THE EVOLUTIONARY IMPACTS OF SYNONYMOUS MUTATIONS

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ABSTRACT

During the 50 years since the genetic code was cracked, our understanding of the evolutionary consequences of synonymous mutations has undergone a dramatic shift. Synonymous codon changes were initially considered selectively neutral, and as such, exemplars of evolution via genetic drift. However, the pervasive and non-negligible fitness impacts of synonymous mutations are now clear across organisms. Despite the accumulated evidence, it remains challenging to incorporate the effects of synonymous changes in studies of selection, because the existing analytical framework was built with a focus on the fitness effects of nonsynonymous mutations. In this chapter, I trace the development of this topic and discuss the evidence that gradually transformed our thinking about the role of synonymous mutations in evolution. I suggest that our evolutionary framework should encompass the impacts of all mutations on various forms of information transmission. Folding synonymous mutations into a common distribution – rather than setting them apart as a distinct category – will allow a more complete and cohesive picture of the evolutionary consequences of new mutations.

INTRODUCTION

"*As I hope to have shown, consideration of natural selection has been a good guide in exposing the amount, the nature, and the consequences of degeneracy of the code, even leading to the revelation of some of its features that add both to its beauty and, in a minor way, to the corpus of genetic concepts*." – T M Sonneborn, 1965

Ever since the degeneracy of the genetic code was revealed, synonymous mutations have played an important role in the development of molecular as well as evolutionary biology. The fact that multiple codons code for a single amino acid immediately implicated synonymous codons as key players during the evolution of the genetic code (Crick et al. 1961; Sonneborn 1965; Woese 1965). This perspective, emphasizing the importance of codon assignment during the evolution of life, remains relevant to date (Koonin and Novozhilov 2016). In the decades that followed, synonymous codon changes have featured in many important developments in evolutionary biology. These include the neutral and nearly neutral theories of molecular evolution, the molecular clock, the organization, composition and exchange of genetic material, the evolution of gene regulation, robustness of information transfer, and methods to identify signatures of selection.

Much of the evolutionary perspective on synonymous mutations has revolved around synonymous codon changes in the context of translation (i.e. with respect to the information

contained within a nucleotide triplet) (Sharp and Li 1986b; Andersson and Kurland 1990; Hershberg and Petrov 2008; Sharp et al. 2010; Plotkin and Kudla 2010). This is because codons read by more abundant tRNAs are often translated faster and with greater accuracy (Precup and Parker 1987; Sørensen et al. 1989; Gardin et al. 2014). However, the role of synonymous changes as carriers and influencers of many different layers of information embedded in the genetic code is increasingly apparent (Chamary et al. 2006; Bailey et al. 2021). These include impacts that are related to translation (Liu 2020), as well as those that do not directly affect translation (Callens et al. 2021). The diverse mechanisms responsible for the consequences of synonymous mutations are described in detail in other Chapters in this book. Here, I focus on the trajectory of the evolutionary perspective on synonymous mutations over the course of the past 50 years. Although I cannot do justice to the many kinds of evidence and developments that drove this arc, I have attempted to give an overview of the major insights that changed the course of the narrative from neutrality to pervasive selection.

EVIDENCE FOR THE EVOLUTIONARY IMPACTS OF SYNONYMOUS MUTATIONS

I first provide a broad introduction to different types of evidence that can be used to infer the nature and strength of selection acting on a focal set of mutations. While these methods can be applied to any class of mutations, below I discuss them in the specific context of synonymous changes, to make it easier to follow the discussion in subsequent sections.

