Comment on "Does constructive neutral evolution play an important role in the origin of cellular complexity?"

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Speijer [1] has provided a critique of constructive neutral evolution (CNE) and its role in the origin and evolution of cellular complexity [2, 3]. Not surprisingly, we disagree with his assertions. Because his description of the CNE model does not precisely conform to our view of CNE, as we [2, 4] and Stoltzfus [3] have elaborated it, we briefly re-state the model before addressing Speijer's objections.

The underlying premise of CNE is a pre-existing, essentially neutral interaction (RNA:RNA, RNA:protein, protein:protein) between component A, which has some activity, and component B. The activity of A is not dependent on the interaction with B, nor is A's activity negatively influenced by this interaction. Thus, B could disappear from the scene without any effect on the "fitness" of A.

We imagine that a mutation occurs in A that compromises its activity and that normally this mutation would be eliminated from the population by purifying selection. However, the pre-existing interaction with B fortuitously suppresses the effect of this mutation, so that selection pressure is relaxed and the mutation may be harmlessly fixed by drift. Thus, A becomes dependent on B for its activity by virtue of the neutral, "pre-suppressive" effect of the A:B interaction. What we submit does *not* happen is that the mutation occurs first, *after* which the interaction with B is positively selected for *because* it suppresses the deleterious effect of the mutation.

Speijer's "Think again" article [1] embraces several misunderstandings about this important process. First, we note that Speijer's critique is considerably longer than was our Perspective in *Science* [3], giving him space to deconstruct several points that he may consider components or at least entailments of our hypothesis, but that we would not. Let us call these misunderstandings of Type A.

Type B misunderstandings reflect Speijer's conflation of micro- and macroevolution, or "levels of selection". He fails to recognize that some features that are neutral or even disadvantageous to individuals – and thus not expected to be fixed by selection operating *within* populations of species – can nevertheless be sufficiently advantageous to species (fostering enhanced speciation or reduced extinction rates) to spread by species or clade selection. Eukaryotic sex might be one of these. Introns could be another. No one really thinks that the insertion of introns was selected for at the level of individuals – that is, that all introns that are currently fixed within a species were fixed *because* individuals that bore them were at a selective advantage compared to conspecifics that lacked them. Indeed, for individuals within species, intron addition could be slightly deleterious. It might nevertheless still be true that species in which many introns have become fixed do better than species with few introns (speciate more frequently or become extinct less often), because introns facilitate exon shuffling or the elaboration of multiple gene products through alternative splicing. There would thus come to be more intron-bearing species (and in consequence more introns) in the world, thanks to species selection. We see many CNE-established features as evolving like this, influencing however subtly the future evolutionary potential – the "evolvability" – of species, and yet their initial establishment was the consequence of the neutral ratchet we describe.

Type C misunderstandings are of a diverse sort. It seems simplest to attempt to correct these as we read

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through Speijer's essay, ending with a few general observations.

We do not recall that suppression by "remaining copies of the nonmutated gene" (Speijer's *Introduction*) was part of our model, nor indeed would we think of that as a form of suppression. Nor did we imagine that "reverse mutation of the single first mutation can not restore the original viable organism". It easily could do that, but if there are additional sites at which further dependency (through mutation) is possible, then a random walk through dependency space will seldom end up back at the doorstep to independence – the initial condition. Similarly, even those who would vigorously deny that life shows a tendency toward complexity would admit that since it began simply and since there are innumerable ways to become more complex, complexity was inevitable. That is the kind of ratchet we envision. Indeed, getting "stuck with it" is an appropriate catchphrase, but otherwise we feel that Speijer is making a Type A mistake here.

In Speijer's representation of our model (Step 2) we are again baffled by the invocation of "asymmetric divisions", which does not form part of the model in any of *our* papers that he cites. Of course if there are multiple copies of the mutated gene, time will be required for segregation: here the fact that such mutations are, in our model, rendered neutral by pre-suppression means that this *can* happen. Maybe Speijer is confused by the phrase "duplicated information" in Lukeš et al. [5]. We think gRNAs arise as duplicates of the original gene, but are transcribed from the opposite strand, producing a complementary RNA. We think this is a Type A mistake on Speijer's part.

In *Origin and use of cellular processes*, we feel that Speijer makes some Type B mistakes. Extra levels of control, fine-tuning, and "evolvability" are indeed possible co-optations of complexities first fixed by CNE, but are not the reason for fixation, at the micro-evolutionary level. Such secondary "functions", which Speijer describes as "fringe benefits", are exaptations or species-level adaptations as we described above, and are by no means excluded by CNE thinking. In this regard, Rabosky and McCune [6] provide a good recent review of the relevant literature.

Speijer asserts, "Using 'neutral' theories to explain highly complex processes is much less straight forward". We only partly concur. We would agree that the case of single editing of a marsupial tRNA is "simple". Indeed it was aptly interpreted in CNE terms by the researchers who first described it, who also noted: "Because in other systems many positions have become dependent on RNA editing subsequent to the initial events, the initial mutations that have caused the evolutionary fixation of editing are probably indiscernible today" [7]. But with an editing system that has already become essential for performing one edit, the conditions are in place for more to arise: it is actually the first cut that is the deepest.

Speijer then invents three criteria for distinguishing CNE versus selection as the cause of a complex feature: first that there should be a "smooth" increase (no rapid bursts) in complexity, second that there should not be much variation between lineages in the extent of complexity, and third that there should not be good adaptationist alternatives. The first two criteria seem to us to be ad hoc in the extreme, and would certainly not be our criteria. Of course such evolution will proceed by fits and starts. Similarly, we will all admit that the rare reintegration of a reverse transcript of a mature edited mRNA can wipe out a whole slew of edits at once, resetting the clock. That this process might vary from lineage to lineage seems a "no-brainer". The third criterion ignores the extensive literature on the ease with which clever biologists can invent evolutionary "Just So Stories" [8], and is highly "verificationist". The principle behind this criterion seems to be "better the widely believed but unprovable hypothesis we have than an equally difficult-to-prove but less popular alternative".

Speijer's section *CNE: Conceptual problems* has its own conceptual problems. We would not be surprised if a population exhibited "exactly the same level of complexity". What determines within-species heterogeneity would be population sizes and the potential number and rate of occurrence of pre-suppressible mutations, which could easily be low enough that we might catch very few CNE interactions in the act of being established. And who would have looked for such a thing?

Speijer then makes three arguable claims. First he states that it is not very likely that neutral changes will "take over the complete population". Most of the enterprise of molecular phylogenetics at the trans-species level is in fact based on such neutral "takeovers". The neutral theory of molecular evolution entails that no single pre-designated neutral mutation is likely to be fixed; at the same time it holds that *some* neutral mutations inevitably will be fixed. Second, Speijer claims that we assert categorically that reversal of changes must be much less likely. This is untrue: we assert only that when there are more open mutational paths to increased complexity than decreased complexity, complexity *will* most often increase by chance. Third, he argues that many neutral changes taken together can be detrimental, because complexity incurs costs. This argument seems to be hiding some belief in optimality, a quality that Speijer surely would not expect of that other prime exemplar of a complex system evolved through both chance and necessity, human institutions such as universities. There are reasons that we have elsewhere described CNE as a theory about "cellular bureaucracy" [2]. "Yes, Dr. Pangloss, there is a downside to complexity", we are tempted to say.

In considering *The creative power of complexity as such*, Speijer discusses two macromolecular machines, the ribosome and mitochondrial respiratory complexes, that we mentioned [2] as possible examples of CNE. Here, we have space to comment only on the latter example; elsewhere we will present detailed CNE scenarios for the evolution of several other complex cellular machines.

Speijer cites the electron transport chain complex I (ETC CI) as an example of "a very complex prokaryotic machine" that experienced a major increase in the number of subunits "during the very rapid development of the eukaryotic cell", with subsequent increases in the number of subunits seeming "like an afterthought". Speijer suggests that the addition of the supernumerary subunits in mitochondrial CI "has all the hallmarks of resulting from a period of intense selection".

Bacterial CI comprises 14 subunits that constitute the evolutionary "bac-

terial core'' of eukaryotic CI [9]. Of 31 additional subunits in bovine CI, 18 are considered, on phylogenetic grounds, to constitute a ''eukaryotic core'' present in the last eukaryotic common ancestor (LECA) [9, 10]. (However, a recent analysis of bacterial CI indicates that three of these eukaryote-core subunits could also have been contributed by the α-proteobacterial progenitor of mitochondria [11].) In bovine CI, the remaining subunits (13) are termed metazoan-specific, as they have not been identified outside of animals [9].

We suppose that ETC complexes such as CI were built up gradually, and it is entirely conceivable that CNE-type interactions played a role in the increase in complexity that accompanied the evolution of CI, both prior and subsequent to the emergence of the eukaryotic cell. Although we can trace a number of eukaryote-specific CI subunits to LECA, there is a ''black box'' interval between the emergence of the eukaryotic cell per se and the emergence of the LECA that we surmise on comparative genomic grounds. Hence, the assumption of a ''very rapid development of the eukaryotic cell'' that has ''all the hallmarks of ... a period of intense selection'' is problematic, to say the least. We simply do not know how many evolutionary dead ends and extinct eukaryotic lineages preceded the emergence of LECA, nor do we know how long this transition took; hence, we can infer nothing about the process and timeline of eukaryotic CI evolution during this period, save for the end result.

In any event, the lineage-specific accessory components of CI and other ETC complexes are of particular interest, as it is these that can be most easily rationalized as having arisen relatively recently via a CNE pathway. These proteins tend to be relatively small, compared to the more evolutionarily ancient subunits in the complexes they inhabit, and to be very narrowly restricted phylogenetically. For example, CV, the mitochondrial ATP synthase, has lineage-specific novel subunits in *Tetrahymena thermophila* [12], but a different lineage-specific novel set in *Chlamydomonas reinhardtii* [13]. Thus, these distinct sets have all the hallmarks of newly evolved proteins that fortuitously happened to be targeted to and imported into mitochondria, where they could engage in interactions with mitochondrial supramolecular assemblages like ETC complexes. This origin model says nothing about how important (or not) these added proteins may now be to eukaryotic ETC complexes, only that their initial interactions with these complexes pre-dated any functional requirement for them.

So, what is CNE and why should it be considered? CNE is a ratchet-like process capable of generating biological complexity that is driven by properties intrinsic to macromolecules and emphasizes the role of neutral evolution, not positive selection. However, once in place, CNE-generated complexities are preserved by negative or ''purifying'' selection, and may later go on to acquire useful functions. Thus, CNE distinguishes between ''origin'' and ''present-day function'' when considering the evolution of complexity. Despite suggestions by Speijer to the contrary, the literature (on RNA editing, e.g. [14]) is rife with ''Just So Stories'' that attempt to link the origin and expansion of complexity with functional advantages. If one doesn't fit, try another. It is our assertion that most molecular biologists first attempt to find adaptive, positive-selection explanations, generally without even considering CNE. Quite the opposite of ''stifling further thought'' on the evolution of complex biological processes, CNE provides a new way to think about it. As a null hypothesis against which adaptationist arguments for the evolution of cellular complexity can and should be compared, CNE should improve the rigor with which we form such explanations.

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