Experimental Evolution

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Abstract

Experimental evolution is the study of evolutionary processes occurring in experimental populations in response to conditions imposed by the experimenter. This research approach is increasingly used to study adaptation, estimate evolutionary parameters and test diverse evolutionary hypotheses. Long applied in vaccine development, experimental evolution also finds new applications in biotechnology. Recent technological developments provide a path towards detailed understanding of the genomic and molecular basis of experimental evolutionary change, while new findings raise new questions that can be addressed with this approach. However, experimental evolution has important limitations, and the interpretation of results is subject to caveats resulting from small population sizes, limited timescale, the simplified nature of laboratory environments and, in some cases, the potential to misinterpret the selective forces and other processes at work.

Experimental evolution as a research tool

Evolutionary theories are usually inspired and tested by studying patterns – of phylogeny, of divergence between species or populations, of variation within populations, of genome structure and sequence – all of which reflect past evolution. Experimental evolution is an alternative research framework that offers the opportunity to study evolutionary processes experimentally in real time. The past decade has seen the fast growth of studies that tap into this potential, fuelled both by an increasing awareness of the power of this approach and by technological advances that facilitate analysis of the genetic and molecular basis of experimental evolution.

We define experimental evolution as the study of evolutionary changes occurring in experimental populations as a consequence of conditions (environmental, demographic, genetic, social, and so forth) imposed by the experimenter (Fig. 1). Thus, we do not consider cases of evolution in action that do not result from a planned and designed experiment. The above definition also excludes artificial selection (c.f. [1]), where breeding individuals are chosen explicitly by the investigator based on phenotypic values of defined traits or genotypes (e.g., at specific marker loci), thus enforcing a predetermined relationship between those traits or genotypes and fitness. By contrast, in experimental evolution selection can act on any and all traits and genes relevant to fitness under the environmental regimes of interest. Experimental evolution is sometimes called "laboratory natural selection"; however, some experimental evolution studies have been conducted in the field [2-4] and, moreover, some others have explicitly focused on other evolutionary forces including mutation, genetic drift, and gene flow [e.g., 5, 6]. Indeed, these other forces almost invariably act along with selection during experimental evolution, just as they do in nature.

Here, we provide an introduction to experimental evolution as a research approach, illustrating its power and versatility, but also highlighting its limitations and caveats. We discuss major aspects of study systems and experimental design, and we summarize recent technological advances that are revolutionizing the study of the genetic and molecular basis of experimental evolutionary change.

Applications

Experimental evolution has been employed to address diverse questions in many areas of evolutionary biology. Below we discuss several major types of questions, keeping in mind that different questions are often addressed in a single experiment. We also address the advantages of long-term experiments and some practical applications of experimental evolution.

Adaptation to specific environments. Many evolution experiments seek to understand how populations adapt to particular environmental conditions, usually defined in terms of a particular factor, such as temperature [7], nutrition [8], other environmental stressors [9], parasites [3], or competition [10, 11]. A few of these studies are specifically designed to test hypothetical links between particular polymorphisms and fitness: they start from a gene pool constructed to be polymorphic at the focal locus or loci and then measure the response in terms of changes in allele frequency (e.g., [12, 13]). Most studies, by contrast, rely on natural (i.e., uncontrolled) genetic variation sampled from a base population or generated de novo by random mutations. Even though these studies are often motivated by specific hypotheses about traits presumed to be relevant for adaptation (inspired, e.g., by patterns of inter-population variation in nature), other traits may evolve and provide additional and unexpected insights. Therefore, what one can learn from experimental

evolution is relatively unconstrained by preconceptions about what traits and evolutionary processes are most important. The traditional focus on phenotypic aspects of adaptation has been increasingly combined with genomic data, facilitated by technological advances (Box 1).

Study of evolutionary trade-offs and constraints. It is widely assumed that many or most adaptations are associated with trade-offs, such that changes in traits that increase fitness in some environments or situations are deleterious in some other environments or situations. Experimental evolution provides ample evidence for widespread (though not universal) trade-offs in general and insights into their mechanisms in specific cases (e.g., [14]); the evidence has been reviewed elsewhere [15, 16]. As one example, experimental populations of *Drosophila melanogaster* that evolved postponed aging showed a decline in their early fecundity relative to populations that were allowed to breed immediately after emerging as adults [17]. That experiment and several similar ones were pivotal in the broader acceptance of an evolutionary explanation for aging [18, 19]. Experimental evolution has also been employed to study constraints imposed by a lack of standing genetic variation for specific adaptation [20] and to address the notion that certain adaptations are unattainable by mutation. In this last category, evolution experiments have falsified the hypotheses that bacteria cannot evolve resistance to amphipatic antimicrobial peptides [21] and that *E. coli* cannot evolve to feed on citrate under oxic conditions [22]. The study of citrate use also throws light on the nature of the constraint: the appearance of the crucial mutation was contingent on earlier evolutionary changes. This contingency explains why this new function only evolved after 31,000 generations of experimental evolution and only in one of 12 replicate populations [22].

Estimating population genetic parameters. Mutation accumulation experiments, in which very small and initially isogenic populations evolve under conditions designed to minimize selection, are one important source of information about the statistical properties of spontaneous mutations affecting fitness and other quantitative traits. These statistics include the rate at which such mutations occur per genome, the distribution of their effects, the way they interact within (dominance) and between (epistasis) loci, and the variance and covariance they contribute to phenotypic variation per generation (reviewed in [6]). Laboratory adaptation experiments with bacteria, coupled with new population-genetic theory, have been devised to estimate the rates and effect-sizes of beneficial mutations [23-25]. In particular, one can estimate these parameters by following the dynamics of a neutral genetic marker seeded into a set of asexual populations, even without identifying the beneficial mutations themselves. Also, by constructing isogenic strains with specific combinations of evolved mutations, the extent and form of epistatic interactions among the beneficial mutations can be measured [26, 27]. Experimental evolution has also been employed to estimate genetic variance in fitness within populations [28] as well as between replicate evolving lines [29, 30]. Finally, selection coefficients acting on alleles can sometimes be directly estimated from their dynamics under the experimental conditions [29, 31].

Testing evolutionary theories. The versatility of experimental evolution as a research framework is apparent in its applications to test predictions from evolutionary theory. It has been employed, for example, to address controversies as to whether particular evolutionary processes, postulated on theoretical grounds, are plausible. Such "proof of principle" studies have demonstrated, for example, that bacteria can evolve a new phenotypic switch (bet hedging) [32], that natural selection may favor male traits that directly reduce the fitness of their mates [33], and that some degree of reproductive isolation can evolve as a byproduct of divergent natural selection in different environments [34-36] or as a consequence of selection against hybrids [37, 38]. Conversely, no unequivocal evidence for

founder-effect speciation has emerged from several experiments designed to test this model (e.g., [5, 39] and references therein); while these failed attempts do not prove that the process cannot occur, they do suggest that it is rare or requires more time to yield a discernible signal than the experiments allowed.

More often, experimental evolution has been employed to test specific predictions concerning the effect of general properties of the environment (e.g., spatial or temporal variability), demography (e.g., population size or structure, extrinsic mortality patterns, transmission rate and mode for parasites), social factors (e.g., relatedness) or other attributes of the population (e.g., mode of reproduction or mating system) on evolutionary processes and outcomes. Some of those hypotheses are listed in Table 1. We emphasize that this is not a comprehensive list and the studies cited are examples, chosen to cover the broad array of research topics to which experimental evolution has contributed. A comprehensive review of evidence for each hypothesis is beyond the scope of this paper.

Long-term experiments. Most evolution experiments start with one specific aim in mind, but as observations accrue new questions arise. A long-term experiment with *E. coli*—now past 55,000 generations—provides a case in point. The initial focus was on the dynamics of adaptation and divergence in 12 replicate populations, with mean fitness in the selection environment being the response of interest [29, 30]. In time, analyses expanded to examine parallelism from morphological [29] to genetic levels [40, 41]; correlated responses, pleiotropy and specialization [42-44]; the evolution of mutation rates [45, 46]; forces maintaining diversity [47-49]; historical contingency [50] and the origin of a new function [22]; evolvability [30] and epistasis [26]; and the coupling between genomic and phenotypic evolution [51]. Long-term evolution experiments in *Drosophila*—some of which have been running for over 600 generations—have also yielded important insight into reversals of correlated evolutionary responses [52], the causes of aging and late-age mortality plateaus [53], and the relative importance of standing versus mutational variance in adaptation [54]. One of the longest running ecological experiments (started in 1856), which was designed to study the effects of fertilization and soil pH on plant community and ecosystem processes, led to insights on local adaptation and the evolution of reproductive isolation [55, 56]. Sufficiently long experiments might also probe the limits of adaptive evolution, at least for simple environments. Many evolution experiments show declining rates of phenotypic change, but does adaptive evolution eventually cease in the absence of environmental change?

Experimental evolution in medicine and technology. For decades, experimental evolution has been the method of choice for the development of live attenuated vaccines against viral and bacterial diseases, such as polio, tuberculosis, yellow fever, measles, mumps and rubella. For this purpose, the pathogens were serially passaged in other host species or artificial media, until their pathogenic effects in humans had attenuated as a correlated response to selection for improved growth in the new environment [57, 58]. Thus, experimental evolution has contributed to saving millions of human lives, beginning even before it was understood that this method of vaccine production involves Darwinian evolution.

More recently, experimental evolution combined with genome sequencing and genetic mapping has been used to identify mutations that confer drug resistance to pathogens, before such mutations appear in nature (e.g. [59]). This approach might facilitate the rapid diagnosis of resistant infections if

they appear in patients, allowing appropriate public-health measures including the development in advance of new drugs that target the resistant mutants.

Experimental evolution also has great potential in other areas of biotechnology. Serial passage of a pathogen on a particular host often leads to increased specialization and higher virulence on that host [57], and this principle has been used to produce more virulent strains of microbial [60] and metazoan [61] agents of biological pest control. Experimental evolution has also been used to produce biocatalysts and biocontrol agents with other desired properties, e.g., high thermal tolerance [62]. For some microbial systems this process can be fully automated [60, 62]. From an engineering perspective, experimental evolution will undoubtedly be employed as a "bottom-up" complement or supplement to "top-down" genetic engineering methods to generate organisms for the production of biofuels [63] and for carbon sequestration.
Experimental evolution can even be extended to artificial living systems including some that are based on molecular processes and others that are computational in nature (Box 2). These artificial systems are being used not only to test basic hypotheses but also to evolve useful new products, from protein catalysts to software and even robots [64-66].

Designing evolution experiments

Study system. Many questions in evolutionary biology apply to a broad range of organisms or even to all. The choice of the study system thus becomes largely a matter of convenience. As a result, most evolutionary experiments have used one of several favorites, in particular *E. coli*, *Pseudomonas*, yeast and *Drosophila* (Table 1). Several phage-bacteria systems and *Daphnia* with its pathogens have been widely used to address questions about coevolution (Box 3). The relative paucity of evolution experiments on vascular plants [67-69] reflects in part their long generation times (even for *Arabidopsis* this is 2 months, compared to 2 weeks in *Drosophila* or *Daphnia*, 3-4 days in *C. elegans*, and hours in microbes). Concentrating on model systems has obvious advantages, such as integration of results from different fields and the availability of genomic information and tools. However, even if the questions an experimenter asks are general in nature, the answers obtained might nevertheless be specific to the taxon under study. Microbes differ from multicellular eukaryotes in many fundamental ways (Box 4), and so extrapolating between these domains must be done with care and may sometimes be problematic. Even closely related species may differ in ways relevant for evolution. For example, populations of *Drosophila melanogaster* are often polymorphic for large chromosomal inversions, which effectively suppress recombination over large regions of the genome; but such inversion polymorphisms are rare in the closely related *D. simulans* [70]. Thus, the evolution of the former species is more likely to be affected by linkage disequilibrium, which may produce more pronounced correlated responses in the absence of pleiotropy. Over time, the differences between general principles and idiosyncratic features of particular systems should emerge if the community of researchers uses a wide range of study systems.

More generally, the common model systems may also be more similar to one another—and unrepresentative of nature—precisely in the ways which make them so easy to study. These species have been chosen because they have short generation times. They all tolerate human-influenced environments, in some cases (e.g., *Drosophila melanogaster*) because they are human commensals. Some of them have recently increased in population size and adapted to the human-modulated

environment, perhaps selecting for higher recombination rates and mutation rates than those in their sister taxa. Compared to their wild counterparts, the strains used in these studies have often already adapted to some laboratory conditions. They may prefer more constant abiotic conditions (e.g. temperature), use a narrower spectrum of resources that require little effort to locate, undergo little or no dispersal, have little need to react to stresses, have reduced capacity to interact with other species, and so forth. Whether these differences between the typical organisms used in experimental evolution and those more broadly representative of nature are important may depend on a particular study's goals. For example, experimental evolution is often well suited to asking whether some particular process or factor (e.g., population size) can be important in evolution, but this approach may not be appropriate to extrapolating parameter estimates (e.g., selection coefficients) to nature without appropriate caveats.

Regimes and controls. Hypothesis testing in evolution experiments typically involves comparisons between sets of populations evolving under different regimes, but originally derived from the same base population or the same ancestral genotype (Fig. 1c,d). Such comparisons quantify the differences in evolutionary response under the various regimes. Sometimes, a distinction can be made between "selection" (or "treatment") and "control" (or "unselected") regimes, perhaps suggesting that the "control" conditions mimic the ancestral conditions to which the base population or ancestral strain was adapted before the start of the experiment (e.g., [8, 71]). However, this assumption is hardly ever fulfilled, because the vast majority of any lineage's evolutionary history occurred outside the laboratory. In any case, the contribution of the different regimes to the observed divergence can be best evaluated if the ancestral population is included in the comparison. The phenotype of evolved populations can be compared in contemporaneous assays to the ancestral population if the latter can be preserved alive but prevented from evolving (Fig. 1a,b), e.g., by freezing or in resting stages such as seeds. At the genetic level, once candidate polymorphisms that may contribute to phenotypic evolution have been identified, allele frequencies in the various evolved populations can be compared with the ancestral population if a sample of genetic material for the latter is available (even if the ancestral organisms are no longer viable). Also, it should be kept in mind that a difference between evolved and ancestral populations might reflect greater inbreeding of the former (for sexually reproducing organisms: see below) or adaptation to aspects of the selection regime other than the factor being tested.

Experimental replicates. Isolated populations derived from the same gene pool will diverge with time even if they are maintained under the same environmental conditions. Such divergence will be driven by random genetic drift affecting pre-existing polymorphisms and the establishment of new mutations, by the order in which mutations appear, and by any uncontrolled environmental variation that affects the direction and intensity of selection. Divergence generated by these stochastic mechanisms can be further amplified by selection if the resulting differences in genetic background influence the fitness effects of alleles [72]. Therefore, genetic divergence between populations cannot be attributed with confidence to different regimes unless this divergence is shown to be greater than occurs in the absence of the imposed differences in regime. Rather, divergence between experimental regimes should be tested relative to variation among independently evolving replicate populations subjected to the same regime. In other words, experimental populations are the units of replication for testing evolutionary hypotheses.

Base population or ancestral genotype. Replicate populations are usually derived from a single base population or ancestral strain. In other cases, however, experimental populations may be paired or

blocked based on their origin, before evolving under different experimental regimes. Evolutionary change is contingent on the initial gene pool, so starting from different ancestors may reduce the statistical power (because the different starting populations may respond differently); on the other hand, having multiple starting populations may increase the generality of any conclusions.

Evolution experiments that rely on pre-existing genetic variation (i.e., most non-microbial studies) start with a base population. That base population was itself typically founded with some dozens to thousands of individuals sampled from nature, then allowed to adapt to the laboratory environment for many generations while being maintained at a large size (e.g. [71, 73]). Such base populations will likely harbor more polymorphisms, including rare alleles, than typical laboratory stocks. An experimenter could also mix individuals from different natural populations or laboratory stocks to increase genetic variation, but doing so would generate linkage disequilibrium, which might be problematic depending on the question of interest.

Experimental population size and number of generations. While evolving microbial populations are usually maintained at sizes of millions, experimental populations in non-microbial systems are limited by practical considerations to thousands, hundreds or even dozens of breeding individuals. Small population sizes have important consequences for several aspects of evolution (Box 5). Of the studies cited in Table 1, many that started from outbred populations have detected divergence in mean trait values or fitness within 10-20, and sometimes as few as 3-8 generations (e.g., [68, 74, 75]). However, experiments that fail to produce an evolutionary response are often not published, and so these numbers should be viewed as optimistic. In microbial experiments, responses may occur within a single day (5-10 generations) when using strong selective agents, such as viruses and antibiotics. With more subtle selection for improved competitive ability, 200 or more generations might be needed before the first beneficial mutations rise to fixation [30]. Experiments designed to detect changes in variance [30, 76], or to observe second-order effects on such traits as mutation rate [45], typically require more generations.

Controlling for maternal effects. Different experimental evolution regimes often involve different environmental, demographic, or social conditions. Conditions experienced by the parents often affect the phenotypes of their offspring (or even grand-offspring) via non-genetic maternal and paternal effects. Such effects can be mediated by egg or seed provisioning, signaling molecules in the cytoplasm, chromatin modification, and other epigenetic mechanisms [77]. Most evolution experiments focus on genetically-based changes. In order to eliminate effects caused by different parental environments, samples of populations from all regimes (and the revived ancestor where applicable) should be reared in a common environment for one or more generations before their divergence is assessed. The choice of this common parental environment can be complicated, however, if there is an interaction between genotypic and maternal-environment effects [78].

Controlling for differential inbreeding. Even if populations under different regimes are maintained at the same census size, the regime with stronger selection will have smaller effective population size [79], leading to a greater degree of inbreeding in sexually reproducing organisms. Greater inbreeding could, in turn, lead to a reduction in fitness components (inbreeding depression), which might be misinterpreted as a correlated response to selection (reflecting pleiotropy or linkage disequilibrium). Crossing replicate populations within selection regimes should restore heterozygosity and thus largely eliminate the inbreeding depression. If crosses between replicate populations within regimes exhibit the same pattern of phenotypic differences between regimes as the original populations,

then the differences can be more safely interpreted as resulting from selection (e.g., [8]). However, such crosses may also show complex patterns if the phenotypically similar responses of replicate populations reflect different genetic mechanisms that interact in a non-additive way (e.g., [80]).

Caveats and limitations

Timescale and serendipity. Numerous success stories notwithstanding, experimental evolution has some limitations as a research approach, and the conclusions from evolutionary experiments are subject to caveats. Although experimental evolution can be extremely fast, some evolutionary processes may be too slow to be seen within the span of a research grant or even a researcher's professional lifetime. Limited insight from experimental evolution into speciation is a case in point; while a measurable degree of reproductive isolation has evolved in several experiments, it has never reached the degree of isolation expected between biological species [72] (except for the special mechanism of speciation via polyploidization of hybrids in plants [81]). Other processes, such as the origin of morphological novelty, may depend on rare sequences of mutational events or improbable outcomes of drift, such that the likelihood of them happening in an experiment is too low to justify the undertaking, especially if other approaches can provide empirical support. Still other questions have been difficult to address with experimental evolution for want of an appropriate study system. In particular, despite some progress (e.g., [82, 83]), efforts to test hypotheses about the short-term advantages of sex have been hampered by the fact that, in species capable of both modes of reproduction, sexual and asexual offspring are usually physiologically or ecologically distinct.

Technical difficulties and laboratory artifacts. Evolution experiments can be compromised by contamination, i.e., inadvertent introduction of "immigrants" into experimental populations [84]. Other unexpected factors may confound the intended regimes. For example, in a study aimed to test the effect of extrinsic host mortality on parasite virulence, the host mortality regime became unexpectedly confounded with multiplicity of infection, completely altering the selective forces on the parasite [85]. Finally, populations may evolve to obviate the intended regimes; e.g., a study concerning the effect of ploidy on adaptation in yeast was thwarted when the initially haploid and tetraploid populations all evolved diploidy [86].

In studies aimed at understanding adaptation to a particular environmental factor, the results may depend on the way in which that factor is implemented. For example, selection for acute starvation resistance in *Drosophila* led to reduced locomotor activity [87]. This behavior was adaptive under the laboratory regime, where flies were deprived of food for a certain time and the survivors were given food later, because reduced locomotion conserves energy. However, food shortages in nature may often favor increased mobility to find new food sources. Indeed, as a plastic (phenotypic) response, flies become highly active when deprived of food [87]. Laboratory environments often confine mobile animals to small space, changing the context of social and sexual interactions; e.g., in contrast to nature, female *Drosophila* cannot escape aggressive sexual interactions, which inflates mating frequency and may amplify sexual conflict [88, 89]. Such considerations indicate the need for caution in extrapolating particular adaptive outcomes from the laboratory to the field.

Population genetics of laboratory evolution. The population genetics of laboratory evolution may differ in important ways from evolution in nature. One reason is the small effective population size in experiments relative to nature, which has manifold consequences for evolution (Box 5). Also, in experiments with outbred populations, evolutionary responses will depend largely on standing

genetic variation present in the base population, at least over the first 100 or so generations [90]. From a population genetic view, such experiments mimic evolution following abrupt environmental changes. The genetics of such responses are expected to differ from evolution that depends on new mutations in several ways; in particular, adaptations to abrupt changes are more likely to involve recessive alleles and alleles with smaller effects (reviewed in [91]).

Finally, owing to the simple environments and strong selection, laboratory evolution may involve alleles with different patterns of pleiotropy from those typical in nature. Many fitness-related traits are presumably affected by many alleles with diverse pleiotropic effects. In nature selection will usually act simultaneously on many aspects of the organism's phenotypes; hence, selection on any particular function or trait will often be weak. Thus, adaptation in nature is more likely to involve alleles that show few or no adverse pleiotropic effects (if such alleles exist). In contrast, experiments often impose strong selection on a single focal factor. Other sources of selection (e.g., suboptimal conditions, pathogens, locomotion and so on) are often absent or minimized. As a consequence, pleiotropic effects that would be deleterious in nature may be neutral or nearly so in the laboratory; as a case in point, about 60% of single-gene deletions in yeast are effectively neutral under optimal laboratory conditions [92]. Furthermore, the availability of alleles with small or no antagonistic pleiotropic effects may be limited by the small sizes of laboratory populations. Laboratory selection may thus more often involve alleles with strong adverse pleiotropic effects than evolution in nature. Therefore, experimental evolution studies may tend to overstate the importance of evolutionary trade-offs.

Experimental evolution in the field

Some of the concerns discussed above can be circumvented by performing evolution experiments in natural environments. A pioneering evolution experiment in the field was initiated in 1976 by transferring a guppy (*Poecilia reticulata*) population between environments with different predation regimes, leading to seminal insights into the evolution of life histories, sexual signaling, mate preferences, and predator-prey coevolution [4, 93-95]. Despite this early and successful start, there have been few experimental evolution studies in the field. Such studies involve moving populations, manipulating natural environments, or both, and these actions impose logistical challenges and may also raise legal, ethical, or conservation issues. Another difficulty is the need to confine experimental populations, which limits the approach to island-like habitats [2, 3, 96-98]. Finally, the environment as a whole is not controlled, making the experiments more likely to fail if, e.g., populations become locally extinct. Many of the hypotheses in Table 1 concern general demographic, genetic, social or other factors, and their predictions are unrelated to a specific environment and its complexity, so it can be argued that field tests in such cases are not worth the additional effort. On the other hand, for reasons discussed in the preceding section, where the question concerns adaptation to specific environmental factors, laboratory environments may introduce artifacts. Furthermore, populations in the field can be much larger. Some experiments are impractical in the laboratory; e.g., by introducing predators to islands, Losos et al. [96] showed that predation drives the evolution of an arboreal lifestyle in *Anolis* lizards. Finally, field experiments allow one to study the direct and indirect effects of evolutionary change on ecosystem processes [94].

Mesocosms (e.g., artificial ponds) offer an intermediate between laboratory and field studies. Experiments performed in parallel in natural habitats and in mesocosms produced reassuringly

similar effects of predation on the evolution of color pattern evolution in guppies [93] and on the advantage of immigrant alleles in genetically depauperate populations of *Daphnia* [2].

Finally, in some cases one can verify the relevance of laboratory-evolved adaptations to fitness in nature by assaying experimentally derived organisms under field conditions. For example, *Drosophila* from populations selected for cold tolerance were more likely than flies from control populations to be recaptured at food sources hours after their release into the field in cold weather, but not at mild temperatures [99]. This finding indicates that the experimental adaptation to cold under laboratory conditions translated into improved ability to survive and find food under cold conditions in the field.

Conclusions

The potential value of experimental evolution as a research approach has long been recognized—already in 1892 a book titled *Experimental Evolution* proposed using this methodology to resolve the controversy between the Darwinian and Lamarckian theories of evolution [100]. The past decade has seen increasing application of experimental evolution to an expanding range of questions, while advances in genomic technology are beginning to provide unprecedented insights into the genetic and molecular bases of evolutionary change. These and other technological advances open new avenues, while discoveries in fields including genetics, developmental biology, and global change pose new questions that can be tackled with experimental evolution (see Box 6, "New Opportunities, New Challenges").

Experimental evolution also offers a unique opportunity to improve science education. While paleontology and comparative studies provide ample evidence for evolution, the fact that scientists can observe evolution in action through manipulative experiments is an eye-opener to people for whom "seeing is believing". Also, many organisms used for research in experimental evolution can be readily deployed in teaching laboratories. For example, a class can quickly evolve bacteria to resist antibiotics [101]. If time permits, students could then compete the evolved and ancestral strains in the absence of antibiotic to test for tradeoffs. Using *Drosophila,* students can, over a semester, observe selection against alleles that are readily scored, such as those that disrupt wing morphology [102]. (Of course, such experiments in classrooms require suitable facilities and appropriately trained teachers, and they must comply with institutional policies and local regulations on biological experiments.) Using digital organisms—computer programs that self-replicate, mutate and compete in a virtual world—students can watch evolution before their eyes as they vary environments and other factors and observe their effects on the emergence of new phenotypes [103]. As the use of experimental evolution continues to expand in the research community, we hope it will also have a growing impact on science education.

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Box 1. Genomics and experimental evolution

The first complete genome sequence was for the phage ΦX174, and its 5,375-bp sequence appeared in 1982. A draft of the ~3,000-Mb human genome was published in 2001. These achievements were remarkable in their day but now, thanks to technical advances, whole-genome re-sequencing is accessible for experimental evolution studies. In 1997 Bull *et al.* [104] sequenced nine ΦX174 isolates that had evolved on two hosts. In 2007 Velicer and colleagues [105] sequenced the genome of a *Myxococcus xanthus* derivative that had evolved from socially cooperative to cheating and back to cooperative. In 2009 Barrick and Lenski [49] deeply sequenced seven whole-population samples that spanned 40,000 generations from an evolving *Escherichia coli* population to find genetic polymorphisms. In 2010 genomics was extended to experimentally evolved eukaryotes, *Saccharomyces cerevisiae* [106] and *Drosophila melanogaster* [54]. The application of genomics to experimental evolution may soon be limited only by the imagination of the investigator and the quality of the study design. Other high-throughput approaches are also increasingly useful for experimental evolution including characterizing an organism's capacity to use diverse resources (e.g., [43]) as well as proteomic (e.g., [107]), transcriptional (e.g., [40]) and metabolic profiling (e.g., [108]).

To date, studies at the interface of genomics and experimental evolution have ranged from descriptive ones that demonstrate new technologies [109, 110] or find genes of interest [105, 106, 111] to quantitative analyses of diverse conceptual issues. How repeatable is evolution at the levels of nucleotides, genes and pathways [27, 51, 104, 112, 113]? How do epistatic interactions and the order of mutations affect evolvability, marginal fitness effects and the origin of new functions [26, 27, 113-115]? What are genomic mutation rates and the spectrum of mutational types, and how do they evolve [49, 51, 116-118]? What are the dynamics of genome evolution in relation to phenotypic change and in terms of hard versus soft selective sweeps [49, 51, 54, 104, 111, 113]?

While these high-throughput methods provide new opportunities, they can also be difficult for analysis and interpretation. In particular, demonstrating causal links between specific changes at the genomic or transcriptional level with divergence in morphology, physiology, behavior or life history remains challenging, especially in non-microbial systems. These methods often identify divergence in allele frequencies at hundreds of loci [54, 111] or in expression of hundreds of transcripts [119]. Owing to linkage, drift, and statistical false-positives, not all of these differences will have been caused by adaptation. Therefore, such data must be interpreted with caution.

Box 2. Experimental evolution with artificial Life

One of the goals of experimental evolution, as a field, is to test general hypotheses about evolutionary processes—in contrast to many comparative studies that seek to understand the evolution of a particular trait in a given phylogenetic context. Yet, all of life on Earth derives from the same primordial ancestors, so how general can evolutionary tests be? As Maynard Smith put it [120]: "So far, we have been able to study only one evolving system, and we cannot wait for interstellar flight to provide us with a second. If we want to discover generalizations about evolving systems, we will have to look at artificial ones."

Artificial evolving systems include synthetic replicators built from organic molecules [121, 122] and digital organisms living in virtual worlds [123, 124]. As Dennett says [125]: "The process of natural selection is substrate-neutral … evolution will occur whenever and wherever three conditions are met: replication, variation (mutation), and differential fitness (competition)." The Avida system is a computational platform developed for this research, one in which digital organisms are programs that replicate, mutate and compete [124, 126]. (Both research and educational versions of Avida are freely available on the web.) The organisms can manipulate bit-strings and, if they perform an operation appropriate to their environment, they obtain additional energy to run their genetic programs. Starting from a simple ancestor that can replicate but not perform other functions, populations can evolve a complex computational metabolism [127]. As in nature, selection acts on the phenotypes of digital organisms, not on their genetic encoding.

Digital systems offer short generations, controllable environments and automated analyses including, for example, lines of descent (showing every intermediate from the ancestor to an evolved state of interest) and genotype-phenotype maps (showing the effect of mutating each genomic position on every phenotype). To date, experiments with digital organisms have addressed diverse issues including the origin of parasites [123], effect of mutation rate on robustness [128], historical contingency [129], origin of complex functions [127], ontogeny and phylogeny [130], evolution of sex [131], role of pleiotropy in ecological specialization [132], recovery from extinctions [133] and selection for altruism [134]. One can even extend this approach to embodied robots by evolving their morphology, behavior, or both in a virtual world constrained by physical laws, then building the computationally evolved robots [64]. In this way, robots can evolve to pursue or evade other robots as predators or prey [135], cooperate [136], and communicate with one another [137, 138].

Box 3. Experimental coevolution

The term "coevolution" refers to two phenomena: (1) the evolution of interacting species whereby evolutionary change in one species induces evolutionary change in another species and vice versa; and (2) a similar process occurring between genes and traits of the same population but expressed in different classes of individuals (e.g., sexes, mother-offspring) or with different modes of transmission (e.g., selfish genetic elements and their suppressors). Most evolution experiments have concentrated on antagonistic coevolution, where the fitness of both parties cannot be simultaneously maximized. Rather than being externally controlled, a crucial aspect of the environment in coevolutionary experiments is the coevolving party and thus a moving target.

Disentangling co- from evolution. Identifying evolved changes that result from the coevolutionary feedback often requires comparing coevolutionary regimes (where the interacting species evolve together) with "unilateral" regimes where only one species is allowed to evolve and the other is kept static [139-141]. An analogous approach to coevolution between the sexes is challenging because the sexes share a gene pool, but inroads have been made using sophisticated breeding designs [142].

Dynamics of coevolution. Antagonistic coevolution proceeds by two fundamentally different modes [143]. (1) Time-lagged negatively frequency-dependent selection favors phenotypes that were rare or absent a few generations ago. This mode often leads to unstable dynamics, such as an arms-race. (2) Selective sweep coevolution occurs when beneficial mutations arise and spread to fixation. Coevolution experiments have been performed to investigate by which mode coevolution proceeds and to test how coevolutionary dynamics are affected by, and in turn influence, genetic and demographic factors [139, 144, 145]. Systems in which samples of coevolving populations can be preserved and revived allow time-shift-experiments [146]. These experiments involve reciprocal transplants in time, where populations of one antagonist (e.g., host) sampled at time t_1 are confronted with the other antagonist (e.g., parasite) sampled at times t_0 (past), t_1 (contemporary) and t_2 (future). Parasites from the future are expected to be more infective to hosts from time t_1 under a selective sweep model, but not under negative frequency-dependent selection models [147, 148].

Coevolution-driven divergence. In a coevolving system, stochastic changes in one antagonist may change selection on the other antagonist, and the resulting evolutionary change may feed back on the evolution of the first species and so on. Coevolution will thus magnify stochastic effects and accelerate divergence between isolated populations, as seen in a several experiments with phage and bacteria [27, 139, 149].

Box 4. Microbes versus macrobes

Microbes were largely ignored by evolutionary biologists for many decades. Eventually, though, the utility of microbes for experimental evolution became clear. Their obvious advantages include short generations, large populations, and the ease with which environments can be controlled and manipulated. Another important feature is that most microbes can be stored frozen in a non-evolving state and later revived (this is also possible for some animals and plant seeds). This property effectively enables travel in time: the experimenter can directly compare organisms from different generations, e.g., by competing derived and ancestral genotypes to measure their relative fitness [30, 150]. Indeed, by performing simultaneous assays with organisms from many different generations, one minimizes the effects of uncontrolled fluctuations in conditions that might confound interpretation of data collected at different times. One can also perform "replays" where evolution is restarted from intermediate generations to test whether an outcome of interest, such as the origin of a new function, was contingent on earlier changes [22, 27, 113].

Evolutionary experiments with microbes and other organisms differ in several ways, the importance of which may depend on the question of interest. Most experiments with microbes start with a single clone and depend on new mutations to generate variation. Evolution often occurs by consecutive selective sweeps, although frequency-dependent interactions and clonal interference (competition between beneficial mutations) also can be important [48, 151-153]. In contrast, experimental evolution in non-microbial experiments is mostly fuelled by genetic variation already present in the initial population, and alleles are regularly recombined by sex. As a consequence, microbial populations may evolve more slowly, at least on a generational basis. Furthermore, some phenomena central to the evolution of many plants and animals, such as sex, sexual selection, parental care and speciation are either absent or involve very different mechanisms in microbes (particularly bacteria or viruses), limiting the utility of the latter as model systems for those phenomena in the former. On the other hand, eukaryotic microbes have been used to study the evolution of reproductive isolation [36, 37] and sexual selection [154]. Furthermore, factors such as mutation rates [46], recombination [83, 155-157] and genetic relatedness [158-162] can be manipulated in some microbes to examine their evolutionary effects.

Finally, microbes are less familiar than the larger organisms we see all around us. As a consequence, most evolutionary biologists have better intuition about what phenotypic traits and environmental factors matter for animals and plants – for example, beak size and seed hardness – than for the physiological traits and physicochemical factors that determine the fitness of microbes. The potential for microbes to exhibit complex life histories [161, 163, 164] and social behaviors [160, 161, 165] has only recently become appreciated. Thus, microbes are now being used as model systems to study the evolution of traits that biologists traditionally ascribed only to multi-cellular organisms.

Box 5. Effects of population size in experimental evolution

By necessity, experimental populations are orders of magnitude smaller than in nature. Therefore, many fewer alleles are available to respond to selection, for two reasons. First, some alleles are lost due to drift in small populations, with the loss rate inversely proportional to the effective population size (N_e). Effective sizes for eukaryotes are usually thought to be up to an order of magnitude smaller than census sizes (N) [166]; factors that reduce N_e compared to N include selection at other loci, random variation in reproductive output, biased sex ratio and fluctuations in population sizes. In microbial populations propagated by serial transfer (e.g. [30, 167]), the effective size depends strongly on the transfer size, not the maximum population size [30, 168]. For diploid organisms, the loss of genetic diversity can also lead to inbreeding depression, which lowers fitness in subsequent generations. Deleterious alleles can fix by random drift if the population is small enough (N_e less than the reciprocal of the selection coefficient); in fact mutation-accumulation experiments rely on this process [6]. The loss of diversity and inbreeding are unlikely to matter too much over the first 10 generations or so, provided the effective population size is a few dozen or more.

Second, the number of new mutations per generation is proportional to the number of genomes in the population, i.e., N or $2N$ depending on ploidy. The relative importance of standing variation and new mutation depends on the size of the population and the length of the experiment. Hill [169] estimates that in long-term experimental selection studies, many fixed alleles arise as new mutations, and of course many experimental populations are started from a genetically uniform stock, making new mutations all-important. Responses to selection can be significantly limited by population size [46, 167, 170, 171].

Small population sizes can affect the progress of evolution in a number of ways. For multi-cellular sexual organisms, the rate of adaptation in a laboratory population is likely to be mutation limited after it exhausts (through selection or drift) the starting genetic variation. Such species typically have long generation times, and few alleles have time to reach fixation during a typical experiment. On the other hand, it may be possible to have much larger populations of single-celled organisms. Indeed, in some cases all one-step point mutations are estimated to have arisen multiple times [22]. In such cases, however, the rate of adaptive evolution still may be limited by small population sizes because some adaptations may require two or more mutations, and the order in which they occur and their epistatic interactions may constrain evolution [22, 26, 113]. Simultaneous double mutations are very rare, and exploring the space of all possible double mutations would require very large populations.