Indirect evidence

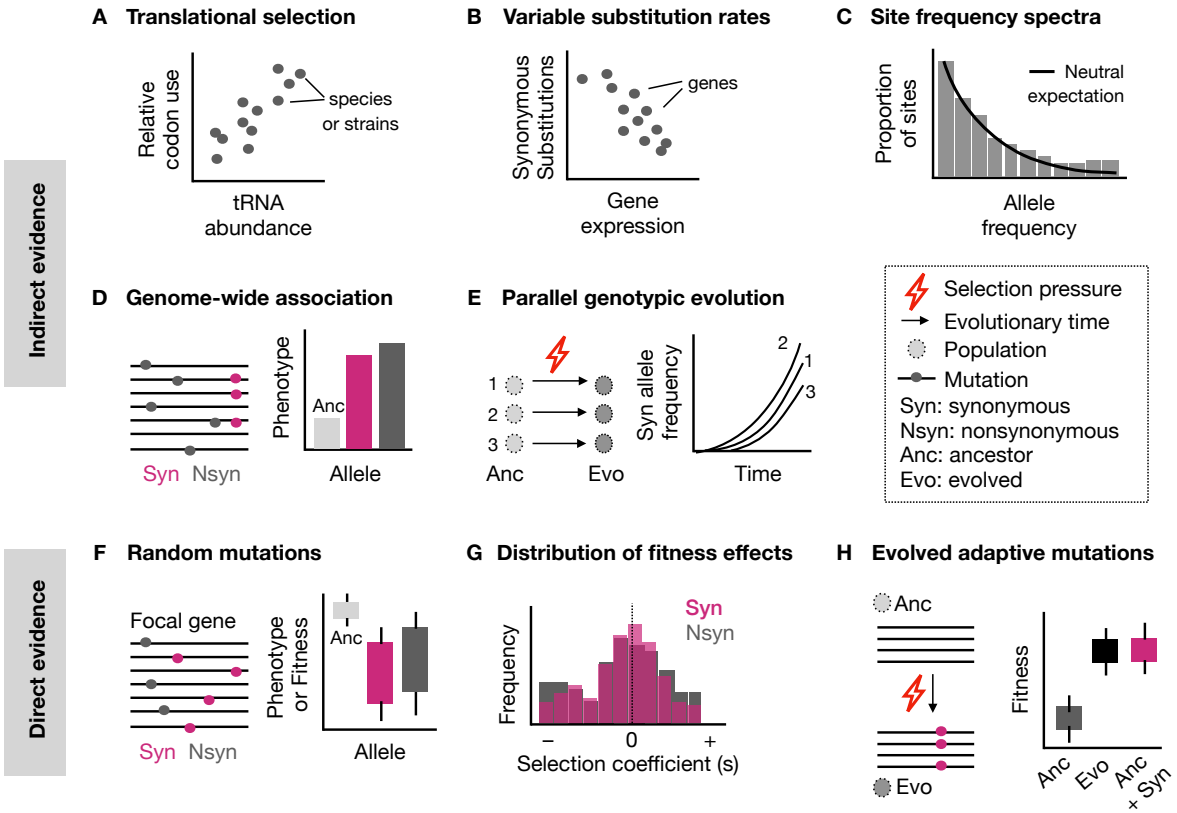
Results from bioinformatics analyses can provide important evidence suggesting the role of genetic drift vs. selection in driving observed patterns of genetic variation. The core idea is that the incidence or frequency of neutral mutations should be consistent with the action of genetic drift. On the other hand, selection either for or against a category of mutations should result in their enrichment or depletion in specific contexts that reflect the source of selection. In case of synonymous mutations, selection for rapid or accurate translation is expected to drive a positive correlation between relative codon use and abundance of cognate tRNAs across taxa (Fig 1A). Within a species, genes that are more important for cell function should evolve under stronger selection. Hence, deleterious synonymous mutations should be removed more effectively from highly expressed genes, reducing the overall synonymous substitution rate (Fig 1B). Although the figure illustrates simple scenarios, more complex and multi-dimensional analyses can allow us to investigate the impact of many factors at once.

Extending this logic to specific mutations, we can ask whether synonymous allele frequency in naturally or experimentally evolved populations is consistent with evolution under selection. Such analyses come in many flavours. Typically, the site frequency spectrum (SFS) – a histogram of derived allele frequencies in a population – is skewed left, so that only a few mutations are observed at high frequency and most substitutions are rare (Fig 1C). Deviations from the expected SFS under neutral evolution are thus indicative of natural selection; e.g. the frequency of mutations at the far right of the distribution in Fig 1C is higher than expected. Such deviations at synonymous sites would suggest selection favouring these mutations. Next, genome wide association studies (GWAS) ask whether a particular phenotype (e.g. a disease) is consistently correlated with the presence (or absence) of specific alleles, including

synonymous polymorphisms (Fig 1D). In SFS and GWAS analyses, evolution has already occurred, and we are asking whether the outcomes (at the level of patterns of genetic polymorphism) are consistent with neutrality or selection. Similarly, an increase in synonymous allele frequency during selection experiments – especially if it occurs repeatedly across independently evolving populations – suggests that the allele may be adaptive (Fig 1E).

A major limitation of these analyses is that it is difficult to control for the effect of multiple known and unknown covariates, and to disentangle complex interactions between them. Ultimately, although these analyses can be powerful, they can only show correlation and not causation. Hence, more direct experimental evidence becomes necessary.

Fig 1: Different types of evidence for the evolutionary impacts of synonymous mutations. Schematics illustrate various kinds of comparative and experimental studies that can demonstrate the role of synonymous mutations in evolution. Panel E shows three independently evolved populations.



Direct evidence

As with the methods discussed above, direct experimental analysis can focus on a class of mutations or specific mutations of interest. A commonly used strategy involves random or directed mutagenesis of a focal gene, followed by identifying the mutation(s) and measuring their impact on phenotype or fitness (Fig 1F). Such datasets can be used to measure the selection coefficient (*s*), which indicates the fitness impact of a mutation relative to the

ancestor. The coefficients can then be used to estimate the general statistical distribution of fitness effects (DFE) of mutations (Fig 1G), which is important for predicting adaptive evolution and the fate of new mutations. For instance, a distribution with fat tails would indicate that many mutations have very large fitness effects and will be rapidly eliminated or fixed by selection.

Finally, the most conclusive evidence comes from experimental evolution studies that directly observe the occurrence of adaptive mutations. Such studies effectively extend the paradigm shown in Fig 1E by engineering the evolved putatively beneficial allele on the ancestral genomic background. If the engineered strain has higher fitness, it is clear that the allele is beneficial and evolved under positive selection (Fig 1H). Whereas the experiments described earlier (Figs 1F and 1G) show that synonymous mutations *could* face selection, studies of the sort exemplified in Fig 1H directly demonstrate that specific synonymous mutations *do* evolve under selection, and are adaptive. This is crucial because various cellular or population level phenomena may buffer or alter the longer-term impacts of mutations, preventing selection from effectively acting on them in proportion to their observed short-term phenotypic effects.

