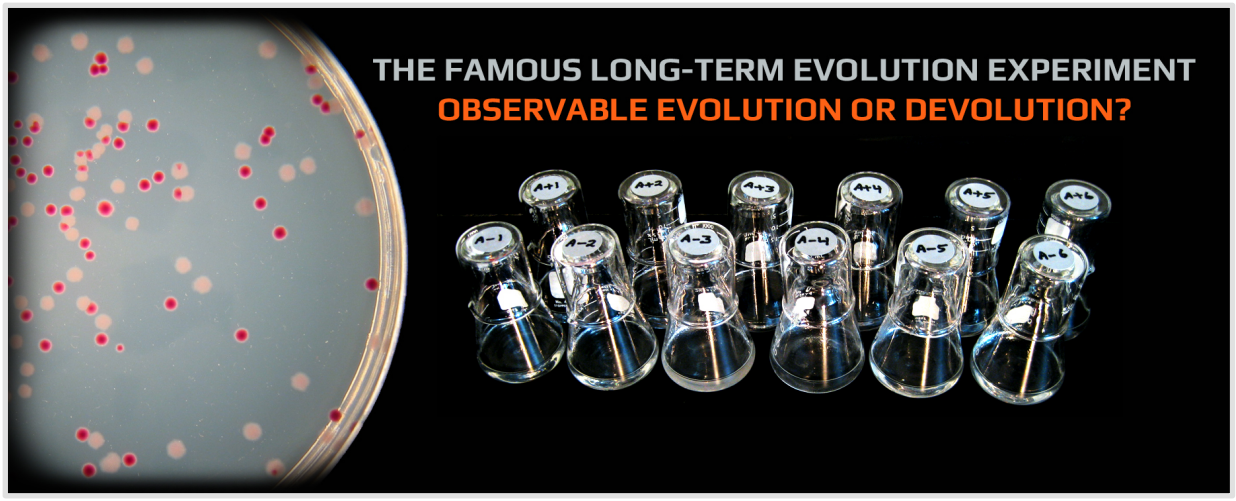

The Most Famous Evolution Experiment of All Time Shows that Evolution Goes the Wrong Way

The most significant evolutionary experiment of all time was started by Dr. Richard Lenski at Michigan State University on February 15th 1988. That experiment continues to this day. It has been argued that this experiment shows that a strain of the E. coli bacterium is undergoing dramatic forward evolution, allowing us to document in the test tube the same type of macroevolution that allows one type of life to morph into a fundamentally different type of life (such as ape-to-man evolution). Some have argued that the resulting bacterial strains have actually morphed into entirely new species. It has been said this experiment lays to rest any doubts about macroevolution. More specifically, it is widely claimed that this experiment proves that the neo-Darwinian mechanism (random mutations plus natural selection) is fully sufficient to explain the origin of all forms of life, including man.

In this paper we will document that this experiment is indeed extremely significant, but for the opposite reasons. The bacteria have not experienced forward evolution, but rather the net effect has been reductive evolution (evolution going backwards). It is true that there has been some adaptation to the new artificial environment, but this has been primarily due to loss-of-function mutations. Such adaptive fine-tuning can at best be called microevolution, and has been accomplished through a net loss of information (broken genes/disrupted gene regulation). In all 12 experimental populations, the functional bacterial genome has shrunk - containing less total information. The resulting bacterial strains are still the same species, but have been seriously damaged. These disabled strains would quickly go extinct in any natural environment.

If any experiment could have validated large-scale macroevolution, it would have been this one. This famous experiment powerfully demonstrates that the mutation/selection process has very serious limitations. Even given huge populations and vast number of generations, all that was accomplished was a trivial amount of adaptive microevolution. Even while some

superficial fine-tuning has been happening at just a handful of genomic sites, significant genetic damage has been accumulating throughout the rest of the genome, due to many slightly harmful deleterious mutations that cannot be selected away. This means that in the long run the net effect will be degeneration. This famous evolutionary experiment proves that in deep time, even given a model population that is optimal for validating evolution, populations do not evolve – but instead devolve.

The Nature of the Long-Term Evolution Experiment (LTEE):

Nearly 30 years ago, Dr. Lenski and his colleagues established 12 genetically identical *E. coli* populations from the same ancestral strain. Since then, each population has been grown in its own separate fluid-filled flask with glucose as the carbon source (and with citrate added as a type of buffer). For all this time, each strain has been transferred to fresh nutrient media daily. Each day the glucose and other nutrients in the medium are depleted, so 1% of each population must be transferred to a new flask with fresh medium – allowing continued growth. The 12 replicate populations have averaged 6.6 rounds of cell division (generations) each day.[7] Every 500 generations a sample of each population is stored in a freezer – creating what Dr. Lenski calls his "frozen fossil record." As of 2014, there have been over 60,000 bacterial generations since the experiment began, with frozen samples filling six freezers. At different times throughout the experiment the original ancestral strain was retrieved from the freezer and was grown together in a mixed culture with each of the 12 continuously growing populations (the so-called "evolving" strains). The purpose of this was to do head-to-head fitness competition tests to determine if the continuously growing *E. coli* populations had developed a competitive edge over the ancestral strain (note: "fitness" was always measured based on growth rate compared to the ancestor in the artificial environment). Lenski and collaborators hoped to experimentally demonstrate that all 12 *E. coli* populations were evolving continuously over time. In an attempt to monitor this, the genomes of the 12 so-called "evolving" strains were periodically sequenced (at generations 2k, 5k, 10k, 20k, 40k, etc.) and compared to the ancestral genome. This gave Lenski and collaborators a chance to observe and analyze the spontaneous mutations that contributed to adaptation and fitness gain.

Over the years since the experiment began, the results have been published in numerous scientific journals (LLEE's full list of publications: http://myxo.css.msu.edu). Mean fitness was reported to increase rapidly in the first few thousand generations and continued to improve, though more slowly, for 20,000 generations with mutations accumulating at a constant rate.[1,10,19,20] During this time the 12 *E. coli* populations became better adapted to the glucose medium and experienced a total fitness gain of 67%; this means that after 20,000 generations, the descendants were able to grow 1.67 times faster than the ancestor.[10,12,13] After 20,000 generations fitness had largely leveled off with few adaptive changes until about 31,500 generations into the experiment. At that time, one of the populations gained the ability to uptake the citrate within the medium into the cell, to be used as a nutrient.[5] Previous to that generation the *E. coli* only utilized glucose as its carbon source. This new adaptation was caused by several complementary random mutations, which allowed unregulated (continuous) expression of the gene controlling citrate uptake.

This was exciting news to evolutionists and generated a lot of hype throughout the scientific community. This mutant feature was said to be a "key evolutionary innovation".[6] *New Scientist* announced, "A major innovation has unfurled right in front of researchers' eyes. It's the first time evolution has been caught in the act of making such a rare and complex trait."[16] Since then the experiment has continued with few noteworthy developments (Figure 1). To date, the experiment has passed the 60,000 generation mark with the 12 *E. coli* populations averaging a total fitness gain of 70% – just a few percentage points higher than the gain of fitness observed at 20,000 generations.[26] In Lenski's mind, these populations will continue to undergo evolutionary improvement indefinitely, with mean fitness increasing "without bound."[33] A recent *Science News* article echoes the same optimism writing, "After 25 years and 58,000 bacterial generations, Lenski's bacteria are still growing, mutating, and evolving."[26]

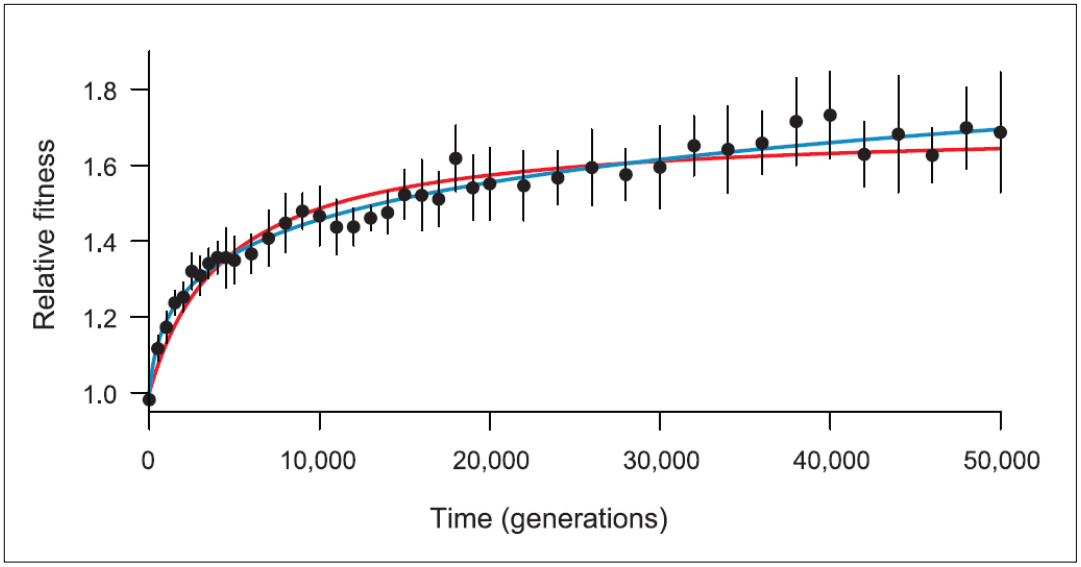

Figure 1. The most dramatic fitness gains occurred within the first few thousand generations.[19] After 2,000 generations meant fitness *increased* 37% *(1.37)*.[20] By 20,000 generations *the* mean fitness increased 67% *(a fitness of 1.67)* and had largely leveled off, with few adaptive changes thereafter. *After* 50,000 generations fitness improved was almost imperceptible (3%). To date, the *E. coli* populations have improved by a total of 70% relative to the ancestor. Note: The red line represents the best-fit curve using the power-law model, based on the complete data set over the course of 50,000 generations. It shows fitness has reached a near maximum and is not expected to significantly increase. The blue line represents an alternate way of interpreting the data (using the hyperbolic model) that is favored by Lenski and colleagues.[33] It suggests fitness will increase without bound, implying uninterrupted evolutionary advance. The problem with this interpretation is that their definition of fitness is growth rate, and obviously *growth rate cannot increase without limit.* (Image from Wiser, *Science*, vol. 342, 2013)

Crucial Questions Regarding the Long-Term Evolution Experiment (LTEE):

In order to understand what the LTEE is really teaching us, we have to ask a series of key questions:

1. Has there been real adaptation? Yes, there has been real adaptation (see Figure 1). This was a foregone conclusion from day one – virtually all biologists know bacteria will undergo some adaption when placed in a new environment. Such adaptation is trivial in nature and

merely *fine-tunes* a few tiny parts of the genome. If mere adaptation was the question, almost 30 years of work, and the careers of many scientists were wasted proving what was already known. At the onset of this enormous experiment the real questions were; "Would there be a net gain or a net loss of total biological functionality?"; "Would there be a net gain or a net loss of genetic information?"; and "Would a significantly new form of life emerge (i.e., would there be macroevolution)?" As we will see, there has consistently been a net loss of function, a net loss of information, and the *E. coli* bacteria clearly remain *E. coli* bacteria – a new form life has certainly not emerged. At best, the genetic changes that have been observed reflect fine-tuning of a few pre-existing genes (microevolution). More fundamentally, in the bigger picture there has been genetic degeneration, which is the consequence of *increasing genetic entropy*.

2. Is the Lenski measure of fitness valid? The best definition of fitness is "total functionality". As we will see, when Lenski et al. examined the "beneficial" mutations that caused adaptation of the 12 "evolving strains" of the bacteria, in each case the mutations involved the loss of a biological function. So all 12 of the "evolving" populations have continuously been losing functions that are required in the real world. In other words, all 12 populations have actually been devolving. The strains superficially have seemed to be getting better – but only in the context of the artificial lab environment, the artificial medium, and the artificial competition assay. Yet even using Lenski's highly artificial definition of fitness, the growth rate advantage of the derived strains over the original strain was very modest (the relative growth rate did not even double). Many evolutionary experiments have shown much larger increases in fitness in much less time.[18,28]

It is important to make the distinction between narrow-sense fitness versus broad-sense fitness (true fitness). Lenski's method of measuring fitness was to test a single trait (growth rate) in a single environment (artificial bacterial cultures, in shaker flasks, on an artificial medium). In this context Lenski's populations have only *increased fitness in a narrow and artificial sense*. However, in the fuller meaning of fitness, all twelve of the populations have experienced a net loss-of-function and so they have reduced *total functionality as needed in the real world*. Real-world total fitness requires functionality in the ever-changing real world, where there are many diverse environmental challenges. Thus, when fitness is defined more broadly to reflect total biological functionality, the 12 so-called "evolving" populations actually experienced an overall decline in fitness.

3. Will the populations grow faster and faster without limit? Most biologists understand that given a new environment, adaptation routinely happens, leading to adaptive optimization and then stasis (or eventual decline). It is remarkable that Lenski and collaborators expect ever-increasing growth rates without limit – because this is biologically impossible. This is especially true in light of their own data, which clearly reveals diminishing returns (Figure 1).[33] As we will see, almost all of Lenski's observed adaptations were due reductive evolution (deleting or silencing genes not essential in the artificial environment). Yes, it will certainly take a long time to fully reduce the genome to its minimal functionality (yielding maximal growth rates), but it should be obvious that there must be a natural limit as to how much of the genome can be stripped away, and how fast the bacteria can grow. Almost all of the

adaptive reductions in the *E. coli* genome appear to have occurred in the very early generations, indicating that most of the significant shortcuts to faster growth rate were quickly exhausted. As we will see, in addition to the very rare reductive mutations that have enabled somewhat faster growth rates, there are a host of *nearly-harmless* mutations accumulating in all parts of the bacterial genome which have no benefit at all, yet escape purifying selection (elimination from the population) - because they have such small effects. Hence the continuous and unabated accumulation of such slightly harmful mutations must eventually result in a very gradual but unavoidable fitness decline, due to continuously increasing *genetic load*.

4. Are the observed adaptive mutations creating or destroying information? To answer this we need to examine each of the beneficial mutations that were responsible for the most noticeable gains in fitness among the 12 so-called "evolving" strains. By sequencing their genomes, Lenski's team of researchers was able to track all the mutations that arose in the experiment. Each mutation was catalogued and reported in various published papers along with their associated fitness effects. This data allowed Lenski and collaborators to identify the beneficial mutations that primarily contributed to fitness improvement – and determine precisely when they arose in the genome (Table 1). In the first 20,000 generations there arose roughly 1.5 billion mutations per population. Of these, only a handful of were shown to be beneficial.[21] These few documented beneficial mutations accounted for almost all (96%) of the gain in fitness observed in this early phase of the experiment.[1] We need to carefully examine these few mutations (especially the mutations that arose repeatedly in multiple populations), because they lie at the heart of the experiment's major claims.[19] These mutations will tell us if the bacteria are actually evolving or devolving. When these key mutations are examined (Table 1), it becomes very clear that these "beneficial mutations" consistently involved loss-of-function (broken genes/disrupted regulation), indicating that LTEE is not undergoing "rapid evolution" but rather, *rapid degeneration*. This is the exact opposite of macroevolution.[19]

Table 1. The ten "beneficial" mutations that caused almost all of the observed fitness gains.
References: 11,1,27,3,35,30,4,34,12,13,31,25,29

Gene or Region	Function	Population(s)	Generation Established	Fitness Gain (%)	Mode of Adaptation
topA	DNA topoisomerase 1	10 of 12	2,000	13.3	Reduction
pykF	Pyruvate kinase	12 of 12	5,000	11.1	Inactivation
spoT	Stringent response regulator	8 of 12	2,000	9.4	Reduction
nadR	Transcriptional regulator	12 of 12	5,000	8.1	Inactivation
glmU promoter	Cell-wall biosynthesis	1 of 12	5,000	4.9	Reduction
fis	Nucleoid-associated protein	10 of 12	10,000	2.9	Reduction
rbs operon	Ribose catabolism	12 of 12	2,000	2.1	Deletion
malT	Transcriptional regulator	8 of 12	5,000	0.4	Deletion/Reduction
pbpA-rodA	Cell-wall biosynthesis	6 of 12	2,000	---	Reduction
citT	Citrate transporter	1 of 12	31,000	---	Loss of regulation

Upon careful review of the LTEE published scientific papers, all of the fitness gains attributed to the 10 documented beneficial mutations were due to deletions, impairments,

inactivations, reduced enzyme activity, reduced gene expression, and loss of regulation (Table 1). By their very nature, random mutations scramble genetic information and lead to functional degradation. But such mutations can still be advantageous in a narrow sense. These loss-of-function mutations were clearly shown to be beneficial to the *E. coli* populations that carried them (Table 1). This is because the bacteria were growing in an artificially stable environment on a glucose-limited diet, which only required the use of a limited number of genes. Any gene functions not required in that static artificial environment could be inactivated, reduced, or deleted to save energy, thereby allowing more rapid growth in that special environment. For example, within the first 2,000 generations, mutations arose in 8 out of 12 of the populations within a gene known as *spoT*, involved in the regulatory control of multiple genes. One of the genes controls the expression of the *flg* operon, which encodes the bacterial flagellum – the tiny whip-like cord that propels bacteria through their aqueous environment. Researchers found that this type of mutation *reduced* the expression of the flagellum-encoding genes, which turned out to be advantageous to *E. coli* bacteria growing in shaker flasks. In the *Proceedings of the National Academy of Science*, Cooper and colleagues explain how the advantage was obtained.

> "First, the array data show that the *spoT* mutation lowers the expression of the flagella-encoding flg operons. The ancestral strain used in the evolution experiment was non-motile, the selective environment lacked physical structure, and the production of flagella is known to be costly. Hence, reducing the expression of these genes could be beneficial."[12]

Since the flagellum encoding genes were unnecessary for the non-motile bacteria and burdensome to maintain in the artificial environment, the reduction mutations conferred a 9.5% improvement in fitness. Other unused genes were also reduced, inactivated, and deleted. Early in the experiment a mutation resulted in the deletion of the genes responsible for the breakdown of the sugar ribose (*rbs* operon) in all 12 *E. coli* populations. In the *Journal of Bacteriology*, Cooper and colleagues report,

> "Twelve populations of Escherichia coli B all lost D-ribose catabolic function during 2,000 generations of evolution in glucose minimal media. … At the molecular level, the loss of ribose catabolic function involved the deletion of part or all of the ribose operon (*rbs* genes)."[11]

Although ribose catabolic function was permanently lost in all 12 populations, the mutations were still considered *beneficial* in terms of their fitness effects. This is because the ribose digesting genes were not necessary for the bacteria while growing on a glucose-only diet. By economizing resources, the deletion of the ribose operon conferred a 2.1% fitness gain. A similar situation occurred with the maltose digesting genes. In 8 out of 12 of the *E. coli* populations, mutations arose in the MalT gene (which encodes a transcriptional activator) resulting in the loss-of-function of the maltose catabolic pathway.[25] Just as with the deletion of the ribose genes, the *malT* knock-out mutations offered a selective advantage to the *E. coli* bacteria. So yes, the catalogued mutations were beneficial (at least based on how fitness was narrowly defined) but it was at the expense of *losing* gene functions. As the *E. coli*

bacteria became more specialized on a glucose diet, they simultaneously suffered functional decay, becoming far *less fit* in virtually every other dimension. In the journal *Nature*, Cooper and Lenski acknowledge this, writing;

> "Note that adaptation to glucose may result from mutations that either improve some aspect of glucose catabolism or eliminate unnecessary functions that are costly to fitness in glucose. In either case, *the mutations improve fitness on glucose while adversely affecting performance on other substrates* [emphasis ours]."[10,]

This is a very revealing statement. It means that if fitness was defined more broadly (as it should), the results of the long-term experiment would have to be interpreted very differently. In LTEE fitness was defined very narrowly based on a single trait – the ability to compete for glucose. This does not reflect the bacteria's true fitness.

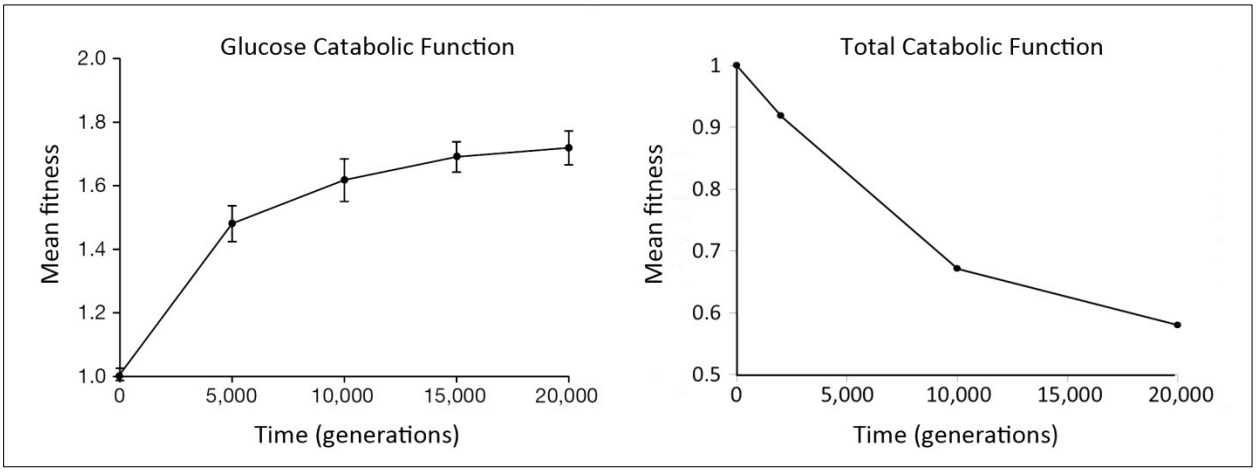

Figure 2. These two plots demonstrate the concept of "trade-offs" in the long-term experiment. The elimination of unused catabolic pathways was advantageous to the *E. coli* growing on a glucose-limited diet (A). But in any other environment, such a drastic decline in catabolic breadth would be detrimental to the so-called "evolved" *E. coli* populations (B). So in terms of total biological functionality (which is a true measure of fitness), the 12 *E. coli* populations had significantly declined in overall fitness. [Trajectories from Cooper & Lenski, *Nature*, 2000; the data from Figure 4 in *Nature* article[10] was re-plotted with a linear Y-axis scale for comparison, shown in image (B).]

Fitness is a multidimensional trait that encompasses total biological functionality – all traits, all genes, and all catabolic pathways – not just proficiency in glucose consumption. Biologists understand this. In the journal of *Genetics*, Pelosi and colleagues write, "…fitness is a complex phenotypic trait that emerges from *all the interactions* among the molecular components of an entire organism."[25] When fitness is defined properly to reflect overall performance (i.e., total catabolic function) a dramatic *decline* can be clearly seen that mirrors the bacteria's improvements on glucose (Figure 2). If the 12 so-called "evolved" *E. coli* populations were removed from their artificially stable environment and placed in the wild, they would almost instantly go extinct. Redefining fitness to reflect total biological functionality means that the 10 or so mutations that were interpreted as "beneficial" can then be seen for what they really are – *deleterious mutations* (having long-term detrimental effects). Lenski recognizes this:

> "...the losses of performance on other resources result from tradeoffs, in which the same mutations that are beneficial in the glucose environment have detrimental effects in other environments."[21]

The 12 *E. coli* populations became better adapted to use glucose only by suffering substantial loss of diverse functions. Leading evolutionary scientists involved in LTEE acknowledge this. In discussing the early generations of the most dramatic improvements, Cooper and Lenski describe the adaptations occurring through "performance losses", "parallel decay", "reductions", and "functional losses".[10] Evolutionary biologist, Robert Holt (PhD, Harvard University) describes the mode of adaptation similarly as "functional degradation".[28] He likens the "evolved" *E. coli* bacteria to blind cave fish. Certain species of fish that dwell in dark caves have been shown to undergo functional degradation through the loss of eyesight. It is costly for the fish to express genes encoding eye apparatuses when they don't need to see. By eliminating or reducing their expression, the fish are able to conserve limited energy and resources that can be allocated to meet more urgent biological needs. Just as with Lenski's *E. coli* bacteria, the blind cave fish have become specially adapted to their habitat by *losing* biological functions. "Use it or lose it" is a fitting expression for what is happening in the LTEE.[17] Scientists in the *American Society for Microbiology* recognize the same *beneficial-decay* phenomenon in the long-term experiment. They write,

> "The nutritional environment of the LTEE consists of a minimal medium with glucose ... Owing to this simple environment, certain functions cannot be lost, including, for example, the production of amino acids. However, some functions are dispensable, including those involved with using alternative resources (e.g., the loss of the ability to grow on ribose) and those necessary for thriving in natural environments. Thus, the simple flask environment – like a host organism – provides environmental constancy and protection that allow certain functions to be discarded. In doing so, the cells may save energy, thereby providing a competitive advantage; even without that benefit, any unused functions tend to decay or be deleted by ongoing mutations."[28]

This makes sense. Roughly half of the *E. coli's* genome consists of conditionally-required genes and alternate metabolic pathways that generally have no utility in a given fixed environment. Such genes can be thought of as being excess weight that can be reduced, inactivated, or deleted to enhance performance in a narrow context. The *E. coli* populations that became glucose specialists may be compared to a racecar that has been stripped down to its bare essentials (Figure 3). Although this may give the car a competitive edge in a drag race (becoming lighter and therefore faster), it is at the expense of removing otherwise useful parts and disabling all non-essential functions. Researchers involved in the long-term experiment have misleadingly called this type of change "adaptive evolution."[12,19] In reality it is nothing more than functional degeneration leading to habitat over-specialization. This only leads to evolutionary dead ends. In the long run this is *adaptive degeneration* – not evolution. Even given millions of years, this type of change will never lead to large-scale evolutionary innovations.

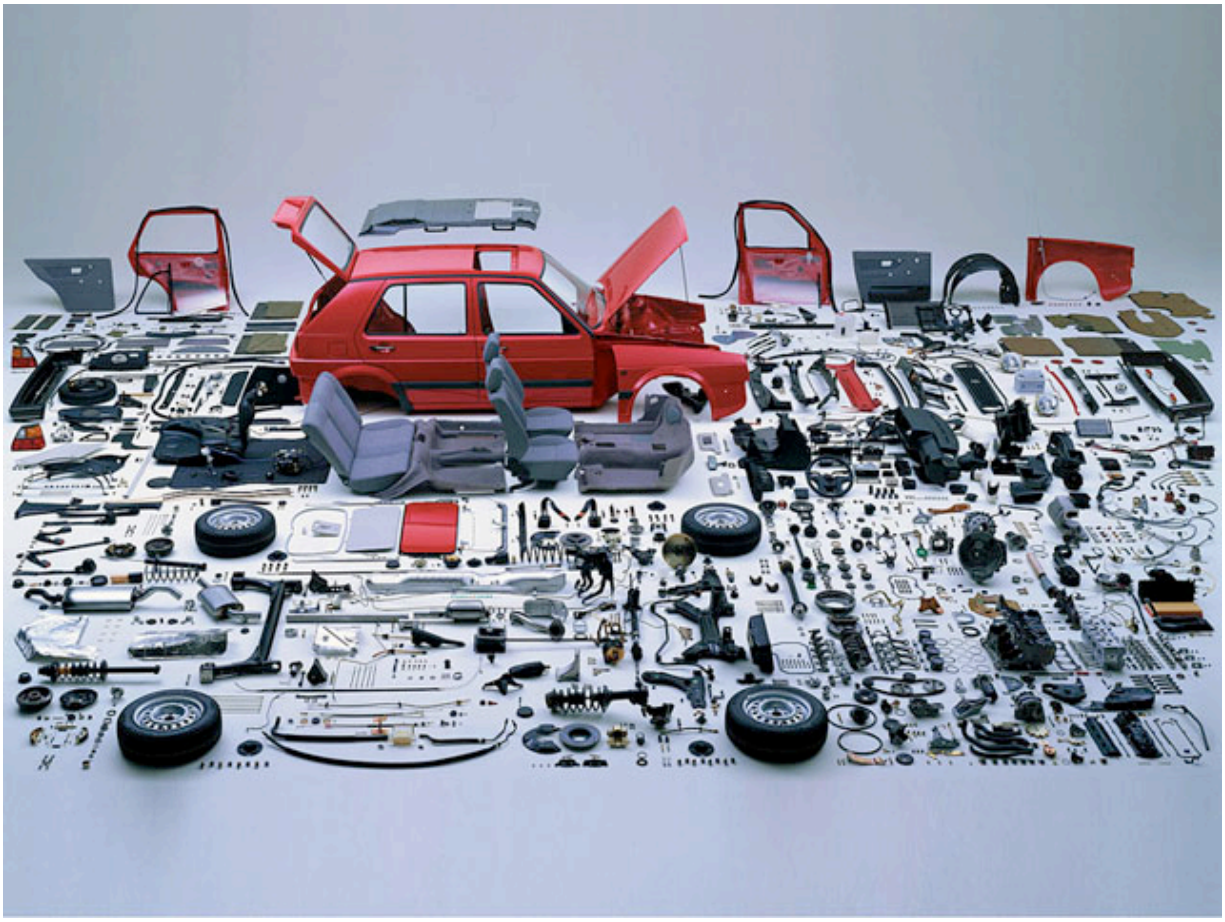

Figure 3a. A car is made up of thousands of functional parts, but a large fraction of these are non-essential in any given circumstance. The same is true of the *E. coli* genome.

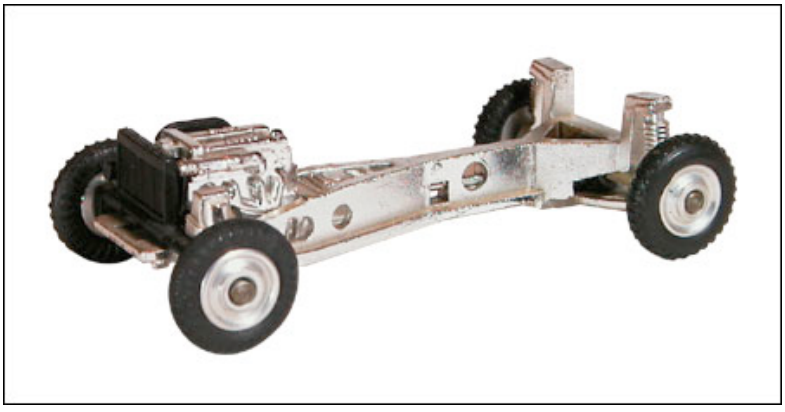

Figure 3b. If speed was the only measure of car functionality, fitness would increase as the car was stripped down to its bare essentials. Even the steering wheel would become expendable. But does this reductive process explain the origin of automobiles? In biology, is this type of reductive streamlining really evolution? Can it explain how the *E. coli* genome arose?

To close this section, we would like to cite the latest paper from Raeside, Lenski, et al. which not only shows that reductive evolution is what is really happening in the LTEE, but that Raeside, Lenski, et al. are fully aware of this fact. The authors state:

> "We identified a total of 110 rearrangement events in the 12 40,000-generation clones, including 82 deletions, 19 inversions, and 9 duplications. Large deletions were the most frequent type of rearrangement, and they were found in all 12 populations, ranging in size up to ~55 kbp."[28]

They go on to report that because of the overwhelming preponderance of deletions, the *E. coli* genome size was reduced in 10 of the 12 clones by amounts ranging from 0.9% to 3.5% of the ancestral genome size. They conclude:

> "...generally, the deleted genes have functions that are not used under the conditions prevailing during the LTEE. These deletions might have conferred higher fitness by eliminating unnecessary and costly gene expression..."[28]

We conclude that there is overwhelming evidence that the famous LTEE merely demonstrates reductive evolution (evolution going backwards).

[**Note:** For a more detailed analysis of the exact nature of the adaptive loss-of-function mutations, see Appendix 1.]

5. Is this type of *reductive evolution* unique to the LTEE? Reductive evolution is widely understood and is very commonly seen. Most of the textbook examples of microevolution involve reductive evolution. We have already described the analogy of a stripped down racing car, and the analogy of blind cave fish. But perhaps the best illustrations of reductive evolution come from other microbial populations. An excellent example of this is found in a recent paper about the Salmonella bacterium, entitled *Selection-Driven Gene Loss in Bacteria*.[18] The authors report:

> "...gene loss is selected because carriage of superfluous genes confers a fitness cost to the bacterium... Approximately 25% of the examined deletions caused an increase in fitness... after serial passage of wild-type bacteria in rich medium for 1,000 generations we observed fixation of deletions that substantially increased bacterial fitness..."[18]
>
> "...pioneering studies of Zamenhof and Eichhorn, Dykhuizen and Koch showed that *reduced expression of certain biosynthetic and catabolic operons... result in an increased fitness* [emphasis ours]."[18]
>
> "...cost of running flagella is 4.5% of the cells total energy expenditure ... corresponds well with the 3.2% fitness increase observed in the non-motile fliG deletion mutant."[18]
>
> "...our results show that a surprisingly high fraction of random deletions introduced into the Salmonella chromosome do in fact increase fitness as measured by

> exponential growth rate. Furthermore, when Salmonella is grown for many generations in a rich growth medium, fitness-increasing deletions accumulate in the population."[18]

Remarkably, even "artificial life" (popular computer simulations) display reductive evolution. Regarding the Tierra artificial life program, Ofria and Wilke (2004) report:

> "Ray witnessed that the organisms were slowly shrinking the length of their genomes, since a shorter genome meant that there was less genetic material to copy, and thus it could be copied more rapidly."[24]

Regarding the Avida artificial life program, Ofria et al (2003) report,

> "Figure 1(A) shows the adaptive drop in average replication time... as sections of the genome that are meaningless in the simple environment are stripped away. In Figure 1(B) we witness the corresponding drop in genome length."[23]

6. What about the gain-in-function mutation that resulted in the ability to utilize citrate?

The LTEE has now generated over 10^{14} cells, and during this time more than 10^{11} mutations have arisen.[26] Less than 100 beneficial mutations have been observed during this time. This is about 1 beneficial in a billion mutations. The ratio of good to bad mutations is not really quite this extreme, because in the first few years of the experiment the very small pool of potential beneficial point mutations within the genome was already becoming depleted. But since that time, the bad mutations have continued to arise recurrently. More conservatively, Lenski and researchers acknowledge that in the LTEE experiment only about 1 in a million mutations was beneficial[14,21], but this estimate only applies to the early stages of the experiment when there were still a significant number of potential beneficial mutations left to be uncovered. The fact that 8 of the 10 documented beneficial mutations arose in the first 5,000 generations clearly indicates that the very small pool of potentially significant point mutations that would be beneficial must have been nearly exhausted very early in the LTEE. Lenski acknowledges that every possible beneficial point mutation had already occurred (many times), in the first 20,000 generations:

> "In fact, with a genome length of 5×10^6 base-pairs and three alternative base-pairs per position, only 1.5×10^7 are even possible. Thus, each population has had most point mutations represented many times over."[21]

The fact that there is only a small pool of potential and substantially beneficial point mutations in the whole bacteria genome, combined with the fact that these rare point mutations are fairly quickly selected in a large population such that this pool is quickly depleted, generally makes the adaptive process inherently short-term and ephemeral in nature. This profoundly limits what mutation/selection can accomplish in the long run.

Within the 100 billion mutations that arose in the LTEE, only one mutant strain arose that might conceivably represent a gain-in-function. Consequently there has been a great deal of hype over this singular mutated gene. Even if this mutant gene really did reflect an isolated

case of a true gain-in-function mutation, in the big picture such a rare gain of information would be much too rare to counterbalance all the genetic damage that is continuously accumulating – so there must still be a net reduction in total functionality. However, upon closer examination (as shown below) it is clear that the nature of this particular genetic change actually does reflect a loss of function.

After around 31,500 generations, a mutation arose in one of the 12 populations that supposedly led to "the evolution of a key innovation".[5,6,15] One of the populations of *E. coli* suddenly began to uptake citrate into the cell, using it as a nutrient and carbon source. This was not due to a point mutation, but was due to a more complex chromosomal rearrangement. This caused a gene to go from a regulated state to an unregulated state. A gene that was normally silent except when it was normally needed was now always "on" (something like a broken light switch that cannot be turned off). This new feature clearly involved loss-of-regulation, and hence was a loss-of-function mutation. However, under the artificial conditions of the experiment, this change was distinctly beneficial. Under the given artificial circumstances the cells could continue to grow using the citrate in the medium, even when the glucose in the artificial medium was depleted. Some commentators have incorrectly claimed that the bacteria had evolved a new metabolic pathway,[9,16] but that was not true – the *E. coli* already had the entire pathway needed to utilize the citrate. Other commentators incorrectly claimed that the *E. coli* had gained a new capability to uptake citrate from the medium,[36] but that was also not true. The *E. coli* already had the gene for citrate uptake, but that gene was normally regulated so that it was only expressed when it was needed (when there was the absence of free oxygen). All the necessary genetic information was *already present* within the ancestral *E. coli's* genome – it was simply being regulated so the function would be expressed only as needed. All that was needed for the *E. coli* bacteria to metabolize citrate in this experiment was for the citrate transporter gene be mutated to "always be switched on." As Lenski himself acknowledged, "The only known barrier to aerobic growth on citrate is its inability to transport citrate under oxic conditions."[6,4,22] This happened through the random duplication (copying) of the *already existing* citrate transporter gene and its re-insertion (pasting) into a new gene region (near the *rnk* promoter) that activated it. The citrate transporter protein could then be continuously expressed. It became an *unregulated* gene that could no longer be turned off.

In short, all that occurred was *the loss of regulatory control* of the citrate transporter gene. This should certainly not be viewed as a "dramatic evolutionary leap"[26] or a "key evolutionary innovation"[5,6] since no new genetic specifications were generated through the mutation-selection process. Arguably, a regulated promoter requires programming to be "on" only when needed – and "off" when not. Loss of such regulation is clearly loss of information. Once again, this is evolution going backwards. The ability to uptake citrate was perhaps the most interesting development that came out of the LTEE. But even this adaptation was essentially reductive in nature, and it does not teach us anything about how genomes arose. LTEE still reflects microevolution at best, and more accurately reflects de-evolution.

7. Will the "evolving" populations eventually display gradual fitness decline and eventual extinction? The LTEE populations have been shown to have substantially faster growth

compared to the starting strain, but this is because of just a handful of significantly beneficial reductive mutations (loss-of-function mutations). But even while these few "beneficial" mutations have been happening, a much larger number of mutations have been accumulating which are not beneficial at all. Most of these non-beneficial mutations should be slightly deleterious. 6 out of 12 of the populations have developed defective DNA repair systems and so now are accumulating such slightly deleterious mutations at a much higher rate than normal (Figure 4).[26] Such populations have by now accumulated well over a thousand such slightly harmful (deleterious) mutations per cell. Lenski et al.[47] acknowledge that this is resulting in growing "genetic load".[32] The accumulation of these mutations is continuous and clock-like (except when mutation rate changes), and reflects the rusting-out of the bacterial genomes. Even in those populations that have not yet started to hyper-mutate, the bad mutations are still accumulating much faster than the good mutations. This fundamental problem has been described as *genetic entropy* (see the book *Genetic Entropy* available at GeneticEntropy.org or Amazon.com). What can stop this type of reverse evolution? It may take decades or even centuries, but this relentless increase in the population's genetic load should eventually cause all 12 populations to experience fitness decline – and eventual extinction. This is the essence of genetic entropy.

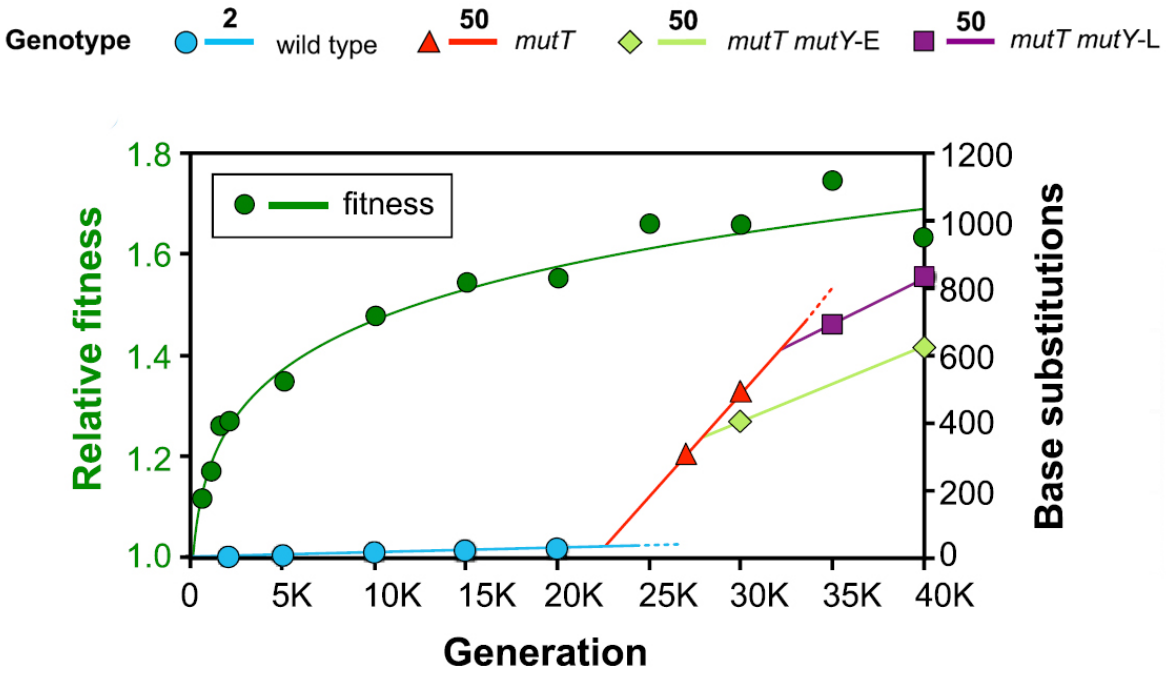

Figure 4. Mutation accumulation within an evolving LTEE population that became a mutator strain during the first 40,000 generations.[32] The green line reflects the fitness increase. The blue line is mutation accumulation before strain began to experience hyper-mutation. Purple and green lines reflect mutation accumulation after compensating mutations slowed hyper-mutation.

8. Numerical Simulations Support the reality of Genetic Entropy in LTEE: We have done numerical simulations to see if the pattern of mutation accumulation, as reported by Lenski et al. (Figure 4), is consistent with deleterious mutation accumulation. We used the computer program *Mendel's Accountant* (free download-version available at MendelsAccountant.info) to simulate deleterious mutation accumulation within an *E. coli* type of bacteria, using bacteria parameters and using the mutation rates described by Lenski et al, and using a biologically realistic Weibull-type distribution of mutation effects (so most mutations having very small effects). We observe the following:

A) Most non-beneficial mutations accumulated without limit, and their accumulation was linear and clock-like (Figures 5 and 6) – just as seen by Lenski et al. (Figure 4). When the mutation rate changed, the slope of the line changed and rate of accumulation changed – but mutation accumulation remains linear and clock-like at the new accumulation rate (Figures 5 and 6).

B) Only the worst mutations were selected away (Figures 5 and 6). This is expected because most deleterious mutations have tiny fitness effects, and are not subject to purifying selection.

C) Just as with Lenski's data (Figure 4), by generation 40,000 there are very roughly 1000 non-beneficial mutations accumulated per cell (Figure 5).

D) When we take this same simulation out to 60,000 generations (closer to the present-day population), we see that this population should have accumulated about 2000 slightly deleterious mutations (Figure 6).

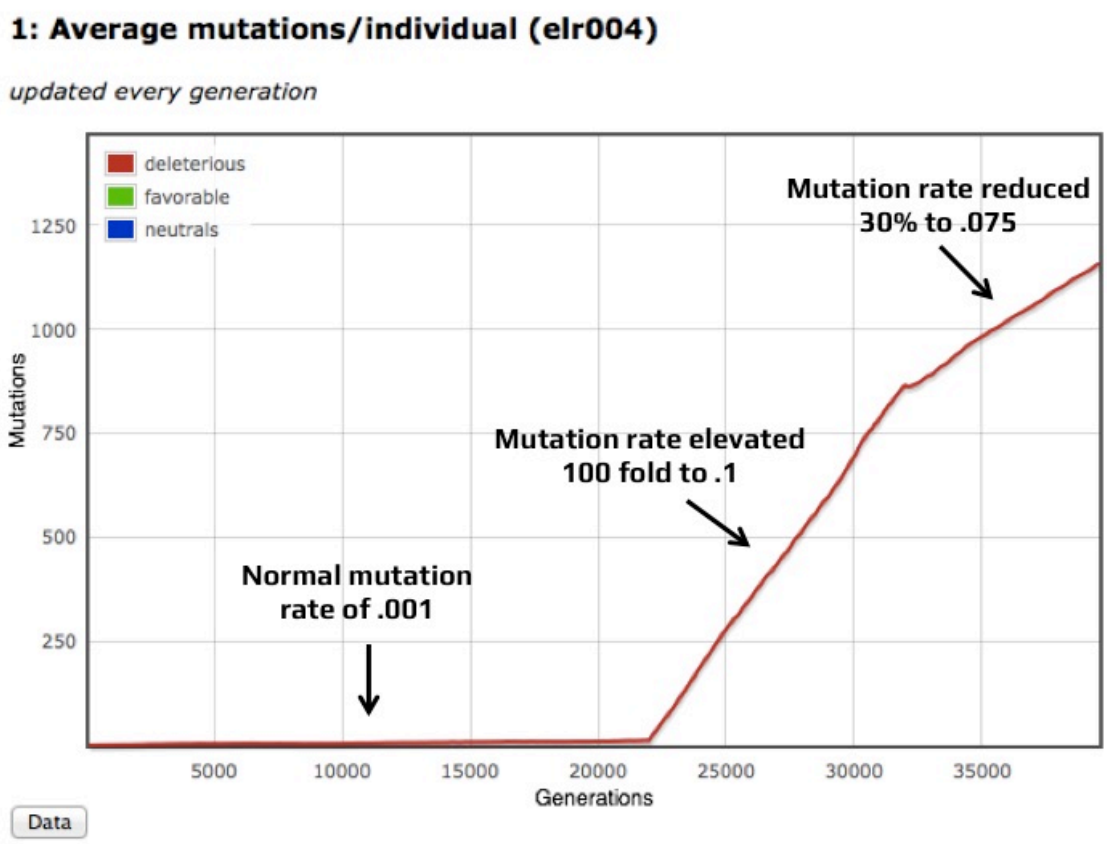

Figure 5. Observed deleterious mutation accumulation per cell using Mendel's Accountant simulations, using parameter settings consistent with the Lenski experiment (Figure 4), and involving similar changes in mutation rate. Note that the observed pattern of mutation accumulation seen by Lenski et al.[32] (Figure 4) perfectly matches the simulated results of Mendel's Accountant, wherein all mutations were deleterious, and the deleterious mutation effects had a natural Weibull distribution. In such a case, most of the deleterious mutations are very slightly deleterious, and so most of the deleterious mutations fail to be selected away, but rather accumulate without limit. Mendel's Accountant parameter settings: Pop = 10,000 Gen = 22,000/10,000/8,000; Deleterious u = .001/.1/.075; Genome size = 4.5Mb; Major mutations = 0.1; Clonal reproduction.

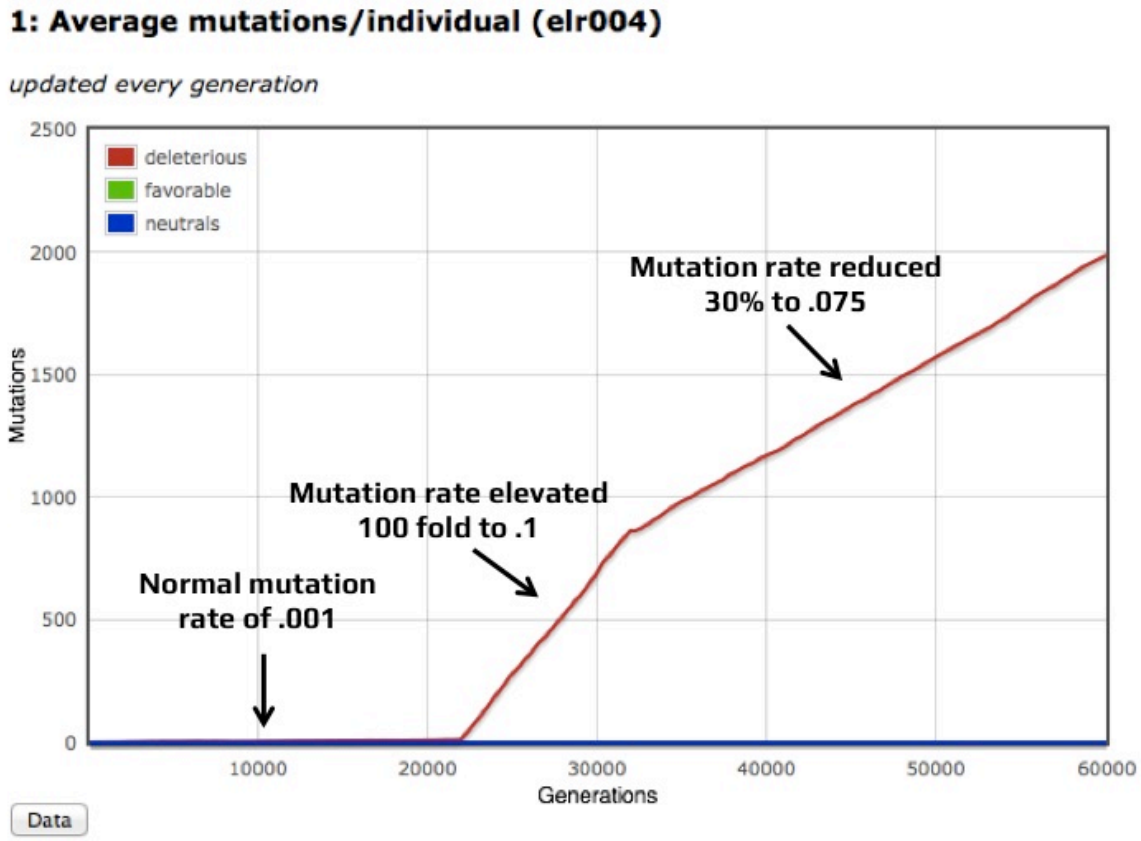

Figure 6. The same population as in Figure 5, but running the simulation out to 60,000 generations. Given the LTEE time frame, by now this particular population should have accumulated roughly 2000 slightly deleterious mutations, even while fitness scores have been increasing due to a handful of beneficial reductive mutations. Mendel's Accountant parameter settings: Pop = 10,000 Gen = 22,000/10,000/28,000; u = .001/.1/.075; Genome size = 4.5Mb; Major mutations = 0.1; Clonal reproduction.

Our simulations are consistent with the concerns of Lenski et al.[32], regarding increasing genetic load. Throughout the long-term experiment there must be a continuous accumulation of slightly deleterious mutations, and such accumulation should increase without limit. These slightly harmful mutations are accumulating much faster than are the "benefical" mutations. Our simulations suggest that slightly deleterious mutations are significantly eroding fitness – however the handful of adaptive mutations have been masking the fitness effects of this accumulating damage. Our simulations suggest that most of the mutations with fitness effects of less that .0005 are accumulating just as if they were neutral.

Lastly, we would like to note that the long-term experiment reveals that the molecular clock dating is not trustworthy (Table 2). Three bacterial strains that are exactly the same age (27 years) will yield radically different molecular clock ages, depending on each one's mutation rate. The frozen ancestral strain should appear to have a molecular clock age of zero (no mutations). A non-mutator strain should have a molecular clock age of about 27 years (and assuming a mutation rate of .001, should have something less than 60 mutations). The

mutator strain, with 2,000 mutations per cell, should have a molecular clock age roughly 33 times older (about 900 years).

Table 2. Molecular clock data: the long-term evolution experiment clearly shows that molecular clock is not a trustworthy way to measure the passage of time.

Sample	Mutations	Actual Age	Molecular clock age
Frozen strain	0	27 yrs	0 yrs
Non-mutator[1]	<60	27 yrs	<27 yrs
Mutator strain[2]	~2000	27 yrs	~900 yrs

[1]Accepted mutation rate = .001 per generation; Mutation count after 60,000 generations < 60
[2]Assuming the mutator strain has a mutation rate 100-fold higher than normal

Conclusions

Contrary to a great deal of hype about the famous Long-Term Evolution Experiment, the actual evidence indicates that this experiment failed to demonstrate macroevolution, or even speciation. Despite the hype, the LTEE is just a very ordinary example of microevolution. What is special about LTEE is not the minor adaptations that were observed, but the very detailed monitoring of the nature of the genetic changes that led to the adaptations. Remarkably, all, or essentially all, of the adaptive mutations were reductive in nature.

Furthermore, the adaptive process observed in this experiment was clearly not perpetual in nature – the bacteria are rapidly moving toward a natural optimum, as is expected in any adaptive cycle. The actual observations indicate nothing more than a momentary *adaptive blip* – it is not even remotely honest to equate this short-term adaptive blip with deep time or macroevolution. Nor is it reasonable to equate a few observed fitness jumps (associated with the fixation a few impactful adaptive mutations), with "evolutionary saltation". A primary reason why the experiment is running out of steam so quickly (a few decades is not deep time) is because the initial pool of potentially impactful beneficial mutations was small and has been significantly depleted. There were only a handful of substantial beneficial mutations available in the whole genome – and that pool of potential beneficial mutations was largely exhausted very early in the experiment.

It is extremely significant that the few documented adaptive mutations were all shown to be reductive in nature (involving loss of function or loss of regulation). It is also very significant that the genomes of most of the populations actually shrank physically. There can be no doubt that in the functional sense every one of the 12 genomes experienced a net loss of information. While some fine-tuning has occurred at a handful of sites within the genome, a much larger number of slightly deleterious mutations have been accumulating throughout the genome. This type of on-going genetic damage causes continuously increasing "genetic load", which must eventually outweigh the transient gains associated with adaptation. Continuous accumulation of slightly harmful mutations is like rust on a car – it is ongoing and

the damage accumulates without limit. Although it will take a very long time, we predict that the LTEE populations will eventually display fitness decline and eventual extinction. This is consistent with our many previous studies involving the general problem of genetic entropy and the associated genetic degeneration of populations (see book *Genetic Entropy* and related peer-reviewed papers at GeneticEntropy.org) In particular, we have shown that the H1N1 human influenza virus (another classic "model evolutionary population") has been undergoing genetic degeneration ever since it emerged within the human population about 100 years ago. The human version of H1N1 went extinct in 2009.[8]

In summation, the most famous evolution experiment ever conducted (LTEE) that is being proclaimed to the world as a dramatic proof of "observable evolution", is ironically one of the most powerful demonstrations of genetic entropy and de-evolution. This is consistent with the Biblical view of origins. The Bible teaches that because of Adam's sin (see LogosRa.org article for genetic evidence for a literal Adam and Eve ancestry) we live in a fallen creation that is subject to the "bondage of decay" (Romans 8:21). Ever since the fall, the genomes of all living creatures have been degenerating due to the accumulation of mutations – this includes the populations of *E. coli* described in this article.

But we are not left without hope. The Bible promises that God will restore our relationship to Him, He will create a new heaven and earth, and He will restore all things (no more death, disease, suffering, and no more degeneration due to mutations). A major weapon that has been used to weaken or destroy people's faith in Jesus has been the powerful deception that Darwinian theory is a scientific fact and that it disproves the Bible (2Thes 2:10-12). By God's grace, The Lord is now giving some of us (those with ears to hear and eyes to see), new evidence to encourage our Faith.

By Christopher Rupe & Dr. John Sanford

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Appendix 1: See PDF attached to article summary page for a more detailed literature review of the mutations recorded in Table 1.