Abstract

In the past three decades laboratory natural selection has become a widely used technique in biological research. Most studies which have utilized this technique are in the realm of basic science, often testing hypotheses related to mechanisms of evolutionary change or ecological dynamics. While laboratory natural selection is currently utilized heavily in this setting, there is a significant gap with its usage in applied studies, especially when compared to the other selection experiment methodologies like artificial selection and directed evolution. This is despite avenues of research in the applied sciences which seem well suited to laboratory natural selection. In this commentary we place laboratory natural selection in context with other selection experiments, identify the characteristics which make it well suited for particular kinds of applied research, and briefly cover key examples of the usefulness of selection experiments within applied science. Finally, we identify three promising areas of inquiry for laboratory natural selection in the applied sciences: bioremediation technology, identifying mechanisms of drug resistance, and optimizing biofuel production. Although laboratory natural selection is currently less utilized in applied science when compared to basic research, the method has immense promise in the field moving forward.

1. Introduction

Empirical studies of natural selection in real time are a fairly recent development in biology (Garland and Rose 2009). Charles Darwin co-founded the theory of evolution via natural selection, but generally believed that, apart from selective breeding, evolution was too slow to observe in real time (Darwin 1859). However, in the last half century we have seen a significant increase in studies which characterize evolution in real time, both in the field and the laboratory (Reznick et al 1990,1997; Losos et al. 1997,2004; Lenski 2017). Direct observations of evolution have contributed to the shift in evolutionary biology from a historical science built on observation into a true experimental science wherein hypotheses are regularly directly tested via experimental manipulation. Laboratory natural selection, in particular, has been able to provide novel insights into several key outstanding hypotheses (Moya et al. 1995; Reboud and Bell 1997; Burch and Chao 1999; Ratcliff et al. 2012) and illuminate the mechanisms underlying fundamental evolutionary processes (Dodd 1989; Meyer et al. 2006; Hollis et al. 2009).

Laboratory natural selection fits into a broader category of experiments we refer to as Selection Experiments, which includes all experiments that utilize selection pressure to change populations over time (Fuller et al. 2005; Kawecki et al. 2012). This category consists of three methods: laboratory natural selection (LNS), artificial selection (AS) and directed evolution (DE) (Arnold 1998; Fuller et al. 2005; Kawecki et al. 2012). All three, while having commonalities, differ in specific methodological mechanisms, and those differences are imperative to identifying which questions are appropriate for each method. We define LNS, sometimes used interchangeably with experimental evolution, as the study of evolutionary changes in experimental populations as a consequence of conditions (environmental, demographic, genetic, social, etc.) imposed by the experimenter (Kawecki et al. 2012; Cooper 2018) (Figure 1). Generally, other forces like genetic drift and mutation are not excluded from operating, however LNS methodologies are biased toward testing or observing adaptive traits, and thus specifically impose natural selection (Cooper 2018). It is important to note that while LNS is sometimes called

experimental evolution, we use LNS to specify experiments which are designed to enable selection to act as the dominant, but not sole, force.

AS can be defined as researcher-imposed selection, where only individuals with desired trait specifications are allowed to reproduce (Hill 2001). AS differs from LNS in that the relationship between a given trait and fitness is determined by the experimenters, as opposed to fitness being determined within the context of the experiment like LNS. Importantly, both LNS and AS can be carried out on populations composed of natural standing genetic variation or standing genetic variation infused via mutagenesis. DE incorporates elements of LNS and AS coupled with frequent periods of mutagenesis to drive evolutionary change. Specifically, DE consists of a core three step procedure of mutagenesis, screening/selection, and researcher-imposed choice of contributing progenitor(s) for the next generation (Bloom & Arnold 2009). A key component of DE is the repeated process of mutagenesis and identification of targets (Lutz 2010; Cobb et al. 2013), as creation of target libraries are a means to generate and test many different variant genotypes from a chosen progenitor (Cirino et al. 2003; Muteeb & Sen 2010; Badran & Lui 2015; Hendel & Shoulders 2021). Thus, DE combines the selection process of LNS with the predetermined fitness relationship of AS, but fuels rapid evolutionary change by frequent mutagenesis.

Although elements could be combined across these methodologies, LNS is most unique relative to AS and DE due to the nature of differential reproduction across the three methodologies (Figure 1). Further, the mechanisms of selection employed by AS and DE often impose stronger selection and limit effective populations sizes relative to LNS, which can significantly alter the evolutionary trajectories of populations under each regime. AS and DE both rely on researcher-imposed truncated selection, where researchers ultimately control which individuals contribute to the next generation based on their measurement or score for some desired trait or ability. This process constitutes a form of specific selection, where all the individuals in a population whose trait do not attain a certain value are removed. In short, researchers are selecting on a specific trait towards a specific direction of phenotypic space, enforcing a pre-determined relationship between those traits and fitness. This method is designed to

artificially facilitate recurrent rapid selective sweeps, which can generate immediate responses to selection but will erode standing genetic variation and reduce effective population sizes. Importantly, population-level, between lineage variation, is lost even with the recurring mutagenesis in DE. Mutagenesis within DE is an effective means of exploring numerous allelic variants originating from a single genotype or lineage, but this process is not sufficient to maintain large effective population sizes. We note that while these definitions are useful for a comparison of selection experiments, the margins of these methodologies can blur in particular systems and contexts. However, the usefulness of these definitions lies in their ability serve as basic conceptual cores which illustrate the primary mechanisms behind the evolutionary changes in each experimental method.

Within the context of AS and DE, fitness is essentially a binomial distribution, where organisms either meet the experimenter determined threshold for a specific trait or they do not. Both AS and DE may involve direct experimenter intervention or experiments may rely on automated systems to carryout selection. Regardless of the mechanism, the experimenter determines the relationship between a given trait and an organism's fitness. Conversely, fitness within LNS is much more continuous, and individuals are selected within the experiment based on relative fitness, regardless of which trait(s) confer that fitness. Importantly, this means populations are being selected on based on their ability to reproduce under particular stimuli rather than their absolute value for a given trait, and regardless of which traits are driving increased fitness. The population genetic consequences of LNS can mirror those of DE and AS under very strong selection, however LNS is often conducted under somewhat more biologically relevant parameters that mitigate the strength of selection and the subsequent population genetic consequences relative to DE and AS. Ultimately, the endpoints of both DE and AS are inherently limited by the vision of researchers and their ability to predict an optimal evolutionary path. While DE and AS are likely to be more efficient than LNS in terms of the magnitude of fitness change per generation, at least initially, LNS is comparatively unencumbered and trades efficiency for evolutionary freedom. This tradeoff permits greater opportunities for neutral evolution, increased opportunity for drift and mutation to operate, greater

liberty for populations to explore the adaptive landscape and ultimately, an increased probability of evolving novel adaptive phenotypes relative to AS and DE (Kauffman 1993; Gavrilets 2004; Poelwijk et al. 2007 Bloom & Arnold 2009).

LNS, AS and DE have all been utilized in multiple fields of the natural and physical sciences, but one key discrepancy exists regarding their usage. LNS is comparatively underutilized in the applied sciences. AS and DE have been mostly used for applied purposes since their inception, with examples like animal and crop domestication (Clutton- Brock 1995; Bruford et al. 2003; Zohary et al 2012; Wilkes 2014), virus attenuation (Badgett et al. 2002), and more recent applications like directed enzyme evolution (Arnold 1997). In fact, evolutionary biology as a whole was once viewed as a field with relatively little non-academic usefulness except for crop and animal improvement (Bull and Wichman 2001). Clearly that view has proven to be inaccurate over the last several decades (Crandall et al. 2000; Fuller et al. 2005; Garland and Rose 2009; Davies & Davies 2010; Hawkins et al. 2019; Chen et al. 2021). Nonetheless, some methodologies in evolutionary biology have not yet been fully utilized in applied biology.

In contrast to AS and DE, LNS has primarily been used by researchers in the basic sciences to answer conceptual questions, like the evolution of multicellularity (Ratcliff et al. 2012), or the evolutionary basis of aging (Rose 1984). While some of this underutilization can be attributed to the history and development of each method, there are multiple niches LNS could fill in the applied sciences, which include bioremediation, biofuels production, and the identification of drug targets. We contend that LNS is currently underutilized in applied science studies, and that by focusing predominantly on basic rather than applied questions we are potentially missing insightful results. Here, we illustrate the strengths and weaknesses of LNS and briefly cover a few notable selection experiments. Further, we detail the main advantages LNS has over other selection methodologies with regards to applied science and address three potential future applications.

2. Why is Laboratory Natural Selection Advantageous for Applied Science?

As with any method or technique, LNS has strengths and weaknesses that need to be considered before usage (Boxes 1 and 2). Many of these attributes help determine which systems and questions are suitable for the technique and will provide insight into the value of the methodology for applied research. Several reviews have been written on LNS and evaluated its strengths and weakness, both in general, and in particular systems (Fuller et al. 2005; Buckling et al. 2009; Burke and Rose 2009; Garland and Rose 2009; Dunham 2010; Kawecki et al.2012; Teotónio et al. 2017: McDonald 2019). Those reviews provide greater detail on some of the concepts mentioned in boxes 1 and 2 (Box 1; Box 2).

While many applied problems could be addressed to some extent by AS and DE, LNS is uniquely suited to provide powerful and unique responses. This can be attributed to two key characteristics and consequences of its methodology: the maintenance of greater genetic and phenotypic diversity in populations and exploring the fitness landscape via neutral and near neutral mutations. These characteristics give LNS the opportunity to explore possibilities that are generally inaccessible to other selection experiment methodologies.

Maintenance of Diversity

In both AS and DE traits are evaluated by experimenters and subsequently selected for propagation based on trait measurements. The end goal of this methodology is to constantly drive the trait toward a particular intended value or magnitude. This means that any variation which arises in the population is quickly eliminated unless it meets one of two criteria: 1) A variation that moves the trait or assay value in the desired direction and with sufficient magnitude or 2) A variation that is selectively neutral and stochastically arises in an individual who also has a variation meeting criterion 1. This paring process generally involves exceptionally strong selection and can functionally dispose of the majority of *de novo* neutral or nearly neutral variation, as screening is biased toward mutations of immediate and large effects. While DE does often include mutagenesis to replenish and/or expand upon, a population's

147 standing genetic variation, the de novo mutations only build off a single or small group of genetic
148 backgrounds. DE experiments seldom use methodologies which allow mutants to contribute to the next
149 generation in a manner proportionate to their fitness (Bloom & Arnold 2009). Therefore, variation is
150 highly restricted and many neutral allelic combinations are not maintained from generation to generation.

151 Conversely, LNS is typically designed to impose moderate to strong selection across a gradient of
152 fitness values, which is permissive to any individual that is sufficiently fit to persist or produce offspring
153 that persist. By often imposing relatively weaker and less specific selection relative to DE and AS (yet
154 still stronger than what is encountered in nature), LNS permits the maintenance of larger effective
155 population sizes, and thus greater overall potential for evolutionary change. The maintenance of neutral
156 or non-additive mutations in particular is important, as those mutations may serve as intermediate steps
157 towards complex novel phenotypes which require some degree of historical contingency in the genome
158 (Blount et al. 2018). In the context of the Breeder's equation (Cite Falconer and Mackay), AS and DE can
159 impose large selection differentials, which can generate rapid and substantial responses to selection.
160 However, such intense selection can also significantly reduce genetic variance across the genome within a
161 population and impede subsequent responses to selection by limiting the heritability of traits (cite Lande).

162 **Exploring the Fitness Landscape**

163 Quantitative traits can be viewed on a fitness landscape, which can be heuristically represented on
164 a topographical map. All possible genetic combinations which confer the trait are visualized on the x and
165 Y axes, and the overall fitness conferred by specific genotypes correlates to the height of the peaks, or
166 depths of valleys, on the Z axis (Wright 1932; Svensson and Calsbeek 2012). In the event the starting
167 population is not proximate to the maps global peak, relying on strong specific selection as occurs with
168 AS and DE, can prevent populations from achieving global peak fitness. This is because movement
169 between peaks likely involves mutations beyond those with immediate fitness benefits. For populations
170 traversing the landscape, the relatively weaker selection used in LNS can permit greater exploration than
171 specific selection by allowing mutations which are effectively neutral to persist. Ultimately, greater

exploration of the landscape may result in the evolution of greater mean fitness, as populations have the ability to evolve toward global fitness peaks as opposed to local fitness peaks (Figure 2). Conversely, the stronger and more specific selection in AS and DE can restrict access to evolutionary pathways by selecting for strong immediate effects and purging variation (Bloom & Arnold 2009). Therefore, AS and DE may ultimately limit the universe of possible phenotypes, while LNS provides researchers with much greater leeway to explore genotypic and phenotypic space as means to identify global fitness maxima. When conducting evolution for the purposes of creating a defined product, whether industrial, medical, or agricultural, reaching these maxima can lead to increased efficacy and thus greater utility.

The Importance of Historical Contingencies

A clear example of the importance of neutral intermediate steps and the traversal of the fitness landscape is the evolution of the CIT+ phenotype in the Long-Term Evolution Experiment (LTEE). Briefly, the LTEE is an ongoing experiment consisting of 12 (initially) genetically identical populations of *Escherichia coli* (*E. coli*) which have been passaged thousands of times in the past 30 years (Lenski et al. 1991). It is one of the most well-known long-term experimental studies and has generated a number of insightful findings (Box 3). CIT+ refers to a novel phenotype which is able to metabolically utilize citrate present in the culture medium (Blount et al. 2008). The evolution of the CIT+ phenotype is perhaps the most impactful finding of the LTEE, as it is among the best experimental evidence for the role of historical contingency in evolution.

Historical contingency refers to evolutionary outcomes which are affected by past events that seem to be inconsequential (Blount et al. 2018). Data has shown that the CIT+ utilizing phenotype required multiple intermediate steps that individually conferred little to no fitness benefit. Further, these mutations accumulated over thousands of generations within a single flask population. To date, no other direct routes to CIT+ have evolved in the other *E. coli* populations, and subsequent experiments have shown that frozen populations from the CIT+ flask which have been reanimated from different time points have different propensities for developing the phenotype (Blount et al. 2008). Considering the

various other evolution experiments which have generated parallel mutations in identical populations (Cooper et al. 2014; Lind et al. 2017), this suggests that other mutational paths to CIT+ are highly unlikely to occur and/or extremely complex. LNS allowed populations to traverse neutral space and reach a presumably distant novel phenotype. Had Lenski and his team decided to artificially or directly evolve populations for citrate usage, relying on specific selection, then CIT+ would likely have never evolved. Given that advantageous mutations are generally thought to be rare (Eyre-Walker and Keightley 2007), populations may routinely find themselves separated from novel phenotypes by neutral fitness space. Thus, LNS may generally lead to more novelty by allowing populations to accumulate these, not only moving in fitness space, but also by building contingencies.

3. Past and Current Uses of Laboratory Natural Selection

Before examining how AS and DE have been used in applied science, and projecting where LNS may be advantageous in that realm, it is important to grasp the history of LNS in its own right. Perhaps the first documented LNS took place in the 1870s , when cleric William Dallinger corresponded with Darwin about his ongoing culture experiments with replicate microbe populations and their ability to adapt to warming temperatures (O'Malley 2006). While not LNS, two of the first experiments studying selection in real time were the 1896 Illinois Corn Experiment (Hill and Caballero 1992) and a study by W.F.R. Weldon on selection in the wild in estuarine crabs (Weldon 1901). These were followed by numerous other studies in the early to mid-20th century (Falconer 1992), which all contributed to the study of evolution in real time becoming more mainstream. Experimental testing of evolution is now so commonplace that it can be done in primary school biology laboratories and undergraduate teaching labs with relatively simple equipment (Krist and Showish 2007; Plunkett and Yampolsky 2010; Fonseca et al. 2012). However, LNS did not develop into a legitimate methodological approach as quickly as other selection experiments. The pioneering work of scientists in the latter half of the 20st century helped to legitimize the method and helped it rise in popularity within the field of evolutionary biology (Lynch

1980, 1994; Robertson and Margarida 2004; Rose 2005). In the past three decades in particular, LNS has experienced a dynamic increase in both the number and profile of studies which rely on the technique. With the rise of genetic methods making it less expensive to gather population-level genomic data and the increase in the speed of the process itself, more and more precise evolutionary studies are possible. Prior to these sequencing techniques, it was difficult to pinpoint the underlying genetic changes in adaptation studies. Researchers can now evolve and resequence populations, providing numerous practical advantages when compared to the methods traditionally used in ecology and evolution (Kawecki et al. 2012).

Much of the usefulness of LNS can be seen in its wide applications in the basic biological sciences, as it has been used to study a diverse array of biological questions. LNS has provided insights into many fundamental evolutionary processes that would have been difficult to unveil using other experimental methodologies. For example, researchers were able to evolve several characteristics hypothesized to be required for the emergence of multicellularity while conducting LNS in yeast (Ratcliff et al. 2012). The genetic consequences of colonization and its ability to facilitate evolutionary divergence were famously shown in the *Anolis* lizards of the Caribbean Islands (Losos et al. 1997), and recent studies have been able to link specific mutations to divergence and its genetic consequences in wild mice (Barrett et al. 2019). Researchers have also elucidated the benefits of sexual reproduction using LNS, demonstrating that mutation accumulation in RNA viruses produced Muller's Ratchet dynamics (Chao 1990) and that coevolving parasites favor biparental reproduction over uniparental reproduction (Morran et al. 2011). Indeed, even fundamental mysteries like aging have been evaluated using LNS, as researchers experimentally increased late- life mortality in *Drosophila* (Rose et al. 2002). Finally, LNS has shown that rapid adaptation is plausible in natural populations (Reznick et al.1997). A more comprehensive list of findings can be found in Kawecki et al. 2012. Studies have also sought to evaluate long term evolutionary effects using experiments which have been ongoing for hundreds of generations like the Park Grass experiment (Silvertown et al. 2005,2006), long term *Drosophila* experiments (Archer

et al. 2003; Phelan et al. 20003; Burke et al. 2010), and the previously discussed LTEE (Box 3). More recently LNS has also been implemented via digital organisms using software like the AVIDA system (Lenski et al. 2003; Misevic et al. 2004; Abi Abdallah et al.2020). Undoubtedly as research continues to progress LNS will be employed in more basic science studies in these fields and many others not listed.

4. Past Artificial Selection and Directed Evolution Experiments

We have, thus far, described the historical and contemporary applications of LNS primarily in the basic sciences. Here, we turn toward the history and contemporary usage of AS and DE within the realms of applied science. This serves to contextualize the place of selection methodologies in the broader realm of methods in applied biology and provide illustrative examples of applications for selection experiments.

Artificial selection has been taking place since the beginning of domestication and human transition to agriculture from nomadic lifestyles. The method works by removing natural selection pressures, like response to predation or fecundity, and instead allowing humans to dictate which traits are favored. Unknowingly in some instances and consciously in others, human ancestors began to artificially select organisms based on traits which benefited humans. Early examples include the domestication of crops like grain and maize (Zohary et al 2012; Wilkes 2014), and various species of farm and pet animals (Diamond et al. 2002; Driscoll et al. 2009; Larson and Fuller 2014). Charles Darwin famously used domestication and breeding as major lines of evidence for natural selection in “The Origin of Species" (Darwin 1859), and subsequently conducted artificial selection experiments with pigeons (Secord 1981; Bartley 1992).

AS is the oldest and most well studied of all the selection experiments and has been discussed in depth in numerous reviews (Falconer 1992; Hill and Caballero 1992; Brakefield 2003; Fuller et al. 2005). Many of these highlight the methods key strengths, including ease of implementation and relatively straight forward methodology. At its core, it only requires a sufficiently heritable trait that can be altered

in a population over time by controlling which parents contribute to the next generation. One useful example of AS is the Illinois Long Term Selection Experiment for Grain Protein and Oil Concentration (ILTSE), which is now one of the longest running experiments in any discipline (Moose et al. 2004). The project sought to test whether selective breeding could produce distinct strains of maize, and strains with altered kernel chemical composition (Hopkins 1899**).** Now with over 100 years and 100 generations of experimentation, the experiment has shown the power of long-term selection experiments as tools of both basic and applied biology.

The experiments began with the analysis of 163 ears of corn, which were screened for oil and protein concentration, and divided into 4 groups based on those analyses. Those groups have been subjected to recurrent directional selection every year since World War II (Moose et al. 2004). Populations now span the extremes of kernel chemical composition, with some measuring over 20 standard deviations from the mean in the positive and 4 standard deviations in the negative for kernel oil content. Furthermore, strains also exhibit much higher levels of genetic variation than expected (Dudley and Lambert 2004). The ILTSE has been studied heavily, especially among geneticists, plant breeders and evolutionary biologists. Results have been used to inform quantitative genetics applied to plant breeding strategies and to develop knowledge on some of the physiological determinants of kernel composition (Goldman et al. 1993; Below et al. 2004). The experiment also has had numerous important contributions to the fields of plant breeding and genetics (Jones 1927; Crabb 1947; Alexander et al. 1967; Hymowitz et al. 1974; Moreno – Gonzalez et al. 1975; Dudley 1994; Lambert et al. 2004). Lastly, the project has shown that: 1) AS can lead to trait values beyond the extreme ranges of the initial starting population, and 2) fairly extreme phenotypic variation can be selected for in crops, which both have potential application in studies seeking crop improvement in various characteristics via breeding (Tracy et al. 2004; Floros et al. 2010).

In contrast, DE can be seen as a combination of LNS and AS. Like LNS, researchers manipulate conditions to invoke an evolutionary response, but like AS, they then choose individuals from the favored

sub populations to reproduce based on a desired trait value. In terms of current applications, DE has made headlines in biotechnology via the development of enzymes for industrial and therapeutic uses (Wang et al. 2021), leading to the 2018 Nobel Prize in Chemistry. The method has also been applied to DNA and RNA among other systems (Cobb et al. 2012). Rather than relying on traditional methods to create new molecular products, directed enzyme evolution combines induced recurrent mutation and selection assays to optimize function over time. In regard to creating new proteins, there are two major considerations: the nature of folding and the subsequent function. A functional protein can lose its function or be impaired by mutations that not only target the enzymatic domain, but also other domains that contribute to proper folding. Conversely, novel mutations may improve the function of a protein by altering the manner in which it folds. Rather than relying on projecting which steps will be advantageous, the combination of creating a mutant library and selectively screening has proven to be more effective and less time consuming method for generating beneficial proteins (Romero and Arnold 2009).

The process begins with the replication of DNA sequences that encode a desired functional protein. Many copies of that sequence are created via replication in microbes and from there, sequences are changed via mutagenesis or recombination. Those microbes then read the DNA and make new proteins based on the mutated sequences. The resulting proteins are then assayed for function and researchers choose which proteins will contribute to the next generation from the pool of successful "parents" (Dalby 2011). Through reiterating this process multiple times and starting with proteins which are good candidates to respond to less complicated changes, researchers have been able to use this "random uphill climb" strategy to optimize proteins. Since its inception, the process has led to the creation of new proteins which have applications in an array of industries. Thus far, the largest successes using DE have been improving the binding affinity of antibodies for therapeutic usage and altering substrate specificity of currently used enzymes to make them more effective (Hawkins et al. 1992; Shaikh & Withers 2008; Toscano et al. 2017).

More recently, DE has been used to increase the efficacy of bacteriophages through repeated rounds of mutagenesis and characterization of the mutation patterns which arise (Favor et al. 2020). Researchers utilized an evolution platform called CAVE which uses iterative mutagenesis, physical characterization, and genomic analysis to steer evolution of a desired trait. After 30 rounds of selection with a thermal-selection filter, researchers were able to generate mutant libraries with highly increased thermal tolerance. The conceptual motivations behind the iterative mutagenesis have also been combined with LNS in lab to speed up dynamics in real time. This was accomplished via the use of a mutator strain of *Aeromonas veronii* in a zebrafish host-microbe system to investigate the role of extra-host factors in the evolution of host-microbe interactions (Robinson et al. 2018).

We note that the use of mutagenesis or mutator strains permits DE to explore the fitness landscape associated with a certain trait much more thoroughly than AS, and potentially LNS as well. Mutagenesis libraries, by definition, represent a large proportion of the genetic landscape within a few allelic changes of the progenitor strain. Therefore, DE is a highly effective means to refine or modify a known trait, yet LNS has the potential to be a more effective means to solve applied problems involving complex traits or for discovering novel beneficial traits. Overall, DE and AS have made significant contributions to applied biology. But, LNS can add to the utility of selection experiments in the applied sciences. Going forward, we focus on current areas of scientific inquiry in the applied sciences which are well suited for LNS, either alone or in collaboration with other selection techniques.

5. Potential Applied Niches for Laboratory Natural Selection

The potential of LNS in the applied sciences is vast. As opposed to AS and DE, LNS methodologies are more likely to be successful in creating complex novel phenotypes due to the nature of selection (figure 1). In the context of applied biology, this could allow for the evolution of phenotypes which are inaccessible to other selection methodologies, and thus products which may not have been

generated (figure 2). While LNS may require more replication and likely generations of experimental evolution, it may often be a more comprehensive approach. Recent work has discussed the potential role applied evolutionary biology could play in addressing global issues related to agriculture, industry and the health of humans, animals, and ecosystems (Carroll et al. 2014; Sandberg et al. 2019; Matthews et al. 2020). We assert that LNS can fill a niche alongside other approaches of applied evolutionary biology in addressing these issues. Here, we highlight three potential areas of research where LNS could make tremendous strides in applied biology: identifying mechanisms of drug resistance, biofuels development, and evolving organisms for bioremediation.

Mechanisms of Resistance & Identifying Drug Targets

The misuse and overuse of antibacterial drugs has caused a surge in drug resistant bacteria in the past half century (Read at al. 2011). Indeed, some pathogen strains have become almost entirely resistant to traditional drug treatments which were effective less than two decades ago. Multiple studies have identified drug resistance as one of the biggest challenges of the future (Neu 1992; Taubes 2008; Laxminarayan et al. 2013; Ventola 2015). The rise of drug resistance, while a public health and medical issue, is one created by well understood host – pathogen interactions, which has led to rapid and intense evolutionary change. While changes in how we prescribe antibiotics can slow the rise of resistant bacteria, it is unlikely to rapidly undo years of evolutionary change. Thus, researchers are tasked with creating new drugs to replace or compliment those currently in use. However, efforts to synthesize new drugs must be combined with effective treatment regimens which are capable of stopping the spread of resistance (Bush et al. 2011). One strategy to help contain the emergence of resistance is the use of combinational therapies, where two or more drugs are used together to increase their efficacy (Tyers & Wright 2019). This can involve either a multidrug cocktail (two antibiotics or an antibiotic and adjuvant) or different drugs given in a sequential pattern. Combination therapies are being pursued as treatments for numerous diseases, ranging from cancer (Bozic et al. 2013; Mokhtari et al. 2017) to COVID- 19 (Asakura & Ogawa 2020; Lauriola et al. 2020), and multidrug combinations have been used to limit the spread of

antibacterial resistance with some success (Joshi 2011). Using LNS, we can develop combinational drug treatments which can help turn the tide, or at least buy time, in the war against drug resistant bacteria.

Antibiotic resistance can emerge quickly, arising within the course of one infection in some cases (Su et al. 2003; Mwangi et al. 2007; Lieberman et al. 2011; Eldholm et al. 2014). Resistance can be intensified by antibiotic driven selection pressure on populations of pathogens, causing a rise in frequency among those with mutations which confer fitness advantages in the face of the antibiotic (J Davies & D Davies 2010). Continual exposure to the antibiotic can result in selective sweeps and higher prevalence of resistance, progressively rendering treatments less effective. Combination therapies are thought to be effective because mutations conferring resistance to one drug may not be sufficiently advantageous when evolving in a multidrug environment (Mouton 1999), generally analogous to the cost of resistance (Andersson & Levin 1999). By changing the fitness landscape the populations are traversing researchers can constrain, or even bias, certain evolutionary paths. An example of this is the use of drugs which impart collateral sensitivity, whereby resistance to one drug confers sensitivity to the other (Barbosa et al. 2017). Indeed, combinational therapies have even been explored as a potential avenue to reverse existing resistance by exploiting various interactions between drugs (Baym et al. 2016). Conducting LNS with medically relevant microbes could be an immensely powerful tool in the creation of combination therapy protocols, as accurately mimicking clinical conditions may result in similar evolutionary outcomes. This would allow LNS to have more predictive power in comparison to other selection methodologies. Further, identifying these mechanisms via LNS can help better inform which combinations of drugs should be prescribed for the given mutations present in a patient.

To identify the specific mutations which confer resistance, microbes are repeatedly exposed to an antibiotic, and surviving individuals are sequenced and/or assayed after each round. Experiments using this, or similar methodologies, to identify resistance genes or constrain their emergence, have become more commonplace as genetic tools have become more available and higher throughput (Kim et al. 2014; Fuentes- Hernandez et al. 2015; Yen & Papin 2017; Santos- Lopez et al. 2019; Hansen et al. 2020). One

example of note is the use of a bioreactor and repeated antibiotic exposure to identify resistance conferring mutations in *Pseudomonas aeruginosa* (Mehta et al. 2018), an opportunistic pathogen with resistance to multiple antibiotics, which the CDC has identified as a serious threat (CDC 2014; Murray 2015). Researchers developed an experimental bioreactor meant to mimic the environment of clinical adaptation, and progressively exposed susceptible *P. aeruginosa* populations to colistin, a last resort drug for gram-negative bacterial infections (Tamma et al. 2012). This adds to a body of work which has attempted to replicate bacterial habitats more accurately and conduct LNS outside of the traditional serial flask transfer method (Zhang et al. 2011; Toprak et al. 2012; Baym et al. 2016). Highly favorable bioreactor conditions were maintained to avoid unwanted adaptive mutations and samples of the entire population were collected everyday (26 days) for deep metagenomic sequencing.

Using this methodology, researchers were able to generate resistance mutations similar to those found in clinical settings, including a hypermutator phenotype (Hammerstrom et al. 2015), and trace multiple adaptive trajectories within the population. The ability of LNS to explore the fitness space of resistance more fully is a clear advantage and one that is not possible with other selection methods. Furthermore, experiments have shown that environments can impact the topography of fitness landscapes (Flynn et al. 2013; Ogbunugafor et al. 2016; Li & Zhang 2018), which support studies showing different evolutionary dynamics under different treatment parameters during antimicrobial evolution (Palmer & Kishony 2013; Baym et al. 2016; Maltas et al.2019). Laboratory experiments like the previously mentioned resistance study (Mehta et al. 2018), may thus be more likely to achieve resistant phenotypes similar to those seen in patients, as they can simulate these conditions. Following this rationale, LNS can also be conducted *in* vivo, as has been done with malaria in mouse models (Mackinnon & Read 2004; Barclay et al. 2011; Acosta et al. 2020). Being able to determine precise evolutionary trajectories and molecular basis of resistance shows the potential advantage of LNS in this realm of research.

Biofuels Optimization

The past few decades we have seen substantial growth in sustainable fuel research. Much of this growth has been driven by two main lines of thinking. One is that fossil fuels are finite and our exploitation of them inevitably will end at some point (Höök & Tang 2013). Secondly, the large increase in greenhouse gasses driven by fossil fuels has caused large scale environmental damage including climate change and ocean acidification (Doney et al. 2009; Pachauri et al. 2014). In the search for reliable sustainable fuel sources, some have proposed the use of carbon neutral biofuels to curb our petroleum appetite in the short term, and potentially replace it totally in the long term. Biofuels are liquids, gas or solid fuels predominantly produced from biomass, which is material originating from plants or animals that is not used for food (Demirbas 2008). Biofuel production utilizes enzymes to break down plant material and convert it into fuel. Commonly, starches in the plant are converted to glucose and then fermented to make ethanol. One difficult portion of the process is that in many crops like corn, glucose is difficult to separate from fibers in the plant due to their resilient nature (Schubert 2006). Separating out the sugar requires treatment with acids or other potentially environmentally harmful processes. Another issue is that in many countries, farmland is limited and crops which could be used for food are instead used to generate fuel. Further, many biofuel products are less energy dense than petroleum and current infrastructure is not built for these fuels (Arnold 2008). Circumventing these difficulties may require growing plants with more easily assessable glucose stores, utilizing more efficient industrial processes, and growing plants dedicated to fuel production.

LNS has the potential to improve the biofuel industry by addressing and improving upon many of these ideas and working in collaboration with other applied strategies like genetic engineering (Snow and Smith 2012). Growing crops with more easily assessable glucose stores (i.e. weaker cellulose structures) could be accomplished via induced mutations and subsequent selection, or by exerting selection pressure to encourage directional selection for increased glucose. Crops with richer stores would reduce the overall area of farmland needed to produce the same yield of fuel, assuming they do not require substantially more resources. Alternatively, LNS could be used to create microorganisms with

more efficient enzymatic processes which may increase overall yields when converting plant matter. Similar to the evolution of a citrate utilizing phenotype in the Long-Term Evolution Experiment, providing a niche within the experiment which favors bacteria with more efficient processing mechanisms may create opportunity for evolutionary change. Both marine and terrestrial plant resources can be subjected to these methodologies (Gaurav et al. 2017). Moving away from more traditional plant fibers and glucose, terpenes, aromatic compounds typically found in plant resin, have been put forward as a genetically manipulatable and readily available alternative (Mewalal et al. 2017). Another possibility for biofuel production would be utilizing fuel sources from genetically engineered microorganisms.

One fuel of note is butanol, which was proliferating prior to the rise of petroleum in the mid 1900's and has received attention again more recently (Rathour et al. 2018; Xue et al. 2019). The production process relies on bacteria to create butanol via metabolic processes, but current output efficiency is not practical for wide scale industrial use. Using LNS, researchers can breed microbes with higher efficiencies in butanol production. Traditionally, butanol fermentation has been studied in *Clostridium* strains, as was first reported by Louis Pasture. However, *Clostridia* have been difficult to engineer and thus a host of other bacteria have been used as surrogates to produce the alcohol (Zhao et al. 2020). While the LNS process may not be the most straight forward method to circumvent these engineering difficulties, it may have greater ability to generate rare genotypes which are inaccessible to other selection methodologies.

Overall, LNS is uniquely suited to address several key issues in biofuel optimization. LNS can widen the scale of possible phenotypes that can be created and alleviate the need to accurately predict specific enzymatic changes to optimize alternative fuels. However, LNS may also be less efficient and more time consuming than AS or DE when optimizing existing phenotypes. Therefore, LNS could be specifically utilized when AS and DE are unable to drive significant progress. In summation, a synergistic research strategy combining all three selection methods may be the most fruitful path forward for biofuel production and usage.

Bioremediation

Bioremediation is the process of using microorganisms to destroy, or reduce the concentration of, hazardous waste (Boopathy 2000). The technique is often used to aid in processes like wastewater, solid waste, and heavy metals removal (Garima & Singh 2014; Ojuederie & Babalola 2017). The idea of using bioremediation to deal with dangerous and/or functionally non-degradable materials is not new, and in some ways, nature has already begun the process through species adaptation to human activities (Sih et al. 2011). For instance, the widespread evolution amongst microbes of the ability to degrade atrazine, a popular herbicide used since the 1950s (Udiković-Kolić et al. 2012). It is not hard to envision scientists breeding organisms to utilize waste products and purge them from the environment. An example which clearly shows the potential for this is the development of nylonase by Flavobacteria. Nylon, a synthetic polymer, was first invented in the 1930s and quickly became widely used around the world. By 1975 scientist had discovered a strain of Flaviobacterium, dwelling in ponds which contained wastewater from a nylon factory in Japan, that could digest nylon products (Kinoshita et al. 1977). Since then, scientists have been able to get other bacterial species to evolve similar capabilities by forcing them to live in resource depleted environments which are rich in nylon (Prijamnada et al. 1995), and by plasmid transfer into *E. coli* (Negoro et al. 1983). More recently, researchers have discovered bacteria which are capable of digesting the common plastic polymer Polyethylene terephthalate, otherwise known as PET (Yoshida et al.2016). As a commonly used plastic, PET is a major source of pollution (Geyer et al. 2017), especially in marine environments (Worm et al. 2017). Researchers are now working with the bacterium and recent studies have shown promising results in characterizing and improving the enzymes responsible for digesting PET (Austin et al. 2018).

The evolution of nylonase by microbes just some 50 years after the development of Nylon has become a textbook example of the power of evolution and the immense potential it has for bioremediation. More recent successes like the aforementioned PET digesting bacterium *Ideonella sakaiensis 201-F6* (Yoshida et.al 2016), continue to show that potential. Bacteria, fungi, and plants are

493 now being researched for their bioremediation potential. In regard to microbes, current efforts to improve
494 bioremediation potential mostly center around molecular techniques like protein and metabolism
495 engineering using both genomics and proteomics (Wood 2008; Shukla et al. 2010; Chovanec et al. 2011).
496 These include the use of transgenic plant species which synthesize enzymes originally found, or
497 enhanced, within bacteria (Peng et al. 2014). Other research approaches via Systems biology or molecular
498 engineering have potential to improve our bioremediation capacities (Dangi et al. 2019) however, LNS
499 should join these techniques on the cutting edge of bioremediation research. Just as researchers were able
500 to use LNS to produce nylon digesting bacteria (Prijamnada et al. 1995), similar techniques could be used
501 to create organisms to degrade plastics and other long-lasting materials. As previously mentioned in the
502 biofuel section, evolving enzymes in their host organism can avoid issues typically caused by using
503 surrogate bacteria in directed evolution. Here, LNS could serve to co-adapt the genome to the engineered
504 products.

505 One of the biggest challenges with bioremediation techniques so far has been the lack of
506 biodegradability for many common products including plastics, oil, and metals (Juwarkar 2010). Rather
507 than waiting for researchers to determine the most efficient way to break down these materials, LNS gives
508 the opportunity for natural selection to engineer chemical processes to deal with these pollutants. This
509 could buy substantial time in preventing environmental damage and wildlife loss. Ultimately, an
510 integrative approach combining the three selection experiment methodologies could be most helpful to
511 bioremediation. Similar to biofuels, in bioremediation LNS would be most helpful with the initial creation
512 of novel phenotypes and optimization, as well as creating a diversity of viable phenotypes. However, once
513 these are created the strong selection imposed by the other selection experiments may be more
514 advantageous. A synergistic approach would ideally use laboratory natural selection to create the needed
515 enzymatic processes, then directed or artificial evolution to further optimize the reactions. Researchers
516 could continue to use LNS to build neutral mutation load and explore genotypic space to find alternative
517 fitness peaks, however this would be purely exploratory unless combined with rational design studies.

Nonetheless, LNS would be vital as the experimental method best suited to create the initial microbial phenotypes for bioremediation.

6. The Future of Laboratory Natural Selection in Applied Studies

Evolution is a powerful process which has shaped the biological world over the course of natural history. Utilizing the power of evolutionary processes for human application is not a new idea. Many organisms we rely on have been altered over the course of time by applied evolution. Selection experiments, which encompass artificial selection, directed evolution, and laboratory natural selection, are all techniques which harness the power of evolution, however, of the three LNS is comparatively underutilized in the applied sciences. This is a missed opportunity, as LNS has the potential to be a useful tool in applied biological science. Unlike AS and DE, which impose strong selection and can create lower effective populations sizes, LNS allows for less stringent selection and thus the maintenance of greater genetic variation over time. By allowing mutations with neutral or small effect sizes to persist and accumulate in the population, LNS can increase the odds of novel phenotypes arising which require multiple negligible fitness conferring mutations. This allows LNS to explore more of the fitness landscape. Functionally, this means that laboratory natural selection can remove the barrier of researcher knowledge or foresight which is needed in biological design studies.

Laboratory natural selection can provide solutions to some of the most important issues in the applied sciences today. Applying LNS strategies to the identification of drug resistance mechanisms, and the development of microbes for biofuels and bioremediation has the potential to yield fruitful results which could greatly impact society. Particularly because these are direct ways of mediating some of the most looming issues we will face in the future: diminishing fossil fuel resources and climate change, emerging antibiotic resistance, and dealing with anthropogenic pollution. Ultimately, AS, DE, and LNS approaches should all be utilized harmoniously, and sometimes collaboratively, to address the broad

issues we face as a society. Generally, not all systems will be well suited for the LNS methods analyzed in this review, and their usage should be carefully analyzed and considered prior to being undertaken. However, the wide variety of systems and conceptual issues LNS has been applied to is indicative of its power as a methodology and the immense potentially it may hold. Just as LNS has been advantageous to researchers in the basic sciences, it can join other selection methods in the forefront of applied studies.

Data Archiving

There are no data associated with this article.

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565 **References**

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Figure & Box Legends

Box 1. Strengths of Laboratory Natural Selection

Figure 1. Diagrams the methodological differences between artificial selection (AS), laboratory natural selection (LNS) and directed evolution (DE). Large circles represent populations, and red triangles represent selection pressure. Blue represents the portion of organisms in the population which are favored based on selection pressure, while green represents those selected by the researcher. In AS the researchers determine which portion of organisms reproduce. In LNS, researchers place selection pressure on populations and the most fit reproduce. In DE, selection pressure manifests through function assays, creating a favored population (blue) and researchers determine which organisms from the favored pool will reproduce (green). Each of these methods can involve mutagenesis to infuse additional genetic variation, but this is most common in DE and LNS.

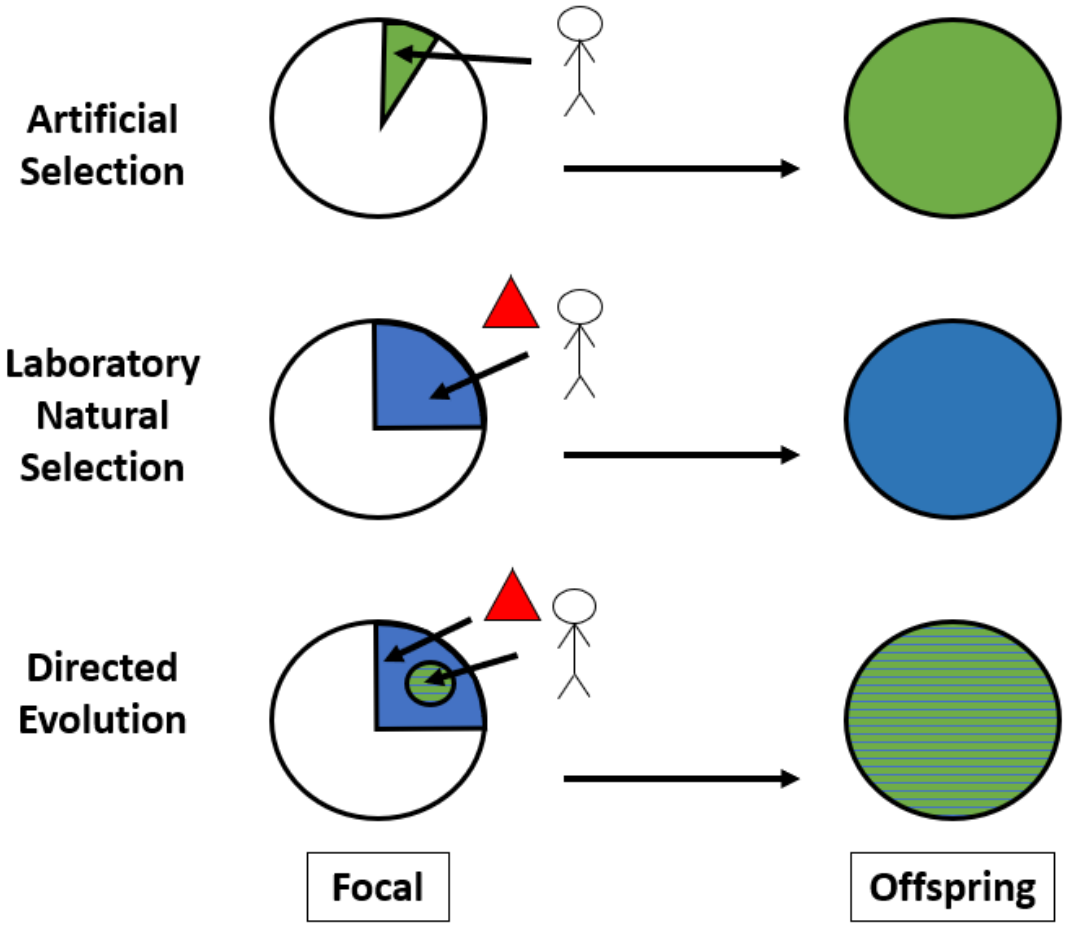
Box 2. Weaknesses of Laboratory Natural Selection

Box 3. Brief recap of the Long-Term Evolution Experiment (LTEE) with methods and main findings

Figure 2. Shows evolutionary trajectories for each of the 3 evolution experiment methodologies. X's represent evolutionary dead ends imposed by the experimenter, while numbers represent generations passed under the respective protocol. In AS and DE (B) existing traits (point 1) are directionally selected to climb that traits fitness peak. With AS using controlled breeding strategies from *de novo* or existing variation and DE using artificially manipulated variation. In both cases the trait being selected for climbs the peak it currently rests on and cannot navigate up the global peak unless it starts there. In LNS (A),

traits can move from local peaks to the global peak via mutation and/or drift, as long as those intermediates are not at a significant fitness disadvantage while traversing the area between peaks. In LNS all dead ends are due to fitness disadvantage, not experimenter-imposed selection. Figure made using **MATLAB 8.0 and Statistics Toolbox 8.1, The MathWorks, Inc., Natick, Massachusetts, United States.**

Figure 1.


Artificial Selection
Laboratory Natural Selection
Directed Evolution
Focal
Offspring

Box 1.

Experimental Control of Isolating Variables. In LNS researchers typically isolate variables, which allows single factors to be evaluated individually, or in combinations with other factors systematically. By "simplifying" the system, LNS practitioners are able to understand the importance of individual aspects to the dynamics of a whole system, and test for synergistic or antagonistic effects in multivariate experiments. When a product is the end goal, isolating variables can allow for efficient directional selection where populations adapt in response to specific pressures.

Population Size & Replicates. Stochasticity is inherent to evolution. Many processes which drive evolution, like genetic drift and gene flow, have effects that scale inversely with population size (Gillespie 2004). The potential effects of these stochastic processes on small populations, when compared to larger populations, can dramatically alter the evolutionary trajectory of those populations over time. Such stochastic events have the power to alter experimental data, and the potential to misrepresent evolutionary mechanisms when comparing them to natural populations (particularly large natural populations). LNS can control for these issues, to an extent, by utilizing model systems with very large population sizes and minimizing the role of stochastic forces in the experiment via randomization. Additionally, researchers can manipulate the impact stochastic processes can have on the population by directly manipulating population size and testing the robustness of results across population sizes.

Fossil Records. Natural fossils can give important information about historical distributions of organisms and morphology changes over time. However, data from fossils is limited due to incomplete records and the inability to determine details related to molecular, behavioral and social processes. In many systems, LNS practitioners can create detailed fossil records via cryogenic preservation of ancestral populations. These techniques are common with multiple systems including *Caenorhabditis* nematodes (Stierngale 1999; Teotónio et al. 2017), *Escherichia coli* (*E. coli*) (Lenski et al. 1991; Elena and Lenski 2003; Blount et al. 2018), various species of yeast (Zeyl 2006; Dunham 2010), and multiple insect species (Leopold & Rinehart 2010; Štětina et al. 2018), including limited success with *Drosophila* (Steponkus et al. 1990; Mazur et al. 1992; Koštál et al. 2016). Populations can be thawed and used for comparative assays and can be "replayed", giving context for odds of certain events and the role of contingencies. This method was used in the LTEE to determine the role of contingencies in CIT+ evolution (Blount et al.2008) (Box 3).

Detailed Genomics. Rather than using techniques post hoc to determine genetic changes that happen in a population, via LNS researchers can completely sequence focal, intermediate, and ending populations (Wichman et al. 2000; Elena and Lenski 2003; Kacar et al. 2017;). This allows detailed information on gene frequency changes and, when combined with fossil records, can uncover the exact sequence of mutations or gene changes that lead to novel or improved phenotypes over the course of the experiment. This can be especially advantageous when attempting to determine the levels and impact of epistasis and hitchhiking, or non-additive mutation buildup over time.

Well characterized Model Systems. Many of the model systems used in LNS are widely used across biology and are well characterized. Many techniques have already been developed in other disciplines and are freely available. This makes voyaging into a new system more assessable for those new to LNS or LNS veterans who wish to utilize new systems. Many are classical systems like *Drosophila* or *E. coli*, which are used in a wide variety of fields and support extensive networks of researchers. For example, the nematode *C. elegans* is used in many areas of the biological inquiry and are well characterized in many respects including genomic data, behavioral and social interactions, lab methods and have diverse strains available (Stiernaagle 1999; Teotónio et al. 2017).

Box 2.

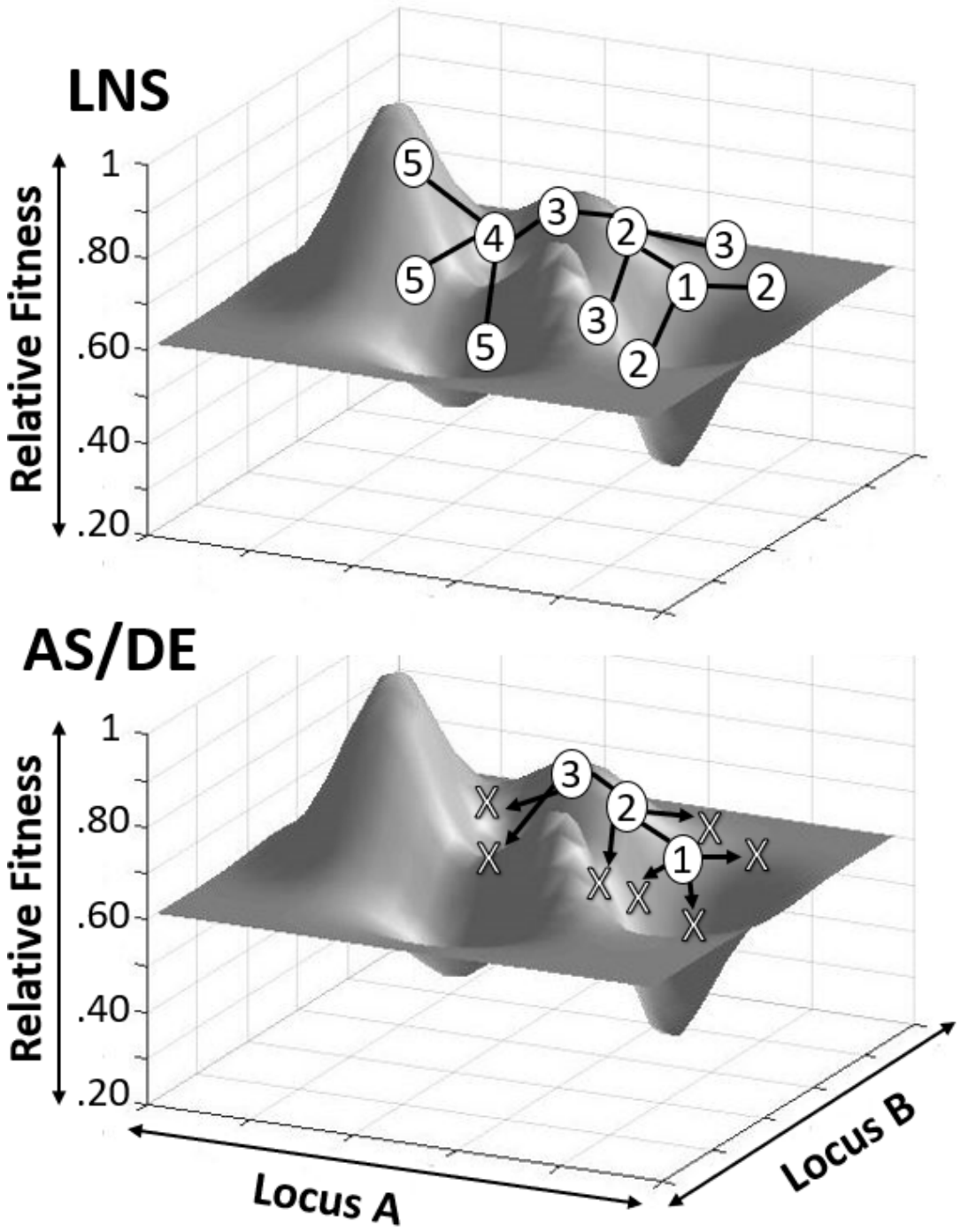
Isolating Variables & Population Size. In many studies, researchers attempt to uncover the processes that govern biological mechanisms or systems. Thus, it can be important that researchers consider all factors affecting a system and how those factors can interact in synchronization. Most experimental environments are typically not equivalent to a microcosm of true conditions, meaning researchers could miss key factors. This is especially true in when researchers are unsure which dynamics are truly influential. Likewise, while researchers can attempt to minimize stochasticity by using large population sizes, for some organisms, experimental population sizes can never approach those seen in nature. This can alter effects of drift and response of selection, making it more difficult to illuminate natural dynamics. This was seen in the case of DDT resistance in *Drosophilla* where lab studies supported polygenic effects, but wild resistance was linked to genes of large effects (Schmidt et al. 2017).

Time Scale. Generation time, defined as the average time between generations in a population or the average age of reproduction in individuals, is extremely important in evolutionary biology. Heritable traits being disproportionally transferred to offspring is the heart of evolution by natural selection, and generation time can determine how quickly changes take place. While evolution can be observed in real time over relatively short timescales, many processes may need longer time to operate. This is especially true when considering the amount of time hypothesized to accompany processes like speciation and macroevolution. Even comparatively small changes in populations, like the creation of novel genotypes, can take thousands of generations to produce (Rozen and Lenski 2000). When comparing this amount of time with the average length of many research studies, it is easy to see the unattractiveness of experimental evolution in many contexts. The constraints of time can also lead to bias in which organisms are suitable to study certain phenomena. Generation time tends to bias studies toward those with shorter average lifespans and generation times like bacteria, phages viruses and fungi. While these may yield more data, it may be more difficult to extrapolate results to more complex organisms. Further, the operating costs of certain systems may make long-term studies prohibitive.

Role of Fortuity. Mutations provide the mechanism through which raw genetic material is altered for natural selection to create disparities in fitness. While mutation rates may increase or decrease between different portions of genomes, there is inherent stochasticity in which loci incur mutations and the nature of those mutations. This is especially true with respect to multiple mutations in a single individual. Due to the nature of the mutational process and the potential for interaction between mutations, via epistasis and non-additive effects, the appearance of novel genotypes may rely on extremely rare sequences of events. Importantly, these effects are more likely to impact asexual species due to clonal interference (Muller 1964; Gerrish and Lenski 1998)

Adaptation, Isolating Selection Pressure & Lab Conditions. Many of the organisms utilized in LNS have been bred in lab for generations and may have adapted to laboratory conditions (Kawecki et al. 2012). Lab induced pressures like social or sexual behavior changes due to confined spaces or numerous other lab conditions can impact results significantly and must be considered (Kawecki et al. 2012). Examples include density dependent mating increase in *Drosophila* (Williams et al.2004) and infection multiplicity from contamination (Ebert and Mangin 1997). This can be problematic when applying lab results to the field. Finally, testing fitness in experiments is typically difficult as fitness is multifaceted and hard to generalize and measure (Rausher 1992; Orr 2009; Wagner 2010).

Figure 2.


LNS
Relative Fitness
1
.80
.60
.40
.20
5
4
3
2
3
5
1
2
5
3
2
AS/DE
Relative Fitness
1
.80
.60
.40
.20
3
2
1
Locus A
Locus B

Box 3.

The Long-Term Evolution Experiment, otherwise known as the LTEE, is a long term experimental evolution project which has been mainly overseen by evolutionary biologist Richard Lenski at Michigan State University. The experiment consists of 12 genetically identical populations of *Escherichia coli* (*E. coli*) (Lenski et al. 1991), which have been passaged thousands of times in the past 30 years. In that time researchers have been able to observe and record data on evolution over the course of over 60,000 generations, along with a detailed and expansive fossil record (Good et al. 2017). Creating over 65 peer reviewed articles, the LTEE has allowed researchers to study multiple elements of evolutionary theory and dynamics. Arguably, the most important thing accomplished by the LTEE has been showing the feasibility and usefulness of experimental evolution (Fox and Lenski 2015). The LTEE has been covered extensively in both academia and general science media, therefore we only provide a brief recap of the methodologies and the main findings of the experiment.

Methodologies. The experiment was started with 12 genetically identical populations of *E. coli*, each in individual containers. Populations only reproduce asexually, have no known plasmids, and no viable prophage, all important details to ensure any changes are due to mutation, drift, and natural selection. Every day (22-26 hours) 1% of each population is propagated via transfer using standard techniques. Every 500 generations (~ 75 days) population samples are frozen in a -80°C freezer for fossil record. Fitness assays are done on evolved populations every 500 generations and compared to ancestral populations (Lenski et al. 1991). This also allows populations to be "re-animated" from previous time points for further experimentation.

Main Findings. Over the course of the experiment all populations showed fitness increases compared to the ancestral strains, with rapid increases taking place within the first 20,000 generations (Lenski 2004). Half of the populations have evolved DNA repair defects which have increased the rate of mutation in those populations (Sniegowski et al. 1997). However, even with this increased mutation rate, Lenski estimates that only 10-20 of the millions of mutations occurring over the first 20,000 generations reached fixation in the population (Lenski 2003). All populations have evolved increased cell sizes which are associated with expression of a gene that is advantageous under the conditions of the LTEE (Philippe et al.2009). All populations have experienced a degree of specialization to the glucose medium in the experiment and now have reduced ability to grow on alternative sources compared to the ancestral strains. Population 2 evolved two distinct variants identifiable through colony morphologies, each with an advantage in varying stages of experiment transfers and co-exist with each other (Rozen and Lenski 2000). Around generation 33,000 population 3 evolved the ability to utilize citrate present in the medium (CIT+), drastically increasing the growth rate of the population. Upon further experimentation, mutants were found as early as generation 31,500. Using the fossil record, experimenters "replayed" evolution and found that cells isolated from after generation 20,000 have a much higher chance of evolving the CIT+ phenotype (Blount et al. 2008). This is considered among the strongest experimental evidence for the importance of historical contingencies in evolutionary history (Blount et al. 2018).