**Experimental evolution and epistasis in variable environments**[1]

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Environmental changes trigger cellular responses, but also impose selective pressures on the underlying regulatory systems. To disentangle this complex interplay we follow a synthetic biology approach. By linking the output of regulatory systems to bacterial growth, quantified temporally variable selective pressures can be applied to regulatory systems. This approach allows one to explore how networks evolve in complex variable environments. Epistatic interactions that underlie evolutionary constraint have mainly been studied for constant external conditions. However, environmental changes may modulate epistasis and hence affect genetic constraints. We investigate genetic constraints in the adaptive evolution of a novel regulatory function in variable environments, using the lac repressor, LacI, as a model system. We systematically reconstructed mutational trajectories from wild type LacI to three different variants that each exhibit an inverse response to the inducing ligand IPTG, and analyzed the higher-order interactions between genetic and environmental changes. We find epistasis to depend strongly on the environment. As a result, mutational steps essential to inversion but inaccessible by positive selection in one environment, become accessible in another. We present a graphical method to analyze the observed complex higher-order interactions between multiple mutations and environmental change, and show how they can be explained by a combination of mutational effects on allostery and thermodynamic stability. This dependency of genetic constraint on the environment should fundamentally affect evolutionary dynamics and phylogenetic analysis.