

Out-of-Equilibrium Selection Pressure Enhances Inference from Protein Sequence Data

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Homologous proteins have similar three-dimensional structures and biological functions that shape their sequences. The resulting coevolution-driven correlations underlie methods from Potts models to AlphaFold, which infer protein structure and function from sequences. Using a minimal model, we show that fluctuating selection strength and the onset of new selection pressures improve coevolution-based inference of structural contacts. Our conclusions extend to realistic synthetic data and to the inference of interaction partners. Out-of-equilibrium noise arising from ubiquitous variations in natural selection thus enhances, rather than hinders, the success of inference from protein sequences.

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Introduction—During evolution, proteins evolve through random mutations of their sequences and natural selection for function. This coupling between sequence and function leads to coevolution between amino-acid sites in multiple sequence alignments (MSAs) of homologous proteins, which share ancestry, three-dimensional structure, and function. Amino-acid identities at contacting positions in a protein's three-dimensional structure are correlated, due to the need to preserve physicochemical complementarity. This has allowed the prediction of structural contacts from sequences using mutual information [1] and Potts models [2–5], which are pairwise maximum entropy models inferred on MSAs. Attention coefficients in protein language models trained on MSAs capture coevolution [6,7], a key factor underlying AlphaFold's success in protein structure prediction [7]. Beyond structure, coevolution allows the inference of interaction partners [8–11] and of functional sectors of collectively correlated amino acids [12–15], as well as the investigation of protein evolution [16–21].

Natural environments often change over time [22–27], causing variable selection pressures on proteins [22]. How do such fluctuations impact inference from sequences? To address this, we consider a minimal model of protein sequences evolving under fluctuating selection to preserve structure. We show that the resulting out-of-equilibrium noise can enhance the inference of structural contacts via Potts models. This conclusion extends to realistic synthetic data. It also holds in a simple model of the onset of a new

selection pressure, where a random ancestral sequence evolves under structural selection with phylogeny. Finally, we show that out-of-equilibrium selection also enhances the inference of interaction partners. Our results suggest that time-dependent external drives can facilitate inference by pairwise maximum entropy models.

Model and methods—We consider a minimal model, where protein sequences are represented as length- L sequences of Ising spins $(\sigma_1, \dots, \sigma_L)$ defined on the nodes of a fixed Erdős-Rényi random graph. Structural contacts are modeled by pairwise ferromagnetic couplings on the graph edges, all set to 1 for simplicity, leading to the Hamiltonian

$$H = - \sum_{(i,j) \in \mathcal{E}} \sigma_i \sigma_j, \quad (1)$$

where $\mathcal{E}$ denotes the set of graph edges; see also [28]. We sample independent equilibrium sequences using a Metropolis-Hastings algorithm by proposing spin flips at random sites and accepting them with probability

$$p = \min[1, \exp(-\Delta H/T)], \quad (2)$$

where ΔH represents the energy variation associated with the flip and T the Monte Carlo sampling temperature; see Supplemental Material Fig. S1(a) for a schematic and Fig. S2 for information about equilibration [29]. The selection strength is controlled by T , with higher T corresponding to weaker selection [28]. We model variable selection strength using a telegraph process that switches between T_1 and $T_2 > T_1$ with equal transition rates $1/\tau$. We start from independent equilibrium sequences at temperature T_1 ; see Fig. S1(b) [29].

To go beyond this minimal model, we generate realistic synthetic data using a Potts model inferred from natural

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sequences via Boltzmann machine Direct Coupling Analysis (bmDCA) [31,32], an approach whose generative power was experimentally validated [32].

Finally, to model the onset of a new selection pressure, we start from a random ancestral sequence and apply selection for structure using the minimal model described above. We evolve different sequences from the same ancestor via independent Markov chains, producing a simple star phylogeny; see Fig. S1(c) [29].

Fluctuating selection enhances contact inference—In our model, selection strength is controlled by the sampling temperature T . At very low T , only spin flips that decrease the energy [Eq. (1)] are accepted, leading to low sequence variability and poor inference. Conversely, at very high T , almost all spin flips are accepted, producing noisy sequences that also hinder inference. As a result, inference is optimal at intermediate temperatures. In the present minimal model featuring a ferromagnetic-paramagnetic phase transition (Fig. S3 [29] and [28,33]), inference is most successful around and moderately above this transition [28,34]. With our parameter values, it occurs at $T_C = 4$ (Fig. S4 [29]). Inference performance also depends on the number of available sequences (Fig. S5 [29] and [33,34]). In natural data, this number is limited by experimental sampling and by biological evolution, leading to finite-size effects that hinder inference [3,4,35]. Thus motivated, we work with finite MSAs throughout.

