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LABORATORY EVOLUTION MEETS CATCH-22:
Balancing Simplicity and Realism

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Everything should be made as simple as possible, but not simpler.

ATTRIBUTED TO A. EINSTEIN

USING LNS TO TEST HYPOTHESES DERIVED FROM COMPARATIVE STUDIES

PROBLEMS ASSOCIATED WITH LABORATORY ADAPTATION

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Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments, edited by Theodore Garland, Jr., and Michael R. Rose.

This book lays out a clear and compelling message: selection experiments are remarkably powerful tools in the armamentarium of evolutionary biologists. We ourselves have often used selection experiments during our careers and certainly expect to use them in the future. In fact, the power and elegance of selection experiments applied to life-history evolution by Rose and colleagues (Rose and Charlesworth 1980; Service 1987) motivated one of us (R.B.H.) to switch from conducting descriptive evolutionary studies on lizards in the field to performing evolution experiments on *Drosophila* in the laboratory.

In this chapter, we look critically at a particular type of experimental evolution, often called laboratory natural selection. In this protocol, stocks of organisms are reared chronically under different conditions (e.g., different thermal or life-history regimes) and allowed to evolve by natural selection over many generations (Rose et al. 1987; Garland 2003). At intervals, phenotypes of population members can be compared in a "common garden" (i.e., reared under identical environmental conditions; Garland and Adolph 1991). Differences between selected and control lines—at least if observed consistently among replicates—represent either direct or indirect responses to the selective regime. This is an old and venerable type of experimental evolution (Dallinger 1887; see box).

Laboratory natural selection (LNS) is distinct from two other types of laboratory evolution (Rose et al. 1996, 1987; Garland 2003; Swallow and Garland 2005; Futuyma and Bennett this volume). In artificial selection, the experimenter actively measures and selects phenotypes to found the next generation. In laboratory culling, organisms are exposed to a lethal condition (e.g., no food, no water), and the longest-surviving individuals are used to found the next generation.

LNS experiments can provide insight into genetic architecture and correlations underlying traits of interest (Rose et al. 1990). They can also be used to evaluate the rate, tempo, and repeatability of evolutionary trajectories (Lenski and Travisano 1994; Ferea et al. 1999; Dunham et al. 2002; Cooper et al. 2003, 2001; Fong et al. 2005; Woods et al. 2006) and to assess how historical contingency (Travisano et al. 1995), sex (Grimberg and Zeyl 2005; Zeyl et al. 2005), sexual selection (Rundle et al. 2006), ploidy (Paquin and Adams 1983; Zeyl and Bell 1997; Zeyl et al. 2003), and life history (Zeyl et al. 2005) influence the outcome of those processes. LNS experiments are especially useful for testing functional hypotheses (Rose and Charlesworth 1980; Bennett and Lenski 1999; Gibbs 1999), as derived lines yield experimental subjects that have "exaggerated" or "novel" phenotypes (Gibbs 1999; Bennett 2003; Garland 2003; Futuyma and Bennett this volume).

LNS experiments are not only broadly applicable but also logistically advantageous (Rose et al. 1987; Futuyma and Bennett this volume). Effective population size can be manipulated over orders of magnitude, especially in microbes, largely eliminating genetic drift, if so desired. Experimental lines can readily be (should be!) replicated (Futuyma and Bennett this volume), and the intensity, frequency, uniformity, and duration of selection can carefully controlled. Selective agents of interest can be applied either singly or in concert. Moreover, selection is accomplished without direct intervention:

Before the experiment was ended by an accident, the infusoria were able to tolerate 158°F! Dallinger noted (p. 199) that nonadapted individuals "are killed at 140°Fahr. But if the adapted organisms at 158°F were taken from that temperature and placed in . . . fluid at even 150°F they were finally destroyed."

Though a pedant could quibble that Dallinger failed to maintain a control line or to control for acclimation and cross-generation effects, all must recognize the pioneering brilliance of his work. Here for the first time was experimental evolution in action!

Interestingly, Dallinger corresponded with Darwin about the preliminary results from his selection results. Dallinger's article (1887, 191) contains an insightful evaluation from Darwin:

> I did not know that you were attending to the mutation of the lower organisms under changed conditions of life; and your results, I have no doubt, will be extremely curious and valuable. The fact which you mention about their being adapted to certain temperatures, but becoming gradually accustomed to much higher ones, is very remarkable. It explains the existence of algae in hot springs.

genotypes that do relatively well in a particular environment simply leave the most surviving offspring (Rose et al. 1987). In contrast, experimenters using artificial selection (AS), especially if family selection is involved (Scheiner and Lyman 1991), must tediously measure and select individuals each generation; the associated logistics can be daunting and may thus restrict sample sizes and increase the likehihood of drift.

