Chapter 1

Experimental evolution to understand the interplay between genetics and adaptation

Jana Helsen and Rob Jelier

Abstract Experimental evolution has grown to be a powerful and versatile tool to study the evolutionary dynamics of genetics. The principle is simple: cells are grown under a specific selective pressure, mutations arise over time, and lineages carrying mutations that increase fitness can outcompete others. Here we discuss how experimental evolution has allowed us to study various evolutionary processes, from clonal interference, to diminishing returns epistasis and genetic hitchhiking. Next, we discuss how experimental evolution can reveal how an organism's genotype affects evolutionary processes, how adaptation can sometimes fix even the most severe fitness defects, and how the approach can be used to learn more about the genetic architecture of complex traits. Finally, we look ahead at how experimental evolution can be used to study genetic networks and, conversely, how the structure of such networks influences evolution.

1.1 Understanding adaptation by experimental evolution

Adaptation is the process by which organisms adjust to their environment and improve their chance of producing fecund offspring. During adaptation, mutations that increase fitness can get selected and fix themselves in the evolving population, a process that is fundamental in shaping the genetic architecture of complex traits. The historical view of adaptation starts with Darwin himself, and was one of gradualism, where adaptation takes place through many small mutations of minor effect. Fisher consolidated this view in 1930 in the form of a coherent but simplified model: the geometric model of adaptation [13]. In this model, Fisher argued that the probability that a random mutation has a beneficial effect drops quickly when the effect size of the mutation increases, according to $1 - \phi(x)$, with x proportional to the effect size and ϕ the cumulative distribution function of a normal distribution. He concluded that small-effect mutations would dominate adaptation. Kimura pointed out in the 60s that fitness effects that were too small would not spread fast enough to fix efficiently in a population, pointing to the importance of intermediate effect mutations [26, 27]. While these models have proven to be very useful, they were in fact based on indirect observations. This changed in the 1980s, when experimental evolution gained traction and allowed us to

directly study and observe the dynamics of adaptation. Since then, the method has yielded many important insights into how adaptation proceeds and what a theory of adaptation should represent [48]. Below we will concisely review seven of the most crucial findings that have been made using experimental evolution.

First, adaptation to a new environment or challenge is typically swift, with a rapid initial fitness increase that depends on a few large-effect mutations [21]. For example, Lenski’s famous long-term evolution experiment (see Box 1) showed a power-law adaptation curve across 12 populations. Fitness improved rapidly over the first ~1000 generations, caused by few mutations of large effect, after which a phase of ever slower adaptation started with mutations showing decreasing fitness benefit [68]. The rate of adaptation, quantified by the increase in fitness over time, therefore depends on how far adaptation has proceeded and is reflected by the current fitness in the given condition. Three factors contribute to explain this phenomenon: a) the overall distribution of fitness effects of mutations (see second point below); b) the mutations that are present at any given time in a population (in terms of both quantity and quality), which in turn depends on population size and mutation rate (see third and seventh points); c) diminishing returns epistasis, the phenomenon whereby beneficial mutations have smaller fitness effects in fitter backgrounds (see fourth point). The potential for rapid adaptation is quite general and has been observed in experimental evolution studies across many conditions, species, and genetic backgrounds [40, 60, 53, 52].

Box 1. The long-term evolution experiment

In the long-term evolution experiment, 12 evolving *E. coli* populations were started from the same ancestral strain in 1988 and have been continuously propagated to this day. The populations grow in a minimal medium (DM25) with glucose as the limiting factor and are passed to fresh medium with a 100-fold dilution every day. Periodically, population samples are frozen so they can be examined in detail at a later time. As of 2020, the experiment has run for more than 73,000 generations. The experiment is remarkable for its duration, but also for the many phenomena observed and studied as the evolutionary process unfolded in the populations (reviewed in [37]). For example, the study has demonstrated how the pace of adaptation changes over time, as well as the repeatability of adaptation between populations and the competition between beneficial mutations in a population (clonal interference). More surprising was the observation that ecological interaction took place between stably co-existing strains. Two distinct lineages arose in one population and co-existed for over 50,000 generations. One strain specialized in rapid growth on glucose, beneficial after transfer to fresh medium, whereas the other specialized in fast growth on acetate, the product of the former’s metabolism and beneficial as glucose supply is limiting. Both strains stably co-existed in a cross-feeding relationship driven by the particular growth conditions during the experiment [36].

Second, though there are many mutations that provide a positive fitness effect, variants of large effect are rare. A crucial part of understanding how adaptation proceeds comes from examining the distribution of fitness effects of genetic variants (reviewed in [12]). Experimental evolution makes it possible for a direct measurement of the distribution of such fitness effects. By following how fast variants rise in frequency in a population, we can estimate their relative fitness as well. Sequencing populations allows to follow variants as they rise and fall during selection

Box 2. Focus on adaptation dynamics with barcoding

Due to cost considerations, whole genome sequencing of populations is normally limited in sequencing depth, i.e. the number of reads that include a genomic position. This means that the detail and resolution for tracking lineages as they rise and fall in a population is limited, especially for new lineages when their frequency is still low. One trick to improve on this limitation is to introduce a highly variable but short barcode into the population, which can then be sequenced at very high coverage. In the paper that introduced this approach, a random 20-nucleotide barcode was synthesized as random primers and then ligated into a plasmid backbone [38]. The plasmid library was subsequently transformed into yeast cells and a Cre-loxP recombination system was used to insert the barcodes into an engineered genome location [38]. The method allowed the researchers to follow lineages arising and competing in a population in unprecedented detail.

[64, 35], but at limited resolution. To overcome this limitation, Levy et al. created a barcoded budding yeast library, where each lineage contained one of ~500,000 random DNA barcodes [38]. Using this system, the evolutionary dynamics could be followed in much higher detail (see Box 2). By measuring the fitness of many lineages using this method, though limited to a single genotype and condition, it was shown that large effect variants are rare, but many variants provide a modest fitness advantage. About 0.04% of the genome's bases (~5,000 bases) confer a larger than 5% fitness benefit when mutated. It is clear, however, that the distribution of fitness effects heavily depends on the condition and current fitness of the strain, as strains closer to the optimum growth rate will by necessity have fewer options for rapid fitness increase.

Third, clonal interference is the norm in large asexually reproducing populations. In a more or less typical microbial experimental evolution setting, the mutation rate will be on the order of $\sim 10^{-10}$ per base per generation [71], effective population sizes can be in the order of $\sim 10^7$, and (for example) the baker's yeast genome has about $\sim 10^7$ bases, so we can expect $\sim 10^4$ new mutations to arise every generation within the population, of which a handful (say $\sim 0.04\%$, see point 2) should be beneficial. Many of these mutations will be lost by chance before they can expand their occurrence rate in the population, but we can expect several beneficial mutations to co-exist and rise in frequency at the same time and so cause competition between alleles. The term clonal interference refers to the effects of this competition, in that some beneficial mutations do not fix, or fix more slowly, because other fit alleles are expanding in the population at the same time. In the absence of recombination by sexual reproduction, only one of the concurrent beneficial alleles can fix, and this will slow down adaptation [18, 15]. This process will also ensure that only large effect variants can fix early in adaptation. Further, the stochastic nature of the mutation rate as well as the chance that additional beneficial mutations are acquired in an expanding lineage can give rise to complex patterns of clonal expansion and shrinkage [35].

A fourth defining feature of adaptation is epistasis. This is the phenomenon whereby the effect of one mutation depends on the presence or absence of other mutations. One particular recurring observation during adaptation is that a beneficial allele will tend to cause less fitness gain when introduced into a strain that is fitter than when introduced into a less fit strain [7, 29, 41, 30, 46, 24, 65]. This is called diminishing returns epistasis, and can also

be described as a generalized negative epistasis between beneficial mutations (i.e. when mutations occur together, their combined effect is worse than what would be predicted by the sum of their individual effects) (see Figure 1.1a). One implication of this is that the order in which beneficial mutations occur in a lineage is important. For example, one particular beneficial mutation that causes a strain to grow twice as fast during early adaptation can have only a marginal beneficial effect on fitness during the later stages of adaptation. At that point, the strain will have already acquired a whole other set of beneficial mutations, and negative epistasis between these mutations and the additional beneficial mutation will result in the new mutation having a smaller effect on growth. This implies that the mutation has a much higher chance of being fixed during early adaptation compared to later on in the evolutionary process. A second observation is that beneficial mutations can show strong sign epistasis when combined. Sign epistasis between mutations occurs when the presence of one mutation (A) causes another beneficial mutation (B) to become deleterious, such that either $f(B) > f(-)$, $f(B, A) < f(A)$ (where $f(B)$ represents fitness in the presence of B, negative epistasis shown here), or the other way around, $f(B) < f(-)$, $f(B, A) > f(A)$ (positive epistasis). In the context of adaptation, reciprocal sign epistasis is of particular interest, as it can lead to a complex multi-peaked fitness landscape [51]. In this special case of sign epistasis, the effect of both mutations is reversed when they are combined: $f(A) < f(-)$, $f(B) < f(-)$, $f(B, A) > \max(f(B), f(A))$. If two beneficial mutations show reciprocal sign epistasis, the lower fitness combination is unlikely to remain in the population. The available mutational routes towards higher fitness will become conditional on existing mutations and hence constrained (see Box 3 and Figure 1.1b). Reciprocal negative sign epistasis has been observed and studied in several experimental evolution experiments [32, 6], and was even found to be common among initial adaptive mutations in a bio-synthetic pathway [47]. In their large-scale study, Szamecz et al. (2014) found two mutations that decreased fitness when they occurred on their own: deleting MDM34 and mutating MGA2. However, the combination of the two mutations resulted in a higher fitness than either of the single mutations, an example of reciprocal positive sign epistasis. Indeed, mutations in MGA2 were selected during evolution in *mdm34Δ* strains [60]. Rojas Echenique et al. aimed to investigate the effect of epistasis on a larger scale [55]. After evolving a set of gene deletion mutants, they tried to reintroduce the originally deleted gene in the evolved strains. For over half of these strains, they did not succeed in reverting the deletion, possibly due to strong epistasis between the deletion and the acquired compensatory mutations. In other words, the compensatory mutations showed reciprocal positive sign epistasis and were strongly detrimental in the absence of the gene deletion.

Fifth, parallel adaptive evolution can be common [54, 2]. Identical mutations or mutations within the same pathway occur much more often during experimental evolution than would be expected by chance. For example, in the adaptation of a cell polarization mutant, Laan et al. found the same gene to be inactivated in all 10 replicate adapting populations [34]. One factor, as noted above, is that large effect mutations tend to be rare, and may be efficiently selected for in a large enough population. Another good example of studies where parallel trajectories are often observed is in those that studied the development of antibiotic resistance in *E. coli*. The parallelism of evolved

Fig. 1.1 Fitness landscapes and epistasis. a. Diminishing returns epistasis. The beneficial effect of a mutation depends on the stage of adaptation of the strain. The same beneficial mutation tends to cause smaller fitness gains when introduced in a fitter strain than in a less fit strain. b. Reciprocal (negative) sign epistasis. This causes a rugged fitness landscape and implies that the order in which beneficial mutations occur in a lineage is important. In this hypothetical example, mutation A and B are equally beneficial when introduced into the initial unfit strain. However, due to negative epistasis between the mutations, their combined effect is worse than their individual effects. As a result, strains that first acquired mutation A will never acquire mutation B and vice versa. Even though they initially have the same beneficial effect, the two mutations direct strains towards different peaks within the fitness landscape. In the illustrated example, strains that first acquired mutation B have the potential to achieve higher fitness values after further adaptation than strains that first acquired mutation A.

antibiotic resistance was found to be high enough at the level of mutated molecular pathways so that simple linear models could be trained to successfully predict the level of antibiotic resistance, using the gene expression of only a few genes [59]. Other large-scale studies found more modest, but still elevated rates of parallel mutation [60, 55], potentially indicating less rare large effect mutations being available. A possible explanation is the relatively high fitness of the adapting strains. When the growth speed approximates optimality, there is limited potential for a large fitness increase with a single mutation (see also fourth point above). Sign epistasis can be another contributing factor. If strong epistasis is present between beneficial variants it can increase diversity between adaptation experiments, as the mutational trajectory becomes dependent on which epistatic mutation arises first in the lineage.

Sixth, hitchhiking mutations are common and highlight the benefit of sex. In asexual populations, all variants in an individual's genome are passed on to its offspring and their frequency will rise and fall together in the population

Box 3. Reciprocal sign epistasis and fitness landscapes

A useful concept to get an intuitive grasp of epistasis and its role in evolution is the evolutionary fitness landscape (see schematic example in Figure 1.11b) [70, 69]. In an adaptive fitness landscape, each coordinate represents a point in the genotypic space, and the value on the z-axis indicates the corresponding fitness of the genotype. In this way, evolution can be visualized as an exploration of the landscape, where selection favours genotypes that climb a fitness peak. Epistasis has a large effect on determining the shape of the landscape. Reciprocal sign epistasis, where the effect of individual mutations is reversed when combined, is the reason why fitness landscapes can show not just one single peak, but multiple local peaks, what is called 'ruggedness' [50, 51]. This ruggedness has been observed in studies using experimental evolution. For example, Kvitek and Sherlock recurrently observed mutations in two loci, MTH1 and HXT6/HXT7, in yeast populations evolving under glucose limitation [32]. Individually, these mutations are both beneficial for fitness. However, the double mutant has a fitness that is lower than even that of the wild-type strain. As a result, strains that carry the MTH1 mutation are unlikely to achieve the slightly higher fitness peak of those with mutations in HXT6/HXT7. In fitness landscape parlance, the two peaks are separated by a deep fitness valley, with a mutational path unlikely to be taken in the face of natural selection.

according to the individual's fitness. This implies that a variant showing a rapid fixation during adaptation may not be beneficial, but is merely hitchhiking with the fixation of a beneficial variant elsewhere in the genome. This complicates analyses of experimental evolution studies, as the fitness effects of fixed mutations need to be confirmed experimentally [35, 64]. One way to get around this is to observe patterns of parallel adaptive evolution, as a hitchhiking mutant should only occur at background rates in independent experiments. The phenomena of hitchhiking and clonal interference differ between asexually and sexually reproducing populations. As budding yeast can grow both asexually and sexually, it can be used to make a direct comparison of adaptation in both scenarios. For example, McDonald et al. compared the adaptation rate between asexually growing populations and those that were forced to reproduce solely by sexual reproduction [45]. They found that sex can indeed allow for faster adaptation, as predicted by theoretical work. It allows beneficial mutations to recombine, which in turn avoids clonal interference, and allows beneficial mutations to dissociate from neutral or mildly detrimental hitchhiking mutations [45].

The seventh and last point is that adaptation can proceed rapidly through relatively crude alterations that only have positive effects within a narrow range of conditions. One such alteration that is often observed during experimental evolution is loss of protein function [34, 60, 33]. This is counter-intuitive given that genes have to be subject to positive selection to be maintained in a genome at all. Adaptation by gene loss does occur in nature (see for example [11]), but it may be observed more frequently in experimental evolution due to the typically controlled and predictable conditions. Indeed, loss-of-function mutations tend to come with negative fitness consequences in conditions other than the controlled environment under which experimental evolution proceeds [60, 33]. For example, in a study to better understand how fitness increased during adaptation using *S. cerevisiae*, mutations were found to be beneficial in only a narrow range of conditions [39]. During the experiment, strains were serially transferred and

the population went through a lag, fermentation, and finally a limited respiration phase after every transfer. The adapted strains had improved respiration and a shortened lag phase, which improved fitness in the experimental conditions. However, there was a trade-off with survivability, which dramatically limited the conditions in which the adaptations were beneficial [39]. Trade-offs are also of crucial importance when strains evolve in fluctuating environments, i.e. where the selecting environment fluctuates over time. Since mutations that enable a strain to grow faster in one environment quite often come with trade-offs for growth in other environments, the extent and speed of evolution when evolving in such conditions can be severely constrained. A related phenomenon is the cost of generalism, where generalists (organisms that are fit in a broad niche) usually have a lower fitness under specific constant conditions than specialists (organisms that are fit in a small niche)[4]. Next to loss of gene function, another common crude adaptation route is aneuploidy [53, 58]. The adaptive aneuploidies tend to have big fitness effects, but only strongly affect the transcription of relatively few genes [58]. As with the loss-of-function mutations, the fitness effects are mostly condition-specific [58]. Both aneuploidies and loss-of-function mutations occur *de novo* relatively frequently when compared to gain-of-function mutations or promoter mutations that specifically alter gene expression responses. Combined with their large, but specific, fitness effects, this may explain their frequent appearance during experimental evolution.

1.2 Experimental evolution to study genes and traits

Experimental evolution can be used to study complex traits and to also improve our understanding of how the genotype of an organism affects evolutionary processes. In classical experimental evolution experiments such as the LTEE, genetic variation arises spontaneously by mutation. However, to be able to systematically explore the interplay between genotype and evolution, genetic variation can also be explicitly introduced at the start of the experiment (Fig 1.2). By crossing and backcrossing divergent strains we can acquire a population with a controlled set of genetic variants. After growing such populations under selection, the frequency of the variants can then be correlated with fitness. Alternatively, different offspring from such crosses can be evolved separately, to explore how their differences in genotype influence adaptation. Another way of inducing genetic variation is to use recombinant DNA technology. This allows us to, for example, evaluate the potential for adaptation under near-fatal gene perturbations. Further, we can study whether and how a specific mutation in an adapting strain can constrain the mutational trajectory during adaptation. A constrained (i.e. parallel) trajectory in a strain that suffered a deleterious gene perturbation could inform us on which cellular processes can alleviate the stress experienced by the cell during adaptation. We will expand upon these two techniques to induce genetic variation and the most important insights they have provided in the three sections below.

Fig. 1.2 Evolving various genotypes to explore the link between genotype and evolution. Variation in genotype can be generated in a controlled manner by crossing and backcrossing divergent strains or by using recombinant DNA technology to, for example, systematically knock down genes. By next evolving the diverse array of genotypes, we can measure the effect of genetic variation on the speed of adaptation, and observe how adaptation proceeds when starting from different parts in the fitness landscape.

1.2.1 Evolving the progeny of crosses to understand the genetic structure of complex traits

Experimental evolution can be used to identify beneficial variants from standing genetic variation. This can be effectively employed to explain the genetic origins of phenotypic differences between individuals. By repeatedly crossing two strains, a synthetic population can be made in which the alleles of the strains segregate in different combinations. Imposing a selective pressure can then increase the relative frequency of beneficial variants (see 1.3). For this approach to work, the population can even be grown for only a handful of generations, just long enough to see that the differences in fitness between lineages are reflected in occurrence frequency in the population. Parts et al. studied the genetics underlying heat sensitivity in *S. cerevisiae* [49]. They crossed a heat-tolerant North American oak tree bark strain and a heat-sensitive West African palm wine strain, and generated between 10-100

million random segregants. They then grew a pool of these segregants at normal and high temperatures for 12 days, to enrich for lineages with beneficial alleles. After sequencing the enriched pools, 21 genomic regions were enriched in the population. They found that the resolution of the regions and their number compared favorably to standard genomic linkage studies. However, this method is limited to selecting for haplotypes that arose during the original crosses, which is only a small fraction of all possible combinations of segregating alleles in the population. Burke et al. (2014) started from a synthetic population with genetic variants stemming from a four-way cross of diploid budding yeast strains from different geographic origins [5]. They switched between mitotic and sexual propagation to break the linkage between alleles, and evolved for over 500 generations with 18 rounds of recombination. This regime enriches the most beneficial alleles efficiently, causing a convergence between independently evolving populations. In theory this should be a more powerful and better resolved way to identify the functional variants that contribute additively to the phenotypic differences between the original strains.

Fig. 1.3 Growing the offspring of crosses under selection to understand complex traits. By repeatedly crossing two strains, a synthetic population can be made in which the alleles of the strains segregate in different combinations. Imposing a selective pressure can then increase the relative frequency of beneficial variants.

The genetic variability in the offspring of a cross can also be used in combination with experimental evolution to study the genetic basis of adaptability itself. Jerison et al. independently evolved 230 individuals descended from a cross between lab and wine yeast strains for 500 generations (see Figure 1.2; here they evolve individual offspring,

not the population) [23]. The aim of the study was to use quantitative genetic tools to identify the genetic basis of adaptability, defined as the speed at which a strain can improve its fitness during evolution. In line with previous work, the authors confirmed that adaptability is highly heritable. A large part of the differences in heritability between strains could be explained by the loci that affect the initial fitness of the strain and indeed the fitness of the founder strains. Strains that are less fit initially will improve their fitness more quickly during evolution, which can be explained by the phenomenon of diminishing returns epistasis as discussed above (Section 1, fourth point). In addition to this, a few genomic regions were found to be specifically associated with adaptability, beyond the effect of founder growth rate [23]. The locus with the strongest signal was explored in more detail. The wine strain allele of a gene within this locus, KRE33, is involved in the biogenesis of the small ribosomal subunit and was detrimental to growth in the experimental conditions. However, further mutations in the same gene or functionally related genes could compensate for the allele’s effect to improve fitness relatively quickly, and the locus was hence associated with increased adaptability.

1.2.2 Gene essentiality and evolvability

In rich medium, a large portion of yeast genes can be removed without having a big effect on fitness. In fact, the deletion of some genes can actually increase fitness or decrease fitness with a wide range of effect sizes [16]. Essential genes are traditionally defined as being critical for survival irrespective of the growth condition. However, the notion of gene essentiality has been found to be dependent on genotype. For example, many synthetic lethal interactions were found in a screen of millions of double gene knockouts in baker’s yeast. Costanzo et al. identified around 3,300 ‘conditionally essential’ genes, about half of the total number of genes, that are essential when they occur in a particular deletion background [9, 8]. Further, Dowell et al. compared gene essentiality between two commonly used *S. cerevisiae* lab strains [10]. They found that both strains contain genes that are essential in one strain, but not in the other. They concluded that the essentiality of some genes depends entirely on the genetic background in which they occur [10].

Experimental evolution has contributed to changing how the concept of essentiality is viewed. Liu et al. were able to recover viable deletion strains by using adaptive evolution for around 9% of the yeast genes that were originally tagged as ‘essential’ [40]. They sporulated heterologous diploid strains, in which one copy of the essential gene was knocked out, and screened a large number of spores. If a strain could yield viable offspring – even if only a diminutive fraction – and contained the gene deletion, that strain would be included in an evolution experiment. The authors showed that these strains could improve their growth rates substantially by adaptation. One perspective on this finding is that there is a problem with the definition of essential genes, and indeed the yeast deletion collection has technical limitations and phenotypic annotations should be interpreted with caution [17]. However, a more

striking insight is that even some of the most serious growth defects can be overcome, or suppressed, by acquiring compensatory mutations.

1.2.3 Compensatory trajectories after gene loss

One way of exploring the effect of genotype on evolution is to evolve strains that only differ in the deletion of one particular gene in parallel and then compare how these strains adapt. Adaptation may be specific to the deleted gene or general for the condition, and follow a single or various adaptive routes. Further, the way the strain adapts may reveal what has been corrected at a molecular level. If deleting the gene results in a growth deficit, one could expect that only a specific set of mutations is able to reverse the strain's fitness loss.

Various small-scale experiments have shown that gene deletion strains can have a characteristic mutational trajectory and a straightforward way of adapting (see also the fifth point in Section 1 about parallel evolution). Wild type *Saccharomyces cerevisiae* cells repeatedly amplify the sulfate transporter SUL1 when they are evolved in sulfate-limiting conditions [19] (introducing aneuploidies, see also the seventh point in Section 1). Strains lacking the SUL1 transporter predictably duplicate the paralogous transporter SUL2. Similarly, variants elsewhere in the genome can change which mutation has the largest beneficial effect, as *Saccharomyces uvarum*, a close relative of *S. cerevisiae*, amplifies SUL2 when it is evolved under the same sulfate-limiting conditions [56]. In other cases, however, adaptation can be complex. Deleting MYO1, the only myosin II gene in budding yeast, results in cells that are unable to divide properly [66]. After serially passaging these *myo1Δ* yeast populations for around 400 generations, the majority of them can adapt and significantly restore cytokinesis even though the evolved strains are still less fit than their ancestor wild type [53]. In this case, though despite evidence of parallel evolution at the level of chromosomal aneuploidies, the populations show a variety of morphologies, mutational and phenotypic profiles, indicating they have followed diverse compensatory trajectories. At least three modes were identified to restore cytokinesis, two of which by mechanisms distinct from the original damaged system.

Studies where a larger number of deletion strains are evolved allow for a general study into the phenomenon of adaptation after gene loss. Teng et al. cleverly made use of the fact that the yeast deletion collection has already undergone several rounds of selection and as such carries compensatory mutations [62]. They first screened the haploid deletion collection for phenotypic heterogeneity within individual deletion strains, under the assumption that heterogeneous populations contain clones with unfixed secondary mutations. After this, they dissected the mutations that were responsible for this variation, and concluded that the gene that was originally deleted is indeed a key predictor for the secondary mutations in a strain. Szamecz et al. performed a more controlled wide-scale study by evolving a large set of deletion mutants in the lab [60]. They generated 180 haploid yeast gene deletion strains with growth defects on rich medium, and evolved them in quadruplicate in this medium for around 400 generations; 65% of the strains recovered $\geq 50\%$ of the fitness lost due to the gene deletion [60]. They sequenced a

subset of evolved clones, and observed that the mutations that were found after evolution tended to be specific to the function of the deleted gene. However, replicate lineages of the same deletion strain did not generally acquire mutations within the same genes. Rojas Echenique et al. selected a set of 37 genes with functions across diverse functional modules, and evolved deletion mutants of these genes on rich medium for about 500 generations [55]. In this case, the majority of compensatory mutations seemed to be non-specific to the original genotype, even though a small but significant correlation pattern was observed between the functional module of the deleted gene, and the functional module of the mutated genes.

In a recent and elegant study involving *Escherichia coli*, McCloskey et al. knocked out five different core metabolic genes and evolved the resulting strains in quintuplicate on glucose minimal medium [42]. By characterizing the mutations that were acquired during evolution, as well as measuring gene expression levels, intracellular metabolite levels, and metabolic fluxes, they showed that each deletion strain followed specific adaptive paths to correct their growth-limiting molecular phenotypes. For example, cells lacking a functional succinate dehydrogenase, which converts succinate into fumarate in the TCA cycle, specifically acquired mutations to reduce the flux through the TCA cycle [43]. In contrast, strains without phosphoglucose isomerase, the catalyst of the second step in glycolysis, experienced increased redox and sugar phosphate stress before evolution. During experimental evolution, cells reproducibly follow mutational paths that result in a reduced flux in those pathways [44].

In conclusion, a strain lacking a specific gene tends to evolve in a genotype-specific way, though this can still be quite variable. If a clear mutational trajectory can be identified, the adaptations can provide insight into the processes perturbed by the gene deletion.

1.3 Outlook: using experimental evolution for systems genetics

As introduced in the previous section, the genetic background of an evolving organism can influence the available mutational trajectories. One way of looking at this is to think of the fitness landscape (see Box 3 and Figure 1.2) as being shaped by the genotype, which determines through epistatic interactions which variants will be most adaptive. Obviously genes are part of an integrated system and interact with each other at different scales; locally at the level of protein interactions or gene transcription, and globally as cellular processes that jointly define organismal properties such as fitness. The solutions found by evolution will always be successful trade-offs given the genotype and environment, and will reflect how different parts of the cellular machinery work together. This means that adaptation experiments have the potential to give us a better general understanding of cellular processes. Below, we will discuss early results and perspectives on learning about genetic networks from experimental evolution experiments.

As mentioned above, recent large-scale assays have systematically mapped genetic interactions between gene deletions. This has resulted in a detailed map of the genetic wiring within a cell [9, 8]. When we look at the

average degree of connectivity in this genetic network, the majority of genes has only interactions with few genes, whereas a small fraction has a very large number of interactions. The genes with the highest number of interactions, the so-called network hubs, usually perform central cellular functions and many of them are essential [22]. As the perturbation of a network hub tends to have large fitness effects [3], and given that genes with more interaction partners tend to show a slower rate of evolution [14, 31, 1], it was thought that changes during evolution primarily occur away from hubs and at the periphery of the network [25]. Through experimental evolution it has been possible to test the effect of perturbing a hub gene on adaptation. For example, Koubkova-Yu et al. investigated the role in evolution of Hsp90, a molecular chaperone and a well-known network hub [28]. They replaced the native *S. cerevisiae* HSP90 with a homolog from *Yarrowia lipolytica* and used experimental evolution to allow the strain to adapt. They showed that perturbing a hub does not preclude adaptation: instead, the strains evolved in a remarkably diverse manner by acquiring mutations in different parts of the genetic network. If we project this situation onto a fitness landscape, then we can say that the perturbation of the hub gene brought the strain into a deep fitness valley from which many distinct evolutionary paths could provide a way out [20].

Further research is needed to test if the finding is generalizable and holds for other highly interacting genes. Specifically, is it just the number of genetic interactions that is important, or is it actually the function of the protein which determines how adaptation proceeds? Does it matter in evolution if a perturbed protein, causing a drop in fitness, is a transcription factor, a chromatin factor, a core metabolic enzyme, or a structural protein? Further, accepting that knocking out a hub gene does not inhibit adaptation, can we identify parts in the network where perturbations are poorly compensated? For example, if heritability itself is affected by a perturbation, does that affect adaptability?

Another feature of genetic and physical interaction networks is that they have a clustered structure, where clusters are groups of genes with a high density of interactions. Such clusters tend to overlap with so-called functional modules, groups of genes that work together to perform a cellular function and whose activity is (partly) self-regulated, such as metabolic pathways or protein complexes [63]. An interesting question is how this modular structure comes into play when organisms evolve. For example, earlier we discussed how generalized diminishing returns epistasis slows down adaptation as fitness increases (fourth point of Section 1). The deeper cause of this widespread phenomenon is not really understood. Clearly, growth speed can increase only up to a point, for example because basic physical processes, such as diffusion, limit the maximal speed of chemical reactions. It is not straightforward, however, to link such ideas to actual biological gene networks. One framework for this is the model of ‘modular epistasis’ [61, 57, 67], which asserts that the fitness of an individual depends on the action of several functional modules. Mutations can improve fitness by improving the functioning of the modules, but only until they reach a state close to their optimal performance. Experimental support for this model is limited. The previously mentioned studies by Szamecz et al. and Rojas Echenique et al. offer a first clue to the role of modular network structure in adaptation [60, 55]. They both show that strains with deletions in the same functional module tend to

evolve in a more similar way than strains lacking genes from other modules. Still, most of the acquired mutations appear not specific to the deleted gene and only a limited amount of parallel evolution is observed. In retrospect, these studies had limited power because of their generic set-up and limited repeats, but also because there was little follow-up on leads to identify exactly what was corrected during adaptation. Nonetheless, these types of experiments can reveal the molecular phenotypes that govern a complex trait, and show which functional modules interact and how they interact.

1.4 Conclusions

Experimental evolution is a versatile method that can be applied to answer many biological questions, and its applications extend beyond what we have discussed here. For example, from an ecological point of view, the method can be used to explore how diverse strains can stably co-exist and how this phenomenon can be emulated and controlled. Experimental evolution is now also being applied in multi-cellular organisms, such as fruit flies and nematodes, which bring their own set of research questions. The method is also frequently being used now in biotechnology, to optimize strains for the production of industrially relevant compounds such as ethanol, and to evolve specific proteins for desired properties, such as brighter and more stable fluorescent protein tags.

In this chapter, we discussed how experimental evolution has allowed us to directly study many factors related to the process of adaptation, which has aided in firmly establishing phenomena such as clonal interference and diminishing returns epistasis. The approach has also shed light on how an organism's genotype influences adaptation. Additionally, it has proved a useful tool in finding the genetic basis of complex traits. The crucial power of the method lies in the fact that it can efficiently select the most beneficial genetic changes out of millions of variants. Even though the method also comes with a number of caveats, such as the fact that adaptation using experimental evolution can be highly specific to the chosen growth regime, with important trade-offs to growth in other conditions, the method holds considerable potential to ask systems-level questions about complex traits and genetic architecture.

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