CHANGING EVOLUTIONARY PERSPECTIVES ON SYNONYMOUS MUTATIONS

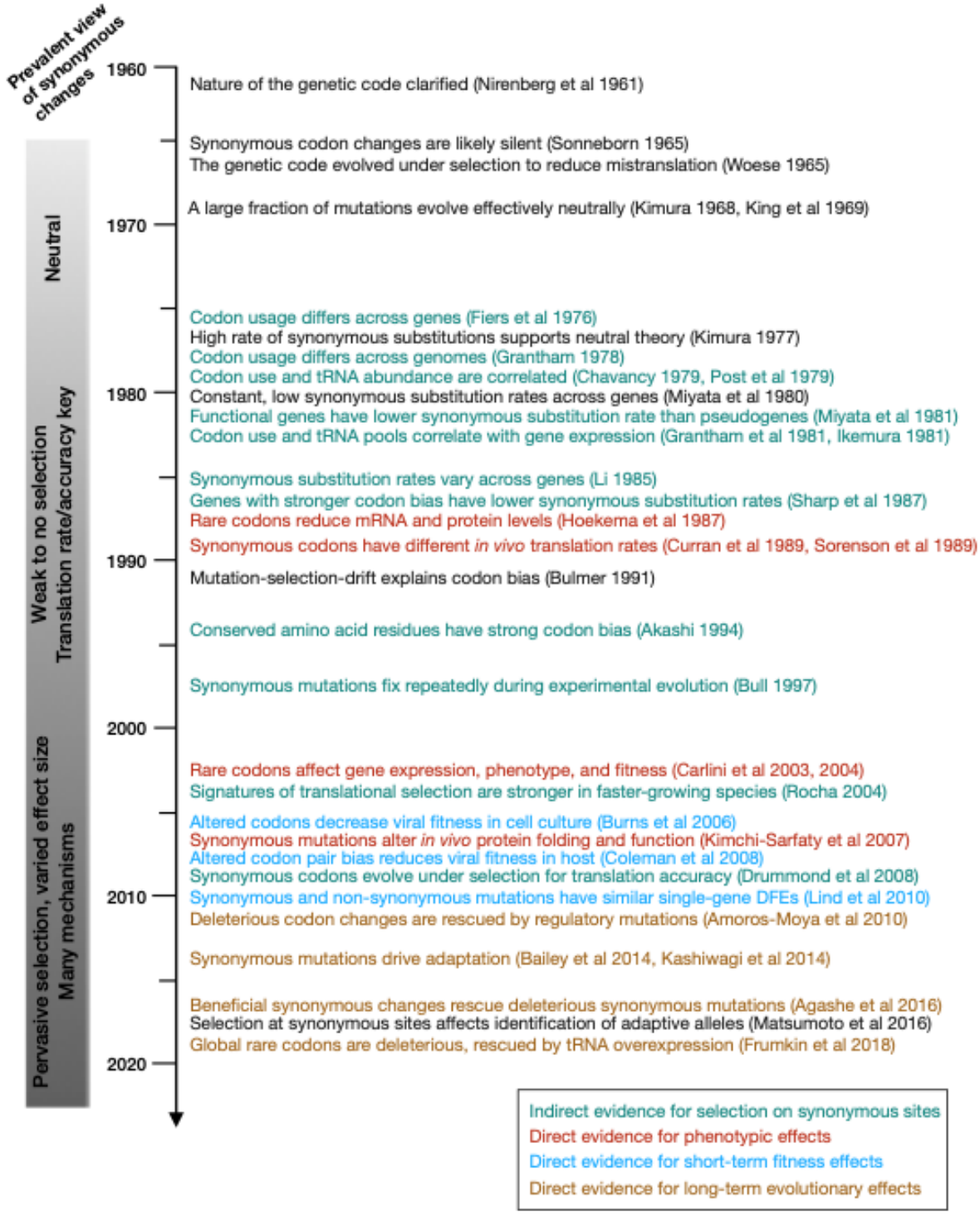
I now provide an overview of the transformation that occurred in evolutionary biology, regarding the role of synonymous changes. As with all transformations, the prevalent thinking changed gradually as evidence accumulated, and perspectives did not fall into neat mutually exclusive bins of time. Nonetheless, for ease of organization and narrative, I have split the timeline into three phases that approximately capture the progression of thought in the field. The timeline is summarized in Fig 2 along with examples of the earliest comprehensive evidence for specific inferences about the evolutionary effects of synonymous changes. Below, I discuss the changing perspectives in more detail, including a larger portion of the body of supporting work.

Phase I: Mostly neutral

The years immediately following the discovery of the nature of the genetic code (Crick et al. 1961; Nirenberg and Matthaei 1961) were heady. Even as biochemists painstakingly assigned meaning to each codon, everyone tried to divine the logic behind the peculiar structure of the codon table. The degeneracy of the code suggested neutrality. Because synonymous changes retained the amino acid identity, they would not face strong natural selection, and were thus christened “silent” (Sonneborn 1965). It also became apparent that when single step non-synonymous mutations did occur, they often encoded amino acids with similar biochemical or biophysical properties. Hence, the structure of the genetic code appeared to have been optimized to reduce catastrophic errors during protein production, and synonymous mutations, due to their neutrality, played a major role in this optimization (Crick et al. 1961; Woese 1965; Crick 1967; Woese 1969). The silent nature of synonymous mutations was also important in the development of the neutral theory of molecular evolution, which posited that most mutations are effectively neutral and evolve under genetic drift rather than selection (Kimura 1968; King and Jukes 1969; Kimura 1977). Intriguingly, in his papers Kimura

cautioned that not all synonymous mutations may be neutral; though he clearly thought that this was a very small proportion and did not discuss it further.