Despite these conceptual and logistic advantages, LNS experiments have inherent problems and limitations. Many have been previously identified (Huey et al. 1991; Rose et al. 1996; Bennett and Lenski 1999; Gibbs 1999; Harshman and Hoffmann 2000; Prasad and Joshi 2003) but are nonetheless worth reiterating. Some are potentially so severe that they can compromise or confound evolutionary and functional interpretations. We describe these problems here and, where feasible, suggest ways to try to circumvent them.

Because LNS experiments have both strengths and weaknesses, researchers contemplating an LNS experiment face the classic catch-22 (or double-bind) situation immortalized in Joseph Heller's (1961) novel of the same name. They may well decide that LNS is the best way to test a given evolutionary hypothesis (Rose et al. 1996:236–238), but simultaneously they must accept the hard fact—and accept it in advance—that some of the inferences they draw from their LNS experiment may be of uncertain validity. Welcome to catch-22, where one is caught "between a rock and a hard place" (Harshman and Hoffmann 2000).

Researchers have several options when faced with such a bind. Yossarians of the world (Yossarian is the protagonist in *Catch-22*) would probably try to solve the problem by switching fields, hoping that they can publish many articles before they (or worse, before others) discover that their new field has its own catch-22s! Or, they can accept the situation

but try to turn it to their advantage (Rose et al. 1996). With a bit of creativity, one can circumvent certain problems inherent in LNS or even use some of them as opportunities for interesting new studies. But despite one's best efforts, some issues are simply likely to remain bedeviling catch-22s. Nevertheless, as Rose et al. (1996:239) note, "from our encounter with this often confusing and unfair world, we can learn about our theories and improve them."

USING LNS TO TEST HYPOTHESES DERIVED FROM COMPARATIVE STUDIES

LNS experiments are often used to test evolutionary hypotheses that have been derived from theory (Futuyma and Bennett this volume) or from observations in nature. We focus here on LNS experiments that are designed specifically to test hypotheses derived from comparative studies in nature. Of course, many issues discussed here will apply to LNS experiments testing theoretical hypotheses. We begin by describing how an LNS study might evolve from a comparative study in nature, and then illustrate some general difficulties.

Many traits show geographic clines. Once a cline is documented, two questions naturally arise (Endler 1977). Did this cline evolve by natural selection? And, if so, what selective factor(s) led to the cline?

Latitudinal clines in the frequency of various chromosomal inversions are well documented in the fly *Drosophila subobscura* (Krimbas 1993). Because these clines are generally in the same direction on three continents (discussed later), they likely evolved by natural selection (Prevosti et al. 1989). But what selective factor is responsible for the clines? Temperature is a reasonable guess: perhaps inversions that are relatively common at low latitudes contain alleles that are adapted to heat, whereas inversions common in high latitude have alleles adapted to cold. Of course, many other abiotic and biotic factors co-vary with latitude (photoperiod, intensity of competition and predation), but temperature is a reasonable first guess for an ectotherm.

To test the role of temperature as a selective agent, one might initially search for "natural experiments" occurring in the field (Endler 1986; Diamond 2001). Climate warming provides just such an opportunity. For decades, evolutionary geneticists have been scoring inversion frequencies of *D. subobscura* at many sites where climate is warming. If inversion clines are driven by temperature, then inversion frequencies at particular sites should shift as climate warms. Specifically, inversions common at low-latitude sites (i.e., presumably warm-adapted genotypes) should increase in frequency as climates warm. This is the case (Rodríguez-Trelles et al. 1998; Solé et al. 2002; Balanyá et al. 2006; see also Levitan and Etges 2005; Umina et al. 2005), consistent with our hypothesis. Nevertheless, because these patterns are still only correlational, we cannot be certain that temperature was indeed the selective factor responsible for the observed clines.