How does the time variability in the selection pressure impact inference from sequence data? To answer this within our minimal model, we start from independent sequences equilibrated at $T_1 = 1$ and evolve them under a time-varying sampling temperature that switches between $T_1 = 1$ and $T_2 = 15$.

Figure 1 shows the true positive (TP) fraction defined as the fraction of true contacts among the top N inferred Ising couplings, where N is the actual number of contacts, versus the number of accepted spin flips representing mutations. Figure 1(a) shows that the average TP fraction increases with time before plateauing, indicating that fluctuating selection strength enhances inference performance. Faster temperature switches further improve inference by increasing the sequence diversity. The effective number of sequences M_{eff} [2] defined by grouping sequences with Hamming distance smaller than 0.2, reaches 1980. This is close to the value for an MSA generated at $T = 15$ (2048), and to the total number of sequences per MSA (2048), and far above the nearly frozen $M_{\text{eff}} = 2$ at $T = 1$. Inference under a variable temperature outperforms both fixed-temperature cases at T_1 and T_2 and matches the best fixed-temperature case (see Fig. S4 [29]) demonstrating that variable selection strength can substantially improve inference.

Figure 1(b) shows that, in single realizations of the process, switching to strong selection (T_1) after a weak selection phase (T_2) yields a transient improvement in

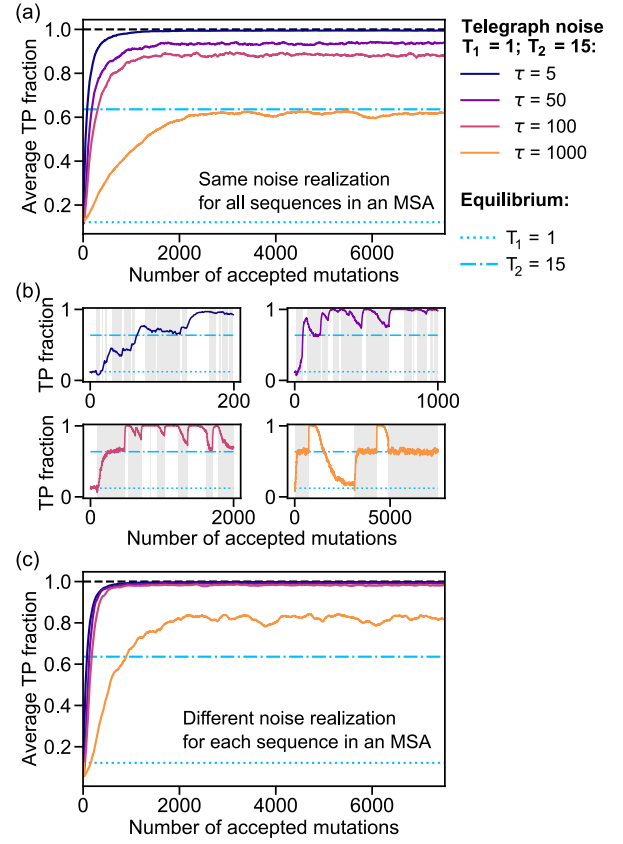


FIG. 1. Impact of fluctuating selection strength on contact prediction. TP fraction versus number of accepted mutations per sequence, while switching selection strength (i.e., the sampling temperature) via a telegraph process with timescale τ . (a) For each value of τ , the TP fraction is averaged over 1000 replicates. In each replicate, the same telegraph process is used for all sequences in the MSA. Different replicates use different realizations of the data generation and of the telegraph process. (b) The TP fraction is shown for single realizations of the telegraph process and data generation. Gray background, T_2 ; white background, T_1 . (c) Same as in (a), except that a different telegraph process is used for each sequence in an MSA. In all panels, TP fractions for equilibrium sequences generated at T_1 and T_2 are shown for reference. Sequences are generated using our minimal model [see Figs. S1(a) and S1(b) [29]], with $T_1 = 1$ and $T_2 = 15$. For each realization, we generate an MSA of 2048 sequences of length 200 using an Erdős-Rényi random graph with probability 0.02 to represent contacts, and infer contacts via mean-field DCA (mfDCA) [3,4], which is computationally efficient and performs well on minimal data [28], with pseudo-count 0.01.

inference performance (see also Fig. S6 [29]). Increasing selection strength suppresses noise that partly obscures structural correlations, before significantly reducing sequence diversity. Thus, switching between weak and strong selection temporarily enhances structural correlations in sequences. If the system is held at a fixed temperature for times comparable to the equilibration times (see Fig. S2 [29]), inference performance relaxes to its

equilibrium value at that temperature. The observed improvement is therefore a transient, out-of-equilibrium effect. Faster temperature alternations, on timescales significantly below equilibration, amplify these effects and further enhance inference performance, as seen in Fig. 1(a).

What is the impact of the temperatures T_1 and T_2 between which the system switches? In our minimal model, inference is optimal around and slightly above the ferromagnetic-paramagnetic transition temperature T_C (see above). Accordingly, alternating between $T_1 < T_C$ and $T_2 > T_C$ improves inference, whereas switching between two temperatures below or above T_C does not (Fig. S7 [29]). Furthermore, for fast switches, the data resemble equilibrium data generated at an intermediate temperature between T_1 and T_2 , and lead to similar inference performance (Fig. S8 [29]).

So far, all sequences in an MSA were subjected to the same fluctuating selection strength. However, in natural data, different sequences (e.g., from different species) may experience different selection histories. Figure 1(c) shows the inference performance when each sequence in an MSA is subjected to an independent realization of the temperature fluctuations. Inference is enhanced even more strongly than in Fig. 1(a), indicating that heterogeneous fluctuating selection further amplifies the effect.

Extension to realistic synthetic data—How do the observations made with our minimal model generalize to more realistic data? To address this question, we infer a Potts model from a natural MSA of the protein family PF0004 (AAA ATPase) from Interpro (Pfam) [36] via bmDCA [31,32]. We then generate sequences from this Potts model, either at equilibrium or under fluctuating selection strength (see the model and methods section).

Figure 2 shows the TP fraction versus the number of accepted mutations for these realistic synthetic data. As in the minimal model, alternating between strong and weak selection improves inference performance, with faster alternations between low and high temperature yielding larger gains. Inference performance then becomes comparable to the best equilibrium performance obtained at temperature $T = 1$ (Fig. S9 [29]), even though both alternating temperatures yield poorer inference at equilibrium. Thus, our conclusions from the minimal model extend to realistic synthetic data.

Impact of selection onset with phylogeny—Beyond fluctuating environments, out-of-equilibrium effects also arise when a new selection pressure emerges. We model this by generating sequences starting from a random ancestral sequence, under selection for structural contacts at fixed T in the minimal model, using a simple star phylogeny (see model and methods section). This setup captures the onset of a specific selection pressure acting on a protein (sub)family, starting from an ancestor that was not subject to this particular constraint.