Fig 2: Timeline of changing evolutionary perspectives on synonymous mutations. The first (or first comprehensive) report of a particular outcome relevant to our understanding of the evolutionary role of synonymous changes is shown here, color coded by the type of evidence (see Fig 1). Subsequent relevant work showing additional support is discussed in the text. CUB = codon usage bias.



Soon after, new comparative and experimental evidence showing biased codon usage began undermining the neutrality of synonymous codons (as outlined in Figs 1A–C). If synonymous codons were indeed silent, one would expect different synonyms to be used in approximately equal proportions. In contrast, organisms seemed to prefer some synonyms over others, suggesting a deviation from neutrality. Early data on the predicted structures of mRNA sequences indicated potential selection for specific degenerate codons to optimize mRNA structure and stability (Adams et al. 1969; Jou et al. 1971; Hasegawa et al. 1979). In addition, comparison of globin genes across a small number of species showed that synonymous substitutions were significantly reduced compared to neutral expectation; since they could not have altered amino acid sequences, their scarcity was proposed to reflect selection on mRNA structure (Kafatos et al. 1977). Further molecular studies continued to find diverse impacts of synonymous codon changes on mRNA structure (Sørensen et al. 1989), codon-anticodon binding affinity (Grosjean and Fiers 1982), and translation rate (Pedersen 1984), though codon usage was suggested to have a stronger effect than mRNA structure on translation (Sørensen et al. 1989). Thus, there was extensive discussion of the idea that synonymous changes could influence the transmission of multiple kinds of information encoded in the genome (not only amino acid sequence but also mRNA and protein structure and stability), and that it was important to tease apart their relative influence on translation (Grosjean et al. 1978; Bennetzen and Hall 1982; Sørensen et al. 1989).

However, in evolutionary biology, the narrative shifted away from selection on mRNA structure specifically, towards broader selection on translation. For instance, mRNA structure could face selection if it allowed cells to regulate translation (Yamamoto et al. 1985), and codon use itself may also evolve under selection due to its direct effects on translation. More sequencing data supported and expanded the view that biases in the use of particular codons were extensive (Fiers et al. 1976; Berger 1978; Grantham 1978; Grantham et al. 1980). At the same time, comparative analyses uncovered a striking correlation between the abundance of specific codons and the availability of tRNAs with matching anticodons, indicating evolutionary optimization to ensure rapid and/or accurate translation (Chavancy et al. 1979; Post et al. 1979; Ikemura 1981; Ikemura 1985). Consistent with the idea of selection driving codon usage bias in proportion to cellular needs, highly expressed genes showed stronger codon preferences (Grantham et al. 1981; Ikemura 1981; Bennetzen and Hall 1982; Gouy and Gautier 1982). Similar correlations across different species suggested that a consistent, common selection pressure acted on genomes to optimize codon use and tRNA pools for translation (Andersson and Kurland 1990). Nonetheless, synonymous codon changes were thought to be largely neutral relative to non-synonymous changes, because the latter could influence both the amino acid sequence as well as mRNA properties (Li et al. 1985). Indeed, early comparative evidence indicated that synonymous substitution rates are constant across genes and genomes, and are higher than rates of non-synonymous substitutions (Miyata et al. 1980), but lower than substitution rates in pseudogenes evolving under minimal functional constraint (Miyata and Hayashida 1981). Since substitution rates should be inversely proportional to the strength of selection, these data indicated that synonymous mutations evolve under substantially weaker selection than non-synonymous mutations.

Population genetic analyses also shed light on the problem of whether and how selection would favor specific codons. Extending the neutral theory, Kimura's model showed that biased

codon usage could also evolve under genetic drift (Kimura 1981). Hence, the existence of codon bias alone was insufficient evidence for selection. The key insight here was that the evolutionary fate of mutations is determined both by their fitness effect (selection coefficient) and the effective population size. Even if the overall codon usage of highly expressed genes had large fitness consequences, an individual synonymous codon change would likely face only very weak selection, because in most cases it would not impede translation significantly (Sørensen et al. 1989). In large populations (such as for bacteria), such weak selective effects are sufficient for the removal of deleterious mutations; whereas in small populations (such as for mammals), most mutations should behave as if they were neutral. Hence, codon bias would evolve under strong selection only in very large populations, where selection could effectively counter the continuous influx of mutations to suboptimal codons (Bulmer 1991). Thus, the dichotomy of neutral synonymous mutations vs. selected non-synonymous mutations continued to shape evolutionary thinking.

Phase II: Weak translational selection

Gradually, analysis of the causes and consequences of codon usage bias developed into a subfield of its own. Part of the motivation here was to explain the large variation in codon choice and strength of codon usage bias observed across genes in a given genome, as well as across genomes (species). Early models suggested that cellular tRNA pools must rapidly adapt to amino acid usage in synthesized proteins (Garel 1974); over long evolutionary periods, codon usage and tRNA pools could thus co-evolve to optimize translation. Important direct support came from the observation that synonymous codons had distinct rates of translation *in vivo* (Robinson et al. 1984; Varenne et al. 1984; Sørensen et al. 1989; Curran and Yarus 1989; Sørensen and Pedersen 1991), as well as differential accuracy (Precup and Parker 1987; Parker 1989). An important development was the codon adaptation index (CAI), which quantified the observed codon bias in highly expressed reference genes (such as ribosomal proteins) relative to the maximum possible codon bias (Sharp and Li 1987a). The expectation was that stronger selection to match the cellular tRNA pool would lead to stronger codon bias and hence higher values of CAI. This metric was a significant improvement over previous ones, because it readily allowed comparisons across genes as well as genomes, while accounting for confounding factors such as gene length, amino acid composition, and distinct codon tables (Sharp and Li 1987a). As a result, the index allowed identification of genes and genomes that had evolved under stronger translational selection relative to others. The CAI also had practical utility to design heterologous genes with enhanced expression in different hosts (Zolotukhin et al. 1996; Kim et al. 1997).

While the impact of translational selection on highly expressed genes was generally well accepted, there was some debate about why genes with low expression level showed weak codon bias. Two main evolutionary hypotheses were proposed to explain this pattern: (1) rare codons serve to suppress expression of these genes (and therefore evolve under positive selection) (Ames and Hartman 1963; Itano 1963; Grosjean and Fiers 1982; Konigsberg and Godson 1983) and (2) genes with low expression level face weaker selection, allowing rare codons to arise and persist due to a combination of mutations and genetic drift (Sharp and Li 1986a; Sharp and Li 1986b). Though the first hypothesis was favoured by those trying to understand gene regulation, translation elongation rates did not seem to be dramatically

reduced by rare codons, due to their low frequency and overall matching between codon usage and the tRNA pool (Holm 1986). Therefore, rare codons would have to occur in large numbers for translation elongation to be significantly altered. Furthermore, genes with low expression level are not enriched in rare codons (Sharp and Li 1986b), even at the beginning of the gene where codon bias is typically weaker (Eyre-Walker and Bulmer 1993). Therefore, while codon bias generally seemed to evolve under selection for smooth translation, rare codons did not appear to evolve under positive selection. Thus, differential strength of selection across genes was better supported as a general explanation for the positive correlation between the strength of codon usage bias and gene expression (Bulmer 1991).

Through the 1980s and 1990s, synonymous mutations continued to be used as exemplars of neutrality (especially at four-fold degenerate sites) as a baseline against which non-synonymous substitutions could be compared to identify genomic regions under selection. Initially this involved simple comparisons between synonymous and non-synonymous substitution rates (Miyata and Yasunaga 1980). As evidence for synonymous rate variation across genes (Li et al. 1985) and correlations with codon use (Sharp and Li 1987b) emerged, these were incorporated to account for variation in important parameters such as unequal rates of codon and nucleotide changes (Li et al. 1985; Nei and Gojobori 1986). These methods were used in interesting ways. For instance, an analysis of enterovirus isolates associated with a conjunctivitis outbreak found identical substitutions in independently evolving lineages at both nonsynonymous and synonymous sites, suggesting that both were similarly constrained by selection (Takeda et al. 1994). Eventually, acknowledging local sequence variation, newer phylogenetic methods included the ability to estimate key evolutionary parameters from sequence data in a maximum likelihood framework (Goldman and Yang 1994; Yang and Nielsen 2000). Nonetheless, tests for signatures of selection included comparing substitution and polymorphism rates at non-synonymous sites (putatively selected) vs. synonymous sites (neutral, evolving under drift) (McDonald and Kreitman 1991).

In 1987, the first experimental report of the impact of rare codons on cellular phenotype was published, showing that increasing the proportion of rare codons towards the 5' end of a highly expressed yeast gene reduced mRNA levels by up to 3-fold, and protein levels up to 10-fold (Hoekema et al. 1987). Though a subsequent study found variable impacts of altering or adding rare codons at different positions in a focal gene (Chen and Inouye 1990), it was clear that synonymous changes could have substantial effects on gene and protein expression. A few years later, the results of Hoekema et al's yeast study were corroborated in flies. Increasing the number of rare codons in the *adh* gene of *Drosophila melanogaster* larvae successively reduced production of the highly expressed enzyme alcohol dehydrogenase (Carlini and Stephan 2003). Importantly, the reduction in enzyme levels reduced larval tolerance for ethanol by ~2% (Carlini 2004). Since the species encounters high levels of ethanol in its environment, these results demonstrated the potential for selection to directly act on local synonymous codon changes. Direct evidence for the global effects of mismatched codon usage and tRNA pools would come much later. Simultaneously replacing a frequently used codon with a rare codon in multiple highly expressed *Escherichia coli* genes led to increased mistranslation and reduced growth rate, which was rescued by overexpression of the cognate (rare) tRNA (Frumkin et al. 2018).

Meanwhile, indirect evidence for the impact of codon usage on translation as well as on protein and sequence evolution continued to accumulate. In Drosophila, evolutionarily conserved amino acids showed stronger codon bias compared to sites that were more labile (Akashi 1994). Selection can also be inferred by comparing patterns of variation in closely related species: greater divergence between species relative to within-species polymorphism indicates that divergence occurred due to selection. Indeed, across closely related species, compared to rare codons, preferred codons showed significantly more divergence relative to polymorphism, suggesting that selection on synonymous changes was prevalent (Akashi 1995). Importantly, mutational biases could not explain the observed patterns of codon usage bias within and across species (Akashi 1994; Duret and Mouchiroud 1999; Eyre-Walker 1999); though in the human genome, mutational biases appeared to better explain codon usage patterns (Urrutia and Hurst 2001). Incorporating mutation-selection-drift parameters into phylogenetic analyses of selection, McVean and Vieira also demonstrated species-specific patterns of selection at synonymous sites (McVean and Vieira 2001), clarifying conditions under which codon preferences could diverge across species.

Subsequent analyses of larger datasets also supported the idea of co-evolved tRNA pools and codon usage bias driven by translational selection for highly expressed genes (Duret and Mouchiroud 1999; Kanaya et al. 1999; Supek 2015), especially in fast-growing species (Rocha 2004; Higgs and Ran 2008; Vieira-Silva and Rocha 2010; Ran and Higgs 2010). Mathematical models and comparative analyses also suggested that different combinations of codon use, tRNA pools, and associated systems (such as tRNA modifying enzymes, which modulate wobble pairing) could evolve under selection (Higgs and Ran 2008; Novoa and Pouplana 2012; Diwan and Agashe 2018). Changes in codon preference could thus be facilitated by periods of genetic drift (Shields 1990), though such weakening of selection is not strictly necessary (Hershberg and Petrov 2009; Sun et al. 2017). Thus, the same global selection pressure could explain diverse patterns of variation in the translation machinery. A broad comparative analysis across model organisms suggested that both synonymous and missense mutations evolve under strong selection to avoid mistranslation, reinforcing the idea of a common selective pressure acting on multiple aspects of protein evolution (Drummond and Wilke 2008). Interestingly, experimental evolution studies with microbes found repeated instances of synonymous mutations rising to high frequency (as outlined in Fig 1E) (Bull et al. 1997; Bull et al. 1998; Holder and Bull 2001; Cuevas et al. 2002; Novella et al. 2004). Although this was rarely discussed seriously, the parallelism suggested that synonymous mutations could impact adaptive evolution. However, direct evidence for the broader evolutionary role of synonymous mutations was still missing.

Phase III: Pervasive (sometimes strong) selection, diverse mechanisms

The period from 2005 onwards would prove to be pivotal, as the number of studies exploded and evidence for major phenotypic and fitness impacts of synonymous changes began to accumulate in various contexts. For instance, selection at synonymous sites became clearer in groups such as mammals (Chamary et al. 2006) and specifically in humans (Comeron 2006), where earlier evidence was weaker. In fact, many synonymous mutations were now shown to be strongly associated with human diseases (Cartegni et al. 2002; Sauna and Kimchi-Sarfaty 2011; Supek et al. 2014; Hunt et al. 2014; Sharma et al. 2019). Hence, a

general consensus began to form that across taxa, synonymous changes evolved under significant selection, with multiple underlying mechanisms (Hershberg and Petrov 2008; Sharp et al. 2010; Plotkin and Kudla 2010; Novoa and Pouplana 2012). Even so, much of this discussion was focused on selection on codon bias and other direct impacts on translation. Gradually, as analyses began to include synonymous mutations outside of the codon context, the broader evolutionary implications of synonymous mutations emerged. For example, mutagenesis of reporter genes expressed in *E. coli* showed large impacts of synonymous mutations on gene expression (as outlined in Fig 1F), with the effects better explained by mRNA structure rather than codon bias *per se* (Kudla et al. 2009; Goodman et al. 2013).

Many studies demonstrated that the phenotypic impacts of synonymous changes also translate into fitness consequences, via mechanisms that are not always directly related to translation. For instance, *in vivo* protein folding and function were altered by synonymous SNPs implicated in genetic association studies of drug-resistant cancers (Kimchi-Sarfaty et al. 2007). Beneficial as well as deleterious synonymous mutations were reported in multiple studies, often with microbes (Kudla et al. 2009; Lind et al. 2010a; Cuevas et al. 2012; Agashe et al. 2013; Bailey et al. 2014; Kershner et al. 2016; Brandis and Hughes 2016; Agashe et al. 2016; Canale et al. 2018; Mochizuki et al. 2018; Zwart et al. 2018; Lebeuf-Taylor et al. 2019). Importantly, these reports include many instances of strong fitness consequences (i.e. high *s* values) of single synonymous mutations (Lind et al. 2010a; Firnberg et al. 2014; Agashe et al. 2016; Lind et al. 2017; Kristofich et al. 2018; Sane et al. 2020). These large-effect synonymous mutations should certainly be visible to natural selection, even in relatively small populations.

A handful of experimental evolution studies with bacteria now provide direct evidence that synonymous mutations can indeed evolve under selection (Bailey et al. 2014; Kershner et al. 2016; Knöppel et al. 2016; Agashe et al. 2016; Kristofich et al. 2018). In these studies, evolved synonymous substitutions were observed repeatedly, and shown to be highly beneficial when engineered into the ancestral genomic background (as outlined in Fig 1H). For instance, *Methylobacterium* strains carrying recoded versions of a highly expressed gene initially suffered a large decrease in fitness relative to the wild type ancestor (Agashe et al. 2013). In fact, an allele carrying only preferred codons was nearly lethal because of the severe reduction in enzyme production. Subsequently, all nine independently evolved replicate populations of this strain rapidly fixed the same synonymous substitution, which elevated fitness to near-wild type levels (Agashe et al. 2016). Similarly, strains carrying other recoded alleles also adapted via synonymous beneficial mutations. Recent studies further demonstrate other characteristics of synonymous mutations that are consistent with observations for non-synonymous mutations. For instance, evolution in synonymously recoded phage genes demonstrates patterns of epistasis that mimic those observed for non-synonymous mutations (Leuven et al. 2020). Evolutionary adaptation to deleterious synonymous mutations also occurs rapidly via similar mechanisms observed for deleterious nonsynonymous mutations, including regulatory or compensatory mutations in *cis* or *trans* (Lind et al. 2010b; Amoros-Moya et al. 2010; Bull et al. 2012; Lind and Andersson 2013; Knöppel et al. 2016; Agashe et al. 2016; Mittal et al. 2018; Mochizuki et al. 2018; Knöppel et al. 2020). Hence, evolutionary adaptation following deleterious synonymous changes does not appear to be special.

Another important line of evidence for the evolutionary impacts of synonymous changes comes from attempts to engineer attenuated viruses that have reduced chances of reversion to virulence, and are hence suitable for vaccine production. Many (though not all) viruses use codons that are favored by their host (Lucks et al. 2008; Carbone 2008; Chithambaram et al. 2014), indicating strong selection on viral genomes for optimizing translation. Due to the expectation of combined large fitness effects but individually weak impacts of synonymous codon changes, it was proposed that introducing hundreds of suboptimal synonymous codons in a viral genome would be an effective attenuation strategy. Indeed, experimental studies showed that synonymous mutations reduced viral activity in human cell culture (Burns et al. 2006) and mouse models (Coleman et al. 2008). However, subsequent work also showed that evolutionary changes and reversions are possible and not rare (Bull et al. 2012; Leuven et al. 2020; Nouën et al. 2021). Hence, understanding the mechanisms through which codon-attenuated viruses recover fitness is important to predict the outcomes of codon deoptimization as an attenuation strategy (Leuven et al. 2020). A broader evolutionary implication is that interactions between organisms can be influenced by synonymous mutations, and this aspect deserves more attention.

Together, recent experimental evidence clearly shows that specific synonymous mutations may evolve under strong selection. But are such mutations with large impacts frequent enough to have significant evolutionary consequences? Important clues come from empirically estimated statistical distributions of fitness effects (DFEs) of synonymous mutations (see Fig 1G). The first such analysis was conducted for two ribosomal protein genes of *Salmonella typhimurium*, finding that the DFEs for synonymous and non-synonymous mutations were remarkably similar (Lind et al. 2010a). An analysis of all possible codon substitutions at specific positions in the Hsp90 protein of *Saccharomyces cerevisiae* also found that most codon changes had very small impacts on fitness, but a few had very large effects (Fragata et al. 2018). The resulting topography of the fitness landscape – which describes fitness peaks and valleys traversed by evolving populations – suggests that synonymous mutations create new fitness peaks, and can therefore alter the course of adaptation. A recent meta-analysis of other DFE studies also shows that large-effect synonymous mutations are not rare (Bailey et al. 2021). There are some notable differences in the DFEs of synonymous and nonsynonymous mutations: the former is unimodal compared to the typically bimodal DFEs observed for nonsynonymous mutations; and overall, synonymous mutations have weaker fitness effects. However, the beneficial part of the DFE – which is most relevant for adaptation – is similar for both classes of mutations, suggesting that they would evolve similarly under positive selection. These patterns for single gene DFEs are also corroborated by genome-wide DFEs described for *E. coli* (Sane et al. 2020).

Recent bioinformatics analyses corroborate these results, showing large variability in the strength of selection acting on synonymous mutations, with a substantial fraction evolving under strong selection. For instance, in some tracts in mammalian genomes, synonymous substitutions are extremely depleted compared to nonsynonymous variation, indicating that the former face strong purifying selection (Schattner and Diekhans 2006). In *Drosophila,* over 20% of four-fold synonymous sites show signatures of evolution under strong purifying selection, and very few sites show signatures consistent with weak selection (Lawrie et al. 2013). This pattern is especially stark when compared to neighboring intronic regions

(Machado et al. 2020). Similarly, in human coding sequences, synonymous mutations at 20-30% of exonic splice regulatory elements show signatures of strong purifying selection (Savisaar and Hurst 2018). Note that these analyses of selection strength lump codon changes across various contexts, and therefore give an overall picture of selection on synonymous mutations regardless of the underlying mechanisms.

SUMMARY AND FUTURE DIRECTIONS

The overview above gives a glimpse of gradually shifting narratives in a field grappling with some of the most fundamental questions in biology. The two parallel lines of inquiry focusing on translational and non-translational impacts of synonymous mutations have effectively merged in the past few years, allowing a cohesive view of the general fitness impacts of synonymous mutations. Multiple initially puzzling patterns of synonymous codon use are now clarified. For instance, variation in codon choice and strength of codon bias across genomes is largely explained by divergent mutational biases (which may themselves evolve under selection). In prokaryotes, variable strength of selection for translation speed also plays a role. Variation in the magnitude of codon bias across genes in the same genome arises from differing strength of selection, approximated by gene expression level. Importantly, it is clear that codon usage is only one of many factors that affect gene regulation in cells. Finally, variation in codon use across different regions of a gene is governed by many molecular mechanisms that underlie information processing and transfer within and between cells. At this scale – of local DNA or mRNA sequence – the translational impacts of codon use *per se* are less important, and the impact of synonymous mutations can be generalized. Below, I highlight ways in which this new understanding of synonymous changes can be leveraged to address outstanding questions in evolutionary biology.

Building a cohesive evolutionary framework

Much like non-synonymous mutations, synonymous mutations appear to evolve under widely variable strengths of selection, depending on the specific context in which they occur. As an illustration, consider that a single synonymous mutation in a functionally important gene may be deleterious because it generates a very rare codon that cannot be efficiently translated. However, given cellular buffering mechanisms (e.g. tRNA modifying enzymes, which may allow other tRNAs to decode the rare codon), the fitness impact of this single rare codon may be very weak. In another position, the same synonymous mutation might be deleterious because it generates or destroys a specific regulatory motif encoded as a secondary layer of information in the mRNA sequence. As a result, it may have a larger fitness impact, depending on the local sequence context. Indeed, in *E. coli*, identical codon changes in adjacent positions in the same gene had opposite fitness effects (Hauber et al. 2016). Conceptually, such context-specific fitness consequences are already well established and rigorously analyzed for non-synonymous mutations; hence, the separation between synonymous and non-synonymous mutational classes is quantitative rather than qualitative. We therefore need to build a single inclusive framework for evolutionary analysis that incorporates the context-specific effects of all mutations, encompassing diverse underlying molecular mechanisms.

Predicting the evolutionary fate of synonymous mutations

As noted recently (Canale et al. 2018; Bailey et al. 2021), there is a surprising mismatch between the short- and long-term evolutionary effects of synonymous mutations, which makes it difficult to predict their evolutionary fate. While many synonymous mutations have very large immediate fitness benefits, there are relatively few cases of beneficial synonymous mutations observed in experimental evolution studies and natural populations. A simple explanation for this pattern is that there are fewer possible synonymous (compared to nonsynonymous) mutations. In addition, in microbial populations, clonal interference (i.e. competition between independent lineages carrying distinct beneficial mutations) can reduce the probability of fixation of beneficial mutations (Gerrish and Lenski 1998). Population genetic simulations show that this effect is stronger for synonymous mutations, because they have slightly weaker fitness effects and are therefore more likely to be outcompeted by non-synonymous beneficial mutations (Bailey et al. 2021). Thus, a combination of sampling bias and weaker fitness effects may limit the evolutionary impacts of synonymous mutations. We now need more extensive theoretical and experimental analyses of the direct and indirect fitness effects of synonymous mutations. The resulting understanding would be important not only in evolutionary biology, but also for practical applications of evolutionary predictions in the context of codon-attenuated viral vaccines.

Developing new methods to identify signatures of selection

As discussed earlier, most of our current methods to identify genomic signatures of selection rely on comparisons with "neutral" sites, typically exemplified by synonymous sites. However, a growing body evidence suggests that accounting for selection on synonymous mutations is necessary to obtain accurate estimates of selection. For instance, Matsumoto and colleagues used simulations to show that weak selection on codon bias can inflate the inferred fraction of adaptive amino acid substitutions (Matsumoto et al. 2016). More generally, commonly used methods to identify sites under selection in focal taxa assume invariant rates of evolution at synonymous sites. However, selection on synonymous sites or local mutation biases can introduce substantial variation in synonymous substitution rates across genes as well as genomes (Rubinstein et al. 2011; Dimitrieva and Anisimova 2014). Assuming constant synonymous substitution rates causes substantial inflation of the number of sites under selection and leads to erroneous evolutionary conclusions (Rubinstein et al. 2011; Davydov et al. 2019; Wisotsky et al. 2020). Therefore, we need to develop new methods to identify selection, that are independent of the oft-violated assumption of weak selection at synonymous sites. Pseudogenes or short introns may be better suited as neutral benchmarks, though their identification and use involves additional challenges that need to be resolved.

Determining the evolutionary history of mechanisms driving selection at synonymous sites

Over the years, the focus on identifying mechanisms underlying the consequences of synonymous mutations has intensified (Hunt et al. 2014). It is now clear that the functional impacts of various kinds of mutations arise via shared mechanisms such as changes in nucleic acid and protein structure, regulation, translation rate and accuracy, and cellular targeting. While understanding mechanisms is undoubtedly important, this does not help us understand the selective pressures that drive evolution. For instance, demonstrating that altered codon bias changes a particular phenotypic outcome (such as intracellular localization) does not mean that codon bias evolved under selection to optimize localization. Instead, across clades,

different mechanisms may have driven selection on synonymous changes at various points during the evolutionary history of life. Hence, we must trace how and when different layers of information were encoded by the primary nucleotide sequence. Although it is challenging to decipher these (presumably) deep and rare evolutionary events, such analyses promise new insights into the evolution of the genetic code in conjunction with the intricate mechanisms of information processing.

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