To challenge our hypothesis further, we might devise an experiment in which we manipulate temperature and then evaluate whether inversion frequencies change in a direction consistent with our comparative hypothesis. Inducing climate warming in the field might prove technically and politically challenging, so a more practical next step would be to induce it in the laboratory. A classical LNS approach would be to start with a large outbred population, set up replicated lines in population cages maintained across generations at different (fixed) temperatures (e.g., 12°, 18°, 22°C), and then at intervals monitor inversion frequencies in the different temperature treatments. Thus, we would use the 22°C constant-temperature treatment as a laboratory proxy of relatively low-latitude environments and 12°C one as a proxy of high-latitude environments. If our temperature hypothesis is correct, then inversions common at low latitudes should increase in frequency in the warm temperature treatment but decrease in frequency in the low temperature one. This experiment has been done (Santos et al. 2004, 2005, 2006), and we will return to it near the end of this chapter.

This LNS experiment seems like a logical and appealing test of our hypothesis, but its validity rests on at least two key assumptions. First, the direct agent(s) of selection in the laboratory must mimic—at least approximately—those in nature. For example, the experiment described here assumes that chronic exposure to high temperature in the laboratory approximates the selective impact of living in warm latitudes in nature. A priori this seems highly improbable. Low latitudes differ from high ones in many ways (Bradshaw and Holzapfel 2006), not just in temperature, and warm terrestrial environments (at least in temperate zones) are never chronically warm. Second, the genotypic variance of the large outbred (experimental) population should be roughly comparable to the combined variance of all natural populations along the cline. This ensures that our lab founder population has sufficient genetic potential for selection to realize phenotypic variance in the trait of interest.

Despite having concerns that these assumptions might be violated, our intrepid experimenters start the selection experiment. Let's imagine how they will interpret their eventual results. If they find that inversion frequencies shift as predicted, they will probably conclude (1) that these results support the hypothesis that latitudinal clines in nature are likely driven by temperature and (2) that their laboratory temperature regimes are a "good enough" approximation of latitudinal environments. No doubt they'll be able to publish their results in a fine journal—provided that they can convince reviewers that they have not committed a Type I error! But if they find patterns contrary to those expected, our investigators will find themselves on decidedly uneasy ground. They might conclude that the temperature hypothesis is false. Alternatively, they might conclude that their experimental design is fundamentally flawed: perhaps inversion frequencies in nature are driven by infrequent cold winter temperatures, not by average ones; or maybe they just chose the wrong temperature levels. Of course, such conclusions might be a Type II error.

LNS experiments have an even more serious problem. To simplify the experiment, our team manipulated only *one* environmental variable: thus, their experimental design

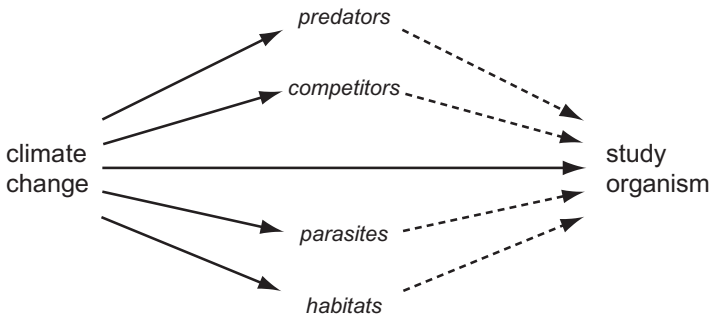

FIGURE 22.2
Effects of climate change on a study organism can be direct (solid arrow) or indirect (dashed arrows), via the impact of climate change on predators, competitors, parasites, and habitat.

tests only for direct effect of temperature on flies (figure 22.2), holding all else equal (in theory). In nature, however, latitudinal patterns of selection might reflect not only the direct effects (solid arrows) of temperature on our study organism, but also the direct and indirect (dashed arrows) effects of temperature on diverse abiotic and biotic factors, which indirectly impinge on our study organism (figure 22.2). Consequently, temperature might well be the selective agent responsible for inversion clines, but the mechanism could be direct or could involve complex interactions, or even all of the above. (And, of course, temperature might be a red herring, such that selection is really driven by other factors that co-vary with latitude [Bradshaw and Holzapfel 2006].) This complex pattern of selection in nature is fundamentally different from the simple and direct pattern of selection in an LNS experiment. Therefore, evolutionary trajectories driven by temperature in an LNS experiment might well differ from those in nature, even if temperature is driving both systems.

We see these problems as inevitable in an LNS experiment attempting to test comparative hypotheses. Although some have argued that "to seek patterns is to do science," documenting patterns is only the first step. We must go further and develop manipulative experiments that enable us to discover causal mechanisms (Paine 1994). But here again we meet a catch-22, for laboratory experiments are inherently artificial and simplistic; and any resulting conclusions must thus be accepted with caution (Rose et al. 1996). However, if we thoroughly understand the assumptions and limitations of our experiments, we should be able to design procedures that reveal rather than obscure mechanism.