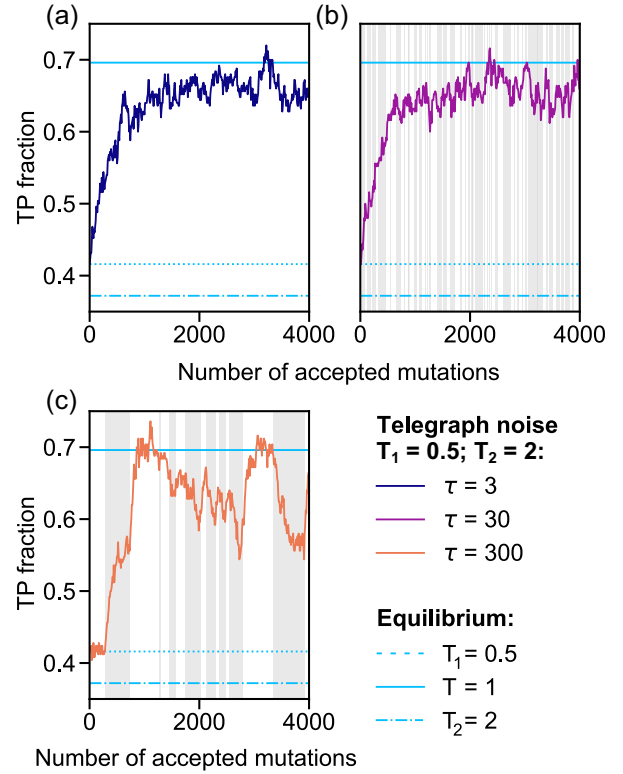


FIG. 2. Impact of fluctuating selection strength on contact prediction: PF0004 family. As in Fig. 1(b), the TP fraction is shown versus the number of accepted mutations under switching selection strength via a telegraph process, but for realistic data composed of MSAs of 70 000 sequences generated from a Potts model inferred on a natural MSA of 39 277 sequences from the PF0004 family (with length 132), following Refs. [31,37], in particular using regularization strengths 0.01. Panels (a)–(c) use telegraph noise with different timescales τ . Gray background, T_1 (alternations not shown in the first panel for readability). Inference performance for equilibrium sequences generated at T_1 , T_2 , and $T = 1$ are shown for reference. Contact inference is performed using pseudolikelihood maximization DCA (plmDCA) [38,39], with regularization strengths set to 0.01 and no phylogenetic reweighting.

Figure 3 shows the TP fraction versus the number μ of accepted mutations per branch in the star phylogeny. For $T < 4$, i.e., below the ferromagnetic-paramagnetic transition of the minimal model, the TP fraction exhibits a maximum at intermediate μ . Inference then performs significantly better than the equilibrium TP fraction approached at large μ . This enhancement coincides with a transient increase of sequence diversity (Fig. S10 [29]), and persists for a balanced binary branching tree (Fig. S11 [29]). Hence, under strong selection, out-of-equilibrium noise originating from the random ancestor enhances contact inference. The beneficial effect of out-of-equilibrium noise evidenced for fluctuating selection thus also applies to the onset of a new selection pressure and remains

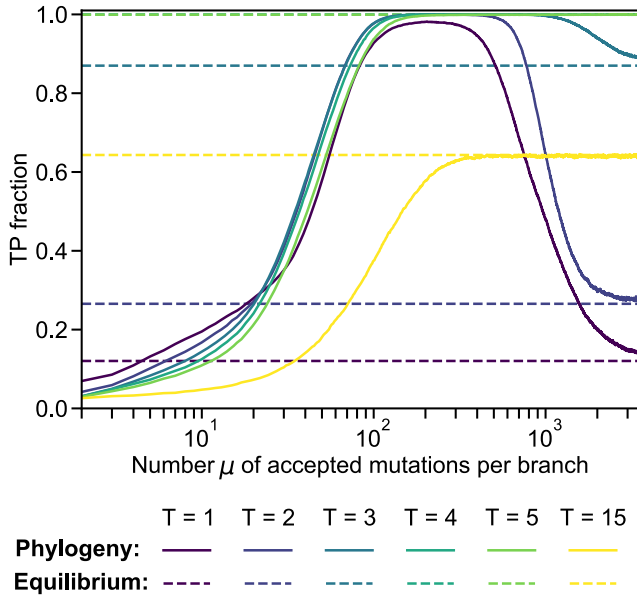


FIG. 3. Impact of selection onset on contact inference with phylogeny. The TP fraction is shown versus the number μ of accepted mutations per branch of the star phylogenetic tree, starting from a random ancestral sequence. Sequences with length 200 are generated as explained in Fig. S1(c) [29], using the same Erdős-Rényi graph as in Fig. 1, along a star phylogenetic tree with 2048 branches. Along each branch, Monte Carlo sampling is performed until μ mutations are accepted, giving 2048 final sequences, which constitute our MSA. We infer contacts via mfDCA [3,4,28] with pseudocount 0.01. Results are averaged over 100 replicates, each starting from a different random ancestor.

effective even with phylogeny, which impairs contact inference performance under constant selection [28].

Extension to interaction partner inference—So far, we considered structural contact inference. Coevolution-based methods are also used to infer protein interaction partners [8–10,40]. To test whether our results extend to this problem, we perform partner inference within our minimal model using the method of [40]. As for contact inference, partner inference performance peaks at an intermediate temperature (Fig. S4 [29]). Figure 4 shows that fluctuating selection strength enhances partner inference, indicating that the beneficial effect of out-of-equilibrium selection extends beyond contact inference.