Box 6. New Opportunities, New Challenges

Automation. Experimental evolution requires a substantial investment of time and labor to maintain the populations under their intended regimes, while the return on this investment - in terms of results and publications - takes months, years, or even decades and is uncertain. The problem may be alleviated to some extent by progress in automation of population maintenance under particular regimes (e.g., www.ksepdx.com/live_transferring.htm and [62]) and of assays of physiological [43], morphological [172], and life history phenotypes [173].

Use of transgenics to verify genetic basis of adaptation. Advances in genetic manipulation techniques offer increasing possibilities to examine the causal links between genomic changes, phenotypes, and fitness. For example, specific point mutations can now be introduced or recombined in several model systems [174, 175]. In *Drosophila melanogaster*, the GAL4-UAS dual technique is now routinely used to express any exogenous transcript in specific tissues or cells [176] or, in combination with RNA interference techniques, to down-regulate endogenous transcripts [177]. Such techniques permit independent tests of the phenotypic and fitness effects of particular mutations or changes in gene expression observed in the course of experimental evolution.

Very long projects. Some ecological experiments (e.g., [55]) and artificial selection projects (e.g., [178]) have now been running for over a century, spanning multiple generations of researchers. Comparable efforts in experimental evolution (already proposed in 1892 [100]) would offer insights into rare events and slow processes, such as the origin of morphological novelties, the functional differentiation of duplicated genes, and perhaps even the speciation process taken to completion.

Epigenetic inheritance. The past decade provided evidence that some quasi-hereditary information can be encoded in patterns of chromatin modification (e.g., DNA methylation). Evidence for such epigenetic inheritance is pervasive in plants [179], but has also been reported to affect longevity in *Caenorhabditis elegans* [180] as well as wing development and possibly reproductive mode in aphids [181]. Epigenetic inheritance is only beginning to be incorporated into evolutionary theory [182] and experimental evolution may contribute to this development.

Protein-coding or regulatory bases of adaptation. Experimental evolution can contribute new data about the relative contributions of mutations in protein-coding versus regulatory regions to adaptive evolution, at least those changes occurring between closely related taxa [183, 184].

Experimental evolution and anthropogenic change. There is growing awareness that evolutionary processes can sometimes be rapid enough to have implications for conservation of species and ecosystems [185-187]. Experimental evolution can contribute to understanding of such processes as species invasions [188] and evolutionary rescue from local extinction [189]. Experimental evolution studies may also influence the scenarios of biotic responses to global change such as evolutionary responses of algae to elevated CO_2 levels [190].

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Table 1. Examples of evolutionary hypotheses with references to selected studies that have tested (but not necessarily supported) those hypotheses using evolution experiments. "F" indicates an evolution experiment in the field.

Hypothesis	Organism and references
Mutation and adaptation	
Adaptation occurs mostly via many mutations of small effect	Bacteriophage φ6 [191]; *Escherichia coli* [23-25]
Fitness effects of beneficial mutations show negative epistatic interactions	Bacteriophage T7 [192]; *Escherichia coli* [26]; *Methylobacterium extorquens* [115]
Mutators (strains with elevated mutation rates) may evolve during adaptation to a novel environment	*Escherichia coli* [45]
Mutators may enhance rates of adaptive evolution	*Escherichia coli* [45]
Genetic drift and inbreeding	
Genetic drift reshapes genetic variance-covariance matrices	*Drosophila melanogaster* [193]
Bottlenecks do not reduce and may increase additive genetic variance	*Musca domestica* [194]*; Tribolium castaneum* [195]; *Drosophila melanogaster* [196]
Offspring of immigrants have high fitness in small inbred populations	*Daphnia magna* (F) [2]
Environmental variability	
Spatial heterogeneity with restricted gene flow favors local adaptation in metapopulations	*Arabidopsis thaliana* [75]
Spatial environmental heterogeneity drives adaptive radiation	*Pseudomonas fluorescens* [153]
Fluctuating environments favor generalist genotypes, and constant environments favor specialist genotypes	*Chlamydomonas reinhardtii* [197] Vesicular stomatitis virus [198]; *Escherichia coli* [43]; digital organisms [132]
Fluctuating environments maintain genetic variation	*Drosophila melanogaster* [76, 199]
Sexual selection and conflict	
Intensity of sexual signals increases under strong sexual selection	*Drosophila pseudoobscura* [200]; *Saccharomyces cerevisiae* [154]
Intensity of sexual signals increases when predation pressure is relaxed	*Poecilia reticulata* (F) [201]
Sexual selection facilitates elimination of deleterious alleles	*Drosophila melanogaster* [31]
Sexual selection leads to reduction (-) or increase (+) in non-sexual fitness components	*Callosobruchus maculatus* (+) [202]; *Drosophila melanogaster* (+) [203], (-) [204]; *D. serrata* (-) [205]

Polygamy favors male traits that reduce fitness of their mates (interlocus sexual conflict)	*Drosophila melanogaster* [33]; *Rhizoglyphus robini* (mite) [206]; *Sepsis cynipseaii* (fly) [207]
Life history and sex allocation	
High extrinsic mortality leads to the evolution of shorter intrinsic lifespan	*Drosophila melanogaster* [208]
High predation favors high reproductive effort	*Poecilia reticulata* (F) [95]
Antagonistic pleiotropy contributes to late-life mortality plateau	*Drosophila melanogaster* [53]
Sex allocation in hermaphrodites evolves towards the Fisherian ratio	*Mercurialis annua* (plant) [68]
Local mate completion favors female-biased sex ratio	*Tetranychus urticae* (mite) [209]
Sexual reproduction and mating systems	
Fitness declines in asexual populations by Muller's ratchet	Bacteriophage φ6 [210]
Sex and recombination accelerate adaptation to a novel environment	*Chlamydomonas reinhardtii* [162]; *Saccharomyces cerevisiae* [83]; *Escherichia coli* [156]
Incidence of sex increases in heterogeneous environments (in species with facultative sex)	*Brachionus calyciflorus* (rotifer) [82]
Self-fertilization evolves under pollinator limitation	*Mimulus guttatus* (plant) [69]
Sexual reproduction favors altered gene interactions and modularity	Bacteriophage T4 [155]; digital organisms [131]
Kin selection and cooperation	
Relatedness favors restraint from cannibalism	*Tribolium confusum* [211]
Limited migration and local extinction promotes competitive restraint	Bacteriophage T4 [212]
Cooperators evolve to suppress social cheaters	*Myxococcus xanthus* [213]
Parasitic mitochondria evolve when among-cell selection is weak	*Saccharomyces cerevisiae* [214]
Single-cell bottlenecks promote cooperation among cells in multicellular organisms	*Dictyostelium discoideum* [215]
Conditions favoring large size may lead to evolution of multicellularity	*Saccharomyces cerevisiae* [164]
Behavior and cognition	
Variation in foraging behavior is maintained by negative frequency-dependent selection	*Drosophila melanogaster* [12]
Opportunity to learn may accelerate genetically-based adaptation (Baldwin effect)	*Drosophila melanogaster* [216]
Host-parasite interactions	
Parasites or predators select for host or prey resistance, and resistance is costly	*Daphnia magna* and microsporidian parasite (F) [2]; *Escherichia coli* and various bacteriophages [14, 27, 44, 217];

	Chlorella vulgaris (algae) and *Brachionus calyciflorus* (rotifer) [218]
Parasites impose negative frequency-dependent selection on the host	*Potamopyrgus antipodarum* (gastropode) and *Microphallus* sp (trematode) [74]
Host-parasite coevolution drives divergence and local adaptation	*Escherichia coli* and bacteriophage φ6 [139, 149]
Vertical transmission and lower virulence evolve under conditions of high host population growth	*Paramecium caudatum* and *Holospora undulate* [219]
Speciation	
Divergent selection leads to premating isolation	*Drosophila pseudoobscura* [35]; *D. serrata* [34]
Divergent selection leads to postmating isolation	*Neurospora sp.* [36]
Hybrid inferiority leads to reinforcement of prezygotic reproductive isolation	*Drosophila yakuba* [38]
Repeated bottlenecks lead to reproductive isolation (not supported)	*Drosophila pseudoobscura* [5, 39]; *Musca domestica* [220]
Repeatability of evolution	
Adaptation in independent populations occurs via parallel changes in gene expression, parallel mutations, or parallel enrichment of pre-existing alleles	*Escherichia* coli [40]; *Saccharomyces cerevisiae* [221]; Various bacteriophages [27, 104, 222]; *Drosophila melanogaster* [54]
Traits less correlated with fitness are more influenced by chance and history	*Escherichia coli* [50]
Ontogeny recapitulates phylogeny	Digital organisms [127]

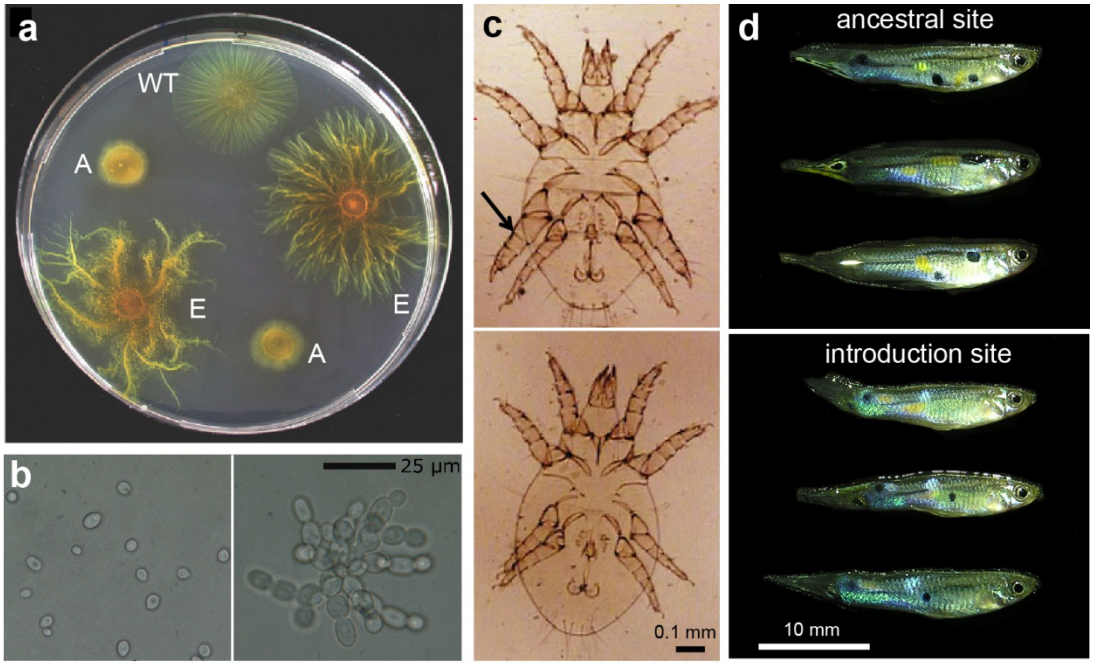

Figure 1. Examples of experimentally evolved phenotypic changes. (a) Cells of the social bacterium *Myxococcus xanthus* cooperate to swarm across solid surfaces in search of food and to form multicellular fruiting bodies. Strains initially unable to swarm due to loss of a necessary gene ("A") evolved alternative swarming mechanisms, leading to novel swarm morphologies ("E") that are markedly different from the ancestral form ("WT") [223]. Each swarm contains many millions of cells. Reprinted by permission from Macmillan Publishers Ltd: *Nature* 425: 75-78, copyright (2003). (b) Multicellular "snowflake" yeast (right) experimentally evolved from a single-celled ancestor (left) under conditions favoring large size (adapted from [164], copyright William C. Ratcliff et al. [164]). (c) Two male morphs in *Rhizoglyphus* mites; "fighter" males (top) use their modified 3rd pair of legs (arrow) to kill rival males but are less mobile than "scrambler" males (bottom). Ten generations of evolution in a complex environment shifted the underlying reaction norm, leading to a substantial decrease in the frequency of the fighter morph [224]. Copyright Jacek Radwan, Jagiellonian University, Kraków. (d) Populations of the guppy (*Poecilia reticulata*) introduced to sites free of the main predator (bottom) evolved brighter male coloration – here, blue dorsolateral spots and stronger blue-green iridescence on posterior body – compared to their ancestors (top) that evolved with visual predators [201]. Copyright Darrell J. Kemp, Macquarie University, Sydney.