A number of more specific issues bedevil LNS experiments. Some have to do with the adaptation of experimental stocks to the laboratory. Some have to do with selection protocols themselves. We itemize these in the following sections and try to suggest ways to circumvent them.

PROBLEMS ASSOCIATED WITH LABORATORY ADAPTATION

SELECTING ON FIELD-FRESH LINES

Nature and the laboratory are different environments, so field-fresh organisms encounter novel selective pressures when transferred to the laboratory (Service and Rose 1985; Matos et al. 2000b; Hoffmann et al. 2001; Simões et al. 2007, this volume). If an LNS experiment begins shortly thereafter, then the subjects will be adapting not only to the specific LNS regime but also to the general laboratory environment. The resulting conflation of selective factors can easily confound interpretations of responses to LNS, for example, yielding falsely positive genetic correlations between traits (see Service and Rose 1985; Clark 1987; Rose et al. 2005).

Service and Rose (1985) propose a solution: start selection only after the study organisms have adapted to the laboratory environment, indicated perhaps by a plateau of the selection response (Gilligan and Frankham 2003; Simões et al. 2007). Their proposition is reasonable in principle, but it leaves open the question of how much laboratory adaptation is sufficient (Harshman and Hoffmann 2000). Unfortunately, we see no easy answer to this question, especially as different traits will adapt at different rates (Matos et al. 2000b). The pace of laboratory domestication is certain to be taxon-specific and to depend on the magnitude and nature of the deviation of the lab environment from that of the field, as well as the amount of standing genetic variation imported with the founder population. Moreover, adaptation to the laboratory may create special problems, as discussed in the next section.

SELECTING ON LABORATORY-ADAPTED LINES

Service and Rose's (1985) proposal solves one problem but creates another. Here's the key issue: laboratory lineages may respond differently to experimental selection than do field-fresh stocks because genetic architecture will change as a result of laboratory adaptation and random genetic drift (Clark 1987; Frankham et al. 1988; Harshman and Hoffmann 2000; Griffiths et al. 2005; but see Promislow and Tartar 1998; Krebs et al. 2001). Changes during laboratory adaptation can be profound. Consider the situation facing a research team contemplating an LNS experiment with the moth *Manduca sexta*. To sidestep the problems associated with field-fresh stocks (Service and Rose 1985), the team might choose experimental lines derived from a base laboratory stock that has been reared in the laboratory now for over thirty-three years (J. Kingsolver, personal communication). This would be an appealing choice, as the lines should by now be well adapted to the laboratory. Moreover, a gold mine of physiological, endocrinological, neurological, and developmental information is available on these lines. So a National Science Foundation panel is likely to look favorably on this choice of stocks.

But if our goal is to learn how wild *Manduca* would respond to a specified type of selection, then we must question whether these laboratory stocks will be remotely

representative of free-ranging *Manduca*. We suspect not. Laboratory *Manduca* have inadvertently been selected for fast growth to a large adult size (L. Riddiford, personal communication). They are now real "porkers," fly clumsily, have degraded vision (J. Sprayberry, personal communication), and have only five larval instars. In striking contrast, their free-ranging ancestors are sleek, agile, and visual, and they can even have six instars (Kingsolver 2007). Consequently, an LNS experiment starting with lab-adapted *Manduca* might yield very different results from those starting with more wild-type stocks. History can matter (Travisano et al. 1995; Moore and Woods 2006).

Again we encounter a catch-22: if we start selection with wild stocks, we conflate domestication with intentional selection; but if we start selection with lab stocks, we may observe unnatural evolutionary trajectories (Harshman and Hoffmann 2000). Experimental masochists might decide to conduct selection both on recently and on long-ago established lines (Harshman and Hoffmann 2000): parallel evolutionary trajectories would be reassuring. Nevertheless, Matos et al. (2000a) have criticized that approach—they see little to gain in studying recently sampled stocks.

LABORATORY ENVIRONMENTS ARE TOO BENIGN

Genetic architecture can be modified by the benign nature of the laboratory itself. Consider our baseline fly experiment simulating climate change. Flies are raised for many generations on ample food and at constant (typically nonextreme) temperature, fixed photoperiod and humidity; they may encounter essentially no variation in the physical environment and have no interactions with predators or parasites or (interspecific) competitors, and food is near at hand (or wing). Life is good. Life is simple.