Discussion—Using a minimal model, we showed that alternating between weak and strong selection enhances structural contact inference from sequences. This effect extends to realistic synthetic sequences. It also extends to the onset of a new selection pressure, even in the presence of phylogeny. Moreover, out-of-equilibrium selection also enhances interaction partner inference.

Under strong selection, limited sequence variability hinders coevolution-based inference. Alternating with weak selection, or starting from an unselected ancestor

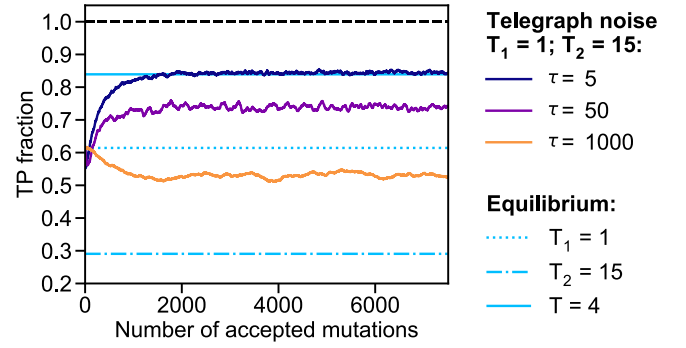


FIG. 4. Impact of fluctuating selection strength on interaction partner prediction. TP fraction versus number of accepted mutations, while switching selection strength (i.e., the sampling temperature) via a telegraph process with timescale τ . For each value of τ , the TP fraction is averaged over 1000 replicates. TP fractions for equilibrium sequences generated at T_1 and T_2 are shown for reference. MSAs comprising 1024 sequences are generated as in Fig. 1(a), and then randomly split into a training set of 400 sequences and a testing set of 624 sequences. The latter is randomly divided in sets of four sequences, each set representing a species, and each sequence is split in two halves of equal length representing two interaction partners. Pairings between partners are blinded in each species and predicted using mfDCA scores with pseudocount 0.01 [40].

transiently increases diversity, enhancing inference. Overall, our results reveal that, far from hindering it, out-of-equilibrium selection can facilitate the extraction of structural and functional information from protein sequences. While we relied on synthetic data, which allowed us to vary selection pressures and control ancestral sequences, the extension to realistic data suggests that these principles are generic and relevant for natural proteins, where variable selection pressures are ubiquitous [22,23]. Hence, our findings suggest that the success of coevolution-based inference in proteins may be partly caused by fluctuating selection intensity.

We considered simple forms of selection variability, either switching between two strengths or adding a new selection pressure. Other forms of fluctuations of selection strengths, including general random walks, are expected to yield similar results, provided that weak and strong selection are included and the fluctuations are faster than equilibration times. In addition, while we focused on structural selection pressures, which are a major constraint on protein evolution [41], our work could be extended to other evolutionary constraints, such as those shaping collectively correlated amino-acid groups [12–15], and to ribonucleic acid structure inference [42]. Additional perspectives include selection pressure variations that cannot be captured by an overall scaling of selection strength, e.g., affecting specific fields or couplings. Finally, although we focused on inference based on Potts models, coevolution also underlies recent deep learning approaches, including

AlphaFold [7], indicating that out-of-equilibrium fluctuations may similarly benefit these approaches.

Beyond natural evolution, our results have implications for directed evolution experiments [43]. In recent studies [44,45], structural prediction was performed using sequences evolved *in vitro* under constant selection strength. It was later shown [46] that tuning this strength can improve contact inference. Our results suggest that dynamically varying selection strength during experiments, e.g., by varying antibiotic concentration, could also enhance inference.

More broadly, pairwise maximum entropy models are widely used for inference beyond protein sequence data. A prominent example is neuronal population activity [47–49]. Neurons are often driven by common input stimuli that vary in time and could play a role analogous to fluctuating selection strength in proteins. Such inputs have been found to impact inference [50], increase inferred couplings in some cases [51], and generate signatures reminiscent of criticality [52,53], which are frequently observed in biological systems [54]. Our results thus suggest a potentially general principle: Time-dependent external drives can enhance inference in systems described by pairwise maximum entropy models.

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Data availability—The code that supports the findings of this article is openly available [55].

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