changed every 12 hours. β-Gal was detected with X-Gal staining [J. R. Sanes, J. L. R. Rubenstein, J.-F. Nicolas, *EMBO J.* **5**, 3133 (1986)]. In 50-μm sections of infected slices, we counted the number of neurons containing β-Gal, and estimated that 68 to 95% of neurons in the injected region were infected, depending on whether central or peripheral regions of virus injection were scrutinized. Stained slices were photographed (Zeiss Axiophot, ×2.5 and ×10 objectives) and imported into Adobe Photoshop 3.0 for graphic presentation.

19. Hippocampal slices (500 μm) were prepared from young adult male Sprague-Dawley rats. Slices were submerged in a stream of artificial cerebral spinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 26.2 mM NaH_3CO, and 11.0 mM glucose), maintained at 22°C to 25°C, and gassed with 95% O_2/5% CO_2. fEPSPs measured independently in SR or SO at a depth of 100 to 150 μm below the slice surface were evoked by two different stimulating electrodes activating the Schaffer collateral-commissural afferents (once every 15 s); the initial (1- to 2-ms) slope was measured. LTP was induced by four trains of high-frequency stimulation (100 Hz for 1 s) separated by 30-s intervals. To quantify I/O relations, the slopes of the regression lines for each experiment were compared. All electrophysiology experiments, with the exception of those noted in (*22*), were conducted with the experimenter being unaware of the experimental condition of the slice. The percents of baseline measurements indicated in the text were taken 50 to 60 min after tetanus. Ensemble average plots represent group means of each EPSP slope, for all experiments, aligned with respect to the time of LTP induction. To assess statistical significance, paired or independent *t* tests were performed. *P* values greater than 0.05 are designated as NS.
20. C. M. Lee, L. J. Robinson, T. Michel, *J. Biol. Chem.* **270**, 27403 (1995). We examined whether the expression of TeNOS inhibits NOS catalytic activity by monitoring the Ca^{2+}-dependent conversion of [^{14}C]arginine to [^{14}C]citrulline in hippocampal homogenates made from infected CA1 regions of hippocampal slices [D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9030 (1989)]. (The NOS activity of Ad–CD8-eNOS was assessed in CHO cells with the same method.) We found that Ad-TeNOS inhibited NOS activity by 14.4 ± 3.2% when compared with Ad-lacZ–infected slices (n = 6 experiments, 3 slices per experiment, for both Ad-lacZ and Ad-TeNOS). This modest but significant inhibition may reflect the relatively small contribution of eNOS to the total NOS activity detected in the hippocampus (*7*) and may account for the observed block of LTP in SR by TeNOS, although alternative mechanisms may also contribute.
21. J. E. Haley and D. V. Madison, *Learn. Mem.*, in press.
22. We combined the simultaneous SR-SO experiments shown in Fig. 3 with an earlier blind study examining LTP in Ad-lacZ– versus TeNOS-infected slices at SR synapses only, because the two sets of SR data were statistically indistinguishable from one another.
23. R. A. J. McIlhinney and K. MGlone, *J. Neurochem.* **54**, 110 (1990).
24. M. J. S. Nadler, M. L. Harrison, C. L. Ashendel, J. M. Cassady, R. L. Geahlen, *Biochemistry* **32**, 9250 (1993).
25. L. A. Paige, G. Zheng, S. A. De Frees, J. M. Cassady, R. L. Geahlen, *ibid.* **29**, 10566 (1990).
26. L. Busconi and T. Michel, *J. Biol. Chem.* **269**, 25016 (1994).
27. Slices were treated with 100 μM HMA dissolved in ethyl alcohol (EtOH) and bovine serum albumin (BSA) (final EtOH concentration, 0.25%; final BSA concentration, 0.25 mg/ml) or with vehicle alone and were maintained in the same MEM described above for 20 to 26 hours before electrophysiological recording. Adjacent slices from one hippocampus were exposed to either experimental or control conditions, and only data from pairs in which the control slice expressed LTP were considered. For experiments in which HMA was applied acutely, a two-pathway design was implemented. HMA dissolved in EtOH plus BSA was added directly to the superfusate at the indicated times, and during the control recording period, slices were perfused with Ringer's containing EtOH plus BSA. In experiments using a NOS inhibitor, L-*N*-monomethyl-arginine (100 μM; Sigma) was applied to slices for at least 3 hours before recording.
28. The half-life of eNOS has been reported to be approximately 20 hours [L. J. Robinson, L. Busconi, T. Michel, *J. Biol. Chem.* **270**, 995 (1995); D. J. Stuehr and O. W. Griffith, *Adv. Enzymol. Relat. Areas Mol. Biol.* **65**, 287 (1992)].
29. NMT activities in homogenates made from the same slices used for electrophysiology were determined as described [M. J. King and R. K. Sharma, *Anal. Biochem.* **199**, 149 (1991)] with a post-hoc assay that measures the transfer of [^{3}H]myristic acid to a synthetic acceptor peptide—the eight NH_2-terminal amino acids of pp60^{src}. [^{3}H]myristoyl coenzyme A was generated enzymatically [D. Towler and L. Glaser, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2812 (1986)], and the NMT activity assay was performed on 50 μg of homogenate protein. Data are expressed as adjusted counts per minute (cpm).
30. P. Sukhatme, K. C. Sizer, A. C. Vollmer, T. Hunkapiller, J. R. Parnes, *Cell* **40**, 591 (1985).
31. D. B. Kantor and E. M. Schuman, unpublished observations.
32. T. Sakoda *et al.*, *Mol. Cell. Biochem.* **152**, 143 (1995).

33. We thank T. Michel for sharing various eNOS cDNAs and unpublished results, B. Seed for CD8 cDNA, A. Berk and L. Wu for Ad-lacZ, and G. Laurent for technical assistance and discussion. Supported by European Molecular Biology Organization grant ALTF 168-1996 (M.L.), National Institute of Mental Health grant 49176 (N.D.), NIH grant NS37292 (E.M.S.), and a Beckman Young Investigator award (E.M.S.).

27 August 1996; accepted 23 October 1996

TECHNICAL COMMENTS

Mechanisms of Punctuated Evolution

In their report, "Punctuated evolution caused by selection of rare beneficial mutations," Santiago F. Elena *et al.* describe (*1*) punctuated changes in a morphological character (cell size) in a population of *Escherichia coli* kept in batch culture with daily transfer for 3000 generations. They state that their observations might explain some aspects of punctuated evolutionary change, a phenomenon often said to be inconsistent with classical neo-Darwinism (*2*). The controversy about punctuated equilibrium involves two questions: (i) How ubiquitous is the pattern of "jerky" evolution in nature? and (ii) Do novel evolutionary processes cause this pattern? Elena *et al.* answer the second question by proposing that stasis in the fossil record arises (as it does in their bacteria) from an absence of genetic variation, while rapid changes in the record reflect the spread of new beneficial mutations.

Although the report (*1*) by Elena *et al.* provides useful evidence of the power of selection to produce rapid evolutionary change, there are substantial problems in relating the results to the fossil record. First, their experiment was designed so that punctuated change was the only conceivable outcome. Second, the phenomenon of punctuation in such laboratory populations is not novel, but has been described many times in the literature. Third, it is unwarranted to extrapolate evolution in a clonal population of *E. coli* to punctuated evolution in sexual eukaryotes.

By beginning their experiment with a genetically uniform clonal population, Elena *et al.* virtually guaranteed a punctuated outcome. Because there was no initial genetic variation, all evolutionary change was constrained to occur by the successive fixation of newly arising mutations (*3*). Many related experiments in flies and mice have shown that, in small or highly inbred populations, the response to artificial selection stops when genetic variation is exhausted, but can undergo sudden jumps on the appearance of new mutations of large effect (*4*). Indeed, periodic selection in bacteria, a phenomenon described by Atwood and his colleagues (*5*), is simply the sporadic occurrence of mutations with a large effect on fitness.

The relevance of observations on clonal or inbred populations to the fossil record is highly questionable; patterns of punctuated evolution in nature can be explained without appealing to the episodic fixation of new mutations. Most fossils come from sexually reproducing populations that are large enough to leave an adequate record (*6*). In such populations, rapid (on a geological scale) evolution in response to environmental changes can be caused by selection acting on genetically variable traits (*7*). There are many well-documented examples of rapid, episodic change in contemporary natural populations (*8*), and artificial selection in large laboratory populations has produced rates of change of orders of magnitude faster than those seen in the fossil record (*9*). Once the environment has stopped changing drastically, stabilizing selection will act to preserve the optimum phenotype, and morphological change will slow down or cease, producing the appearance of stasis. Given the unpredictable nature of environmental change, it is thus not unexpected that evolution often goes in fits and starts, a fact that has long been recognized (*6*, *10*).

The mechanism suggested by Elena *et al.* for punctuated evolution is one that was explicitly rejected by the originators of that theory, Gould and Eldredge (*11*)

> Punctuated equilibrium is a theory that attributes this pattern of spurt and stasis neither 1. to imperfections of the fossil record in a truly gradualistic world, nor 2. to such theories of occasional anagenetic rapidity as Simpson's important hypothesis of quantum evolution, but to speciation as a process of branching, characteristically occurring at geologically instantaneous rates—with trends then explained not as anagenetic accumulation, but as differential success by species sorting.

Gould and Eldredge (*2*) have repeatedly emphasized that morphological stasis is caused by developmental constraints that can be broken only by a restructuring of the genome through genetic drift in small, sexually reproducing populations. Such nonadaptive genetic changes were said to accompany the appearance of reproductive isolation, producing a pattern of morphological change accompanied by speciation. Finally, the punctuated changes in the fossil record are said to occur via "species selection," in which descendant species rapidly supplant their ancestors (*1*). These conditions do not exist in the study of Elena *et al.*: Their population is large and asexual; the stasis cannot be a result of developmental constraints (it is overcome by selection); and there is neither speciation nor species selection.

Much of the attention given to punctuated equilibrium was based on its supposed mechanism, which was novel and non-Darwinian. Shorn of this mechanism, the theory reduces to the noncontroversial statement that morphological evolution sometimes occurs episodically.

Jerry A. Coyne
Brian Charlesworth
Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637, USA

REFERENCES

1. S. F. Elena, V. S. Cooper, R. E. Lenski, *Science* **272**, 1802 (1996).
2. S. J. Gould and N. Eldredge, *Paleobiology* **3**, 115 (1977); S. J. Gould, *ibid.* **6**, 119 (1980); *Science* **216**, 380 (1983); _____ and N. Eldredge, *Nature* **366**, 223 (1993).
3. L. R. Ginzburg, *Theory of Natural Selection and Population Growth* (Benjamin/Cummings, Menlo Park, CA, 1983), p. 144; J. R. G. Turner, in *Patterns and Processes in the History of Life*, D. M. Raup and D. Jablonski, Eds. (Springer, Berlin, 1986), pp. 183–207.
4. R. C. Roberts, *Genet. Res.* **8**, 347 (1966); L. P. Jones, R. Frankham, J. S. F. Barker, *ibid.* **12**, 249 (1968); M. A. Lopez and C. Lopez-Fanjul, *ibid.* **61**, 107 (1993).
5. K. C. Atwood, L. K. Schneider, F. J. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **37**, 146 (1951).
6. G. G. Simpson, *Tempo and Mode in Evolution* (Columbia Univ. Press, New York, 1953).
7. B. Charlesworth, R. Lande, M. Slatkin, *Evolution* **36**, 474 (1982).
8. J. Bishop and L. Cook, *Genetic Consequences of Man-Made Change* (Academic, London, 1981); B. R. Grant and P. R. Grant, *Proc. R. Soc. Lond. B* **251**, 111 (1993).
9. P. D. Gingerich, *Science* **222**, 159 (1983); K. E. Weber, *Genetics* **125**, 579 (1990).
10. C. Darwin, *On the Origin of Species*, (John Murray, London, ed. 6, 1872), p. 375.
11. S. J. Gould and N. Eldredge, *Nature* **368**, 407 (1994).

3 July 1996; accepted 24 September 1996

Response: We are in complete agreement with what seems to be the main point made by Coyne and Charlesworth. In particular, the form of punctuated evolutionary dynamics that we reported evidently arose by the simplest and most orthodox mechanism in population genetics, namely natural selection for rare beneficial mutations (*1*). As such, our findings cannot be taken as support for any other theory of punctuated evolution that depends on more complex—and more controversial—evolutionary processes, such as population bottlenecks or species selection. In fact, demonstrating the simplicity of the population genetic mechanisms that could give rise to seemingly complex punctuated dynamics in evolving populations of bacteria was the central point of our report.

We strongly disagree, however, with many specific points raised by Coyne and Charlesworth. First, as we emphasized in our report, some 10^6 new mutations occurred every day in the experimental population (*1*). This can hardly be characterized as an absence of genetic variation; instead, stasis resulted from the rarity of mutations having large beneficial effects. Had there been many beneficial mutations, each with a small effect, then we would not have detected such conspicuous punctuated dynamics. We did not, as experimenters, exert any direct control over the distribution of mutational effects.

Second, we did not state that the mechanism we put forward to explain the phenomenon of punctuated evolution was novel in any way; in fact, we emphasized its orthodoxy. But we do not know of any other study that provides such clear and unambiguous support for the role of this mechanism in producing punctuated dynamics for a conspicuous morphological trait. We agree that the phenomenon we showed is theoretically equivalent to so-called "periodic selection" that was first described by Atwood *et al.* (*2*), who studied bacteria. We emphasized that we had previously reported similar dynamics for fitness in our experimental populations (*3*). However, we pointed out that these earlier studies, including our own, had not dealt with quantitative morphological characters of the sort that are preserved in the fossil record. Thus, we sought in our report (*1*) to extend these studies to show that this simple mechanism could indeed give rise to punctuated dynamics in a conspicuous morphological trait. Some other studies (including the ones cited by Coyne and Charlesworth) are *consistent* with the hypothesis that sudden jumps in traits depend on the appearance of new mutations of large effect, but these studies with flies and mice are much less definitive in several important respects. In particular, statistical tests for punctuated change are generally lacking, and a series of unambiguous steps is not apparent. (One must "squint hard" to see any sign of punctuated dynamics in some of these other studies.) Moreover, except for studies begun with completely homozygous populations, an alternative explanation for any jumps that do occur is that rare alleles were present in the base population, but their effects were only seen after most of the initial variation was exhausted. This is an important point, because macroevolutionists have implicitly criticized traditional population genetic studies as being concerned only with the fate, and not the origin, of genetic and phenotypic novelty (*4*). Unlike typical studies with flies and mice, experiments with bacteria can rigorously discern the consequences of the origin of novelty for evolutionary dynamics. Our study also differs from the reliance of these earlier studies on artificial selection (whereby the investigator decides which traits will determine an individual organism's reproductive success) in that we allowed natural selection to proceed in the laboratory so that any genotype that gained a reproductive advantage could proliferate (irrespective of the specific identity or number of traits that might be involved in giving an advantage).

Third, in our report, we pointed out that clonal reproduction in our experimental system "may have increased our ability to resolve punctuated changes" (*1*). But we also pointed out that both sexual and asexual populations may show similar dynamics when adaptive evolution depends on rare beneficial mutations of large effect. We did not intend to suggest that the selection of rare beneficial mutations was the sole explanation for punctuated dynamics; we cautiously concluded (*1*) that "to the extent that [certain] conditions are fulfilled in nature, then the selective sweep of beneficial alleles through a population might explain some cases of punctuated evolution in the fossil record." In our study (*1*), environmental inputs were held constant, so that environmental change was not responsible for the punctuated dynamics we reported. We have no strongly held opinion on whether

selective sweeps of rare beneficial mutations—in environments that are essentially constant in terms of selection for those mutations—might account for few or many cases of rapid evolutionary change in nature. But we also see no compelling reason to dismiss the role of a mechanism for punctuated evolution that requires only the two most basic evolutionary processes of mutation and natural selection.

Santiago F. Elena
Vaughn S. Cooper
Richard E. Lenski

Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824, USA

REFERENCES

1. S. F. Elena, V. S. Cooper, R. E. Lenski, *Science* **272**, 1802 (1996).
2. K. C. Atwood, L. K. Schneider, F. J. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **37**, 146 (1951).
3. R. E. Lenski and M. Travisano, *ibid.* **91**, 6808 (1994).
4. S. J. Gould, *The Panda's Thumb* (Norton, New York, 1980), chap. 18.

29 July 1996; accepted 24 September 1996

Dating the Cenancester of Organisms

Russell F. Doolittle *et al.* (*1*) argue that their evidence indicates that the most recent common ancestor (cenancestor) of present-day organisms occurred about 2 billion years ago (Ga). This is considerably more recent than is commonly believed. Doolittle *et al.* recognize that the estimate of that time is related to a distance that in turn depends on the fraction of the amino acid positions of the sequence that are variable. They determined this as the fraction of the sites that have varied in at least one sequence. This is sufficiently large, 90 to 95%, so that a correction for the invariable positions would not materially affect their conclusions. But this assumes that any site that has varied once anywhere is forever variable. There is considerable evidence that the positions at which amino acid replacements are accepted are different in different taxonomic groups, or that there may be a sizable number of invariable positions, or both (*2*–*6*). This could not be correct unless positions in the sequences could sometimes be variable and at other times invariable, the point of the covarion model of protein evolution.

This description means that many of the varied sites may nevertheless have spent a considerable portion of their time in the invariable category. The estimates of the percent of the sequence that are covarions (percent of the sequence that is variable at any one time) include cytochrome c (*2*, *3*), α hemoglobin, β hemoglobin, ribonuclease, fibrinopeptide A, and insulin C peptide (*4*), as well as six mitochondrial proteins (*5*). In these proteins there are a total of over 2000 codons, only 614 (30%) of which are covarions, implying that 70% of the sites are not variable at any one time and for which many might rarely be variable. Doolittle *et al.* indicate that their dating of the cenancestor would be in line with current beliefs if 35% of the sites were invariable. If the figures for the proteins we just cited are representative of the proteins that Doolittle *et al.* used, the *effective* number of invariable sites may well be large enough to demand a correction which would make the current estimated 3.5-Ga date for the cenancestor within the error of their method. Because Doolittle *et al.* have not estimated the number of covarions nor how fast their composition changes for their sequences, one cannot be sure that the effective size of the invariable group is not as large as 35%. Their Poisson correction cannot address this problem.

This criticism does not depend on the correctness of the covarion model. As long as some positions are more variable than others, the distribution of mutations across sites is often much better matched with a gamma function. Had Doolittle *et al.*'s distances been corrected in this manner, their estimated time for the cenancestor would have been greater, possibly giving a date of 3.5 Ga.

Note added in proof: M. M. Miyamoto and I (W.M.F.) (*7*) have now shown that the data from the report by Doolittle *et al.* can be fit with a gamma distribution and, depending on the parameter chosen, obtain dates for the cenancestor ranging from the data proposed by Doolittle *et al.* of 2 Ga to values larger than 3.5 Ga.

Masami Hasegawa
Institute of Statistical Mathematics, 4-6-7 Minami-Azabu, Minato-ku, Tokyo 106, Japan
Walter M. Fitch
Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92717, USA

REFERENCES

1. R. F. Doolittle, D.-F. Feng, S. Tsang, G. Cho, E. Little, *Science* **271**, 470 (1996).
2. W. M. Fitch and E. Markowitz, *Biochem. Gen.* **4**, 579 (1970).
3. W. M. Fitch, *ibid.* **5**, 231 (1971).
4. ———, *J. Mol. Evol.* **8**, 13 (1976).
5. J. Adachi and M. Hasegawa, *ibid.* **40**, 622 (1995).
6. J. H. Reeves, *ibid.* **35**, 17 (1992); A. Sidow, T. Nguyen, T. P. Speed, *ibid.*, p. 253.
7. M. M. Miyamoto and W. F. Fitch, *Syst. Biol.* **45**, 566 (1996).

19 April 1996; accepted 28 August 1996

Doolittle *et al.* (*1*) calculate dates for two major events in the early evolution of life: the last common ancestor of all now-living organisms and the ancestor of eukaryotes and prokaryotes. These dates are derived from the molecular records stored in the various proteins of extant organisms. To calibrate the molecular clock, Doolittle *et al.* rely on data for metazoan evolution derived from the fossil record. They date the prokaryote-eukaryote ancestor and the last common ancestor to about 2000 Ma. These calibrations are wrong because: (i) they do not properly consider horizontal gene transfer, and (ii) they significantly underestimate the effect of differing substitution rates among amino acid positions on their calibration of the molecular clock.

1) Doolittle *et al.* mention (*1*) horizontal gene transfer as a possible complication; however, in most cases the reported analyses include too few bacterial species to allow for the detection of horizontal gene transfer events. For example: if more bacterial triose phosphate isomerase (TPI) sequences are added to the data set used by Doolittle *et al.*, one recognizes that the eukaryotic TPIs group with their counterparts from purple bacteria (*2*). P. J. Keeling and W. F. Doolittle have interpreted this relation to indicate that the eukaryotic TPI was contributed to the eukaryotic cell by the endosymbiont that evolved into the mitochondria (*3*). If this interpretation is correct, then the prokaryote-eukaryote split determined for this enzyme does not reflect the origin of the eukaryotes, but rather the uptake of mitochondria into the eukaryotic cell.

The same argument can be made for those enzymes found in archaea that indicate a close relation between archaea and eubacteria. Several molecular phylogenies have been reported that indicate such closeness—in particular, between gram positive bacteria and archaea—whereas other enzymes and especially functions involved in DNA duplication, transcription, and translation indicate a close association between the archaea and the nucleocytoplasmic component of the eukaryotic cell (*4*). Including more prokaryotic species in the analyses (*5*), we found the representatives of the archaea grouping within the eubacteria in the several cases (*6*). If the enzymes that group the archaea within the eubacterial domain were obtained by the archaea through horizontal transfer from eubacteria, then this event (or these events) of hori-