Such benign laboratory environments will likely weaken selection on many traits that in nature must deal with fluctuating physical environments, predators, parasites, or competitors (figure 22.2). As a result, the performance capacities for those traits might decay over time, as a result of either energy conservation (Regal 1977) or mutation accumulation (Mueller 1987; Promislow and Tartar 1998). Natural isolates of *C. elegans* show classical thermoregulatory behavior on a laboratory gradient, whereas the standard lab stock (N2) decidedly does not, suggesting that N2 has lost thermoregulatory abilities during laboratory adaptation at constant laboratory temperatures (Anderson et al. 2007). However, degradation in performance may not always be readily apparent. Kondrashov and Houle (1994) found that some fitness differences between control and mutation accumulation lines of *Drosophila* were apparent only in harsh environments. This problem may not be general, however, as Chang and Shaw (2003) observed no exaggerated decline in mean fitness of mutation accumulation lines of *Arabidopsis* when challenged in low-nutrient environments.

Moreover, benign environments permit evolutionary trajectories that would likely be maladaptive in nature (Gibbs 1999). For example, flies selected for starvation resistance (Chippindale et al. 1996; Hoffmann and Harshman 1999) quickly evolve enhanced

levels of lipids and effectively become "butterballs": in one experiment (Harshman et al. 1999), starvation-selected lines were 21 percent heavier than control lines! Similarly, flies selected for desiccation resistance accumulate body water (table 22.1; Chippindale et al. 1998; Gibbs et al. 1997). Accumulating resources (lipids or water) during the larval period may be a viable evolutionary response for flies experiencing selection for starvation or desiccation resistance in the lab, but not for flies in nature: butterballs and water melons would be easy and tempting targets for predators, and furthermore, they probably have reduced ability to disperse (Gibbs 1999, 2714). Moreover, stress-selected lines had low preadult viability, suggesting that resource sequestration during the larval period has an associated cost (Chippindale et al. 1998). Not surprisingly, real desert flies do not accumulate water (table 22.1; Gibbs and Matzkin 2001). Therefore, mechanisms of adaptation to starvation or desiccation resistance in the laboratory may involve very different solutions than in nature (Gibbs 1999). Such "unnatural" LNS trajectories are still of academic interest and certainly may offer insight concerning genetic and physiological mechanisms, but they may not always be relevant to testing comparative hypotheses generated from field observations.

Laboratory adaptation can lead to other changes that might influence selective trajectories. When flies first are brought into the lab, they often pupate on the surface of the medium. But the remaining larvae continue to work the medium, such that pupae on the surface of the medium often die, presumably because they become buried. As a direct consequence, selection favors larvae that pupate on the walls of vials, especially in high-density regimes (Mueller and Sweet 1986). The shift is dramatic and rapid. But the shift means that LNS and wild pupae experience different environments, and this might (or might not) result in inadvertent selection on pupal traits (or on wandering larvae), altering diverse aspects of the genetic architecture and confounding overall evolutionary trajectories. A vial or bottle or even a population cage is not the field.

Among model microorganisms such as *Escherichia coli* and Baker's yeast, laboratory conditions appear to select against certain "wild-type" traits as well as select for others

TABLE 22.1 Increased Resistance for Desiccation Can Potentially Be Achieved Several Ways

Possible Response	Experimental Response of Lab-Selected Flies	Comparative Response of Flies from Nature
Store more water	Yes	No
Lose water more slowly	Yes	Yes
Tolerate greater water loss	No	No
Modify behavior	Not possible in the lab	Probably

NOTE: Desert flies from nature rely primarily on losing water relatively slowly. However, flies selected for desiccation resistance in the laboratory rely mainly on storing more water, which would probably be disadvantageous in nature. Data summarized from Chippindale, Gibbs, et al. 1998; Gibbs et al. 1997; Gibbs and Matzkin 2001.

not commonly observed in the wild. Mikkola and Kurland (1992) imposed LNS on a set of natural *E. coli* isolates that were highly variable in their growth rate and translation efficiency. In fewer than three hundred generations, these diverse strains converged on growth and translation phenotypes that characterize laboratory "wild types."

Genome architecture can differ markedly between natural and laboratory *E. coli* strains. For example, the core genome of natural isolates is estimated to range from 2,800 (Fukiya et al. 2004) to 3,100 open reading frames (Dobrindt et al. 2003), but that of the nonpathogenic laboratory strain K12 contains 4,288 predicted open reading frames, many of which have unknown function (Kang et al. 2004). Even commensal natural isolates show enormous variation in the presence or absence of many virulence factors (Escobar-Paramo et al. 2006). Among natural isolates, genome size may vary by as much 20 percent between strains adapted to endocellular and extracellular lifestyles (Bergthorsson and Ochman 1999; Perna et al. 2001). These discrepancies point to the need for caution in generalizing results of LNS experiments using model lab strains.

By contrast, genome content appears remarkably conserved among *Saccharomyces* congeners (Kellis et al. 2003). And in *Saccharomyces cerevisiae,* systematic deletion of "nonessential" genes does not appear to confer competitive advantage (Sliwa and Korona 2005). Indeed, genomic studies have revealed widespread anueploidy among laboratory strains, including the widely used yeast "knock-out" collection (Hughes et al. 2000; Scherens and Goffeau 2004). Still, laboratory populations of *S. cerevisiae* differ from their wild conspecifics in many key respects, including pheromone response, as well as in the timing and location of daughter cell separation. Intriguingly, much of the variation in these particular features has been attributed to polymorphisms at the trans-acting regulatory loci GPA1 and AMN1 (Yvert et al. 2003; Ronald et al. 2006).

Environmental differences between nature and the lab can result in major differences in phenotypes. Free-living yeast and bacteria face the prospect of prolonged resource limitation and the threat of dehydration, and they mitigate these hazards by forming biofilms. However, microbes in the laboratory generally don't face these hazards and have evolved changes in both colony morphology and the associated transcriptional program that supports this quasi-multicellular habit (Kuthan et al. 2003; Palkova 2004).

Finally, and of perhaps greatest concern for LNS experiments, laboratory and wild microbes potentially differ in mutation rate. In *E. coli*, a gene's mutation rate differs according to chromosome location (Hudson et al. 2002). Because genome size and organization vary so widely among stocks, mutation rate in essential genes might well differ between lab and natural isolates, as well as among natural isolates. In *S. cerevisiae* strain S288c, recurrent bottlenecks and an overall relaxation in selection intensity are hypothesized to underlie its higher rates of nonsynonymous substitutions relative to its wild conspecific, YJM789 (Gu et al. 2005). Further analysis, using an additional wild isolate, has challenged the generality of this (Ronald et al. 2006).

LABORATORY ENVIRONMENTS ARE TOO STRESSFUL

As just noted, it is easy to conclude that LNS environments are too benign to be ecologically realistic—after all, predators or parasites are mercifully absent. But laboratory environments can be surprisingly stressful and potentially pathological in unexpected ways. It's a catch-22 all over again!

Unless replenished continuously (as in a chemostat), food quality will change over time. In a standard fly experiment, food is replaced at intervals; and food deteriorates as waste products accumulate and as food itself is depleted. In *D. melanogaster*, this can lead to a stable genetic polymorphism (Borash et al. 1998). One genotype evades these problems by evolving early emergence, which it achieves via elevated feeding rates. The other genotype feeds and grows more slowly, but it evolves greater tolerance of the waste product ammonia. This fascinating example shows that laboratory environment may not always be benign and can modify evolutionary trajectories in unanticipated—and unwanted—ways, including the evolution of enhanced (or possibly blunted) phenotypic plasticity (Garland and Kelly 2006).

When organisms are evolving in chemostats, their growth and reproduction are continuously substrate limited (Novick and Szilard 1951; Kubitschek 1970; Adams and Hansche 1974; Dykhuizen 1990). Under these conditions, one might expect populations to evolve by periodic selection of fittest clones, so that only one clone is likely common at any given time (Muller 1931; Williams 1975). However, chronic nutrient deprivation can promote stable genetic polymorphism (Helling et al. 1987). For example, when *E. coli* evolve on limited glucose, subdominant clones can quickly evolve the capacity to scavenge acetate, a fermentation by-product secreted by the dominant clone growing best on the limiting substrate (Treves et al. 1991; Rosenzweig et al. 1994). So both clones persist.

Laboratory environments are less than benign in other ways. Most LNS experiments (but see Bennett and Lenski 1993; Riehle et al. 2005) are conducted at constant temperatures. But to some organisms, constant temperatures appear to be physiologically pathological (Huey 1982) and can yield aberrant results (Brakefield and Mazzotta 1995). Similarly, light levels in incubators are low and often have nonnatural spectral qualities (G. Gilchrist, personal communication). Insects perceive the flicker of AC lights, and so their world resembles a "continuous disco" (J. W. Truman, personal communication). Dim light might suppress visual cues (important to behavioral interactions), modifying selection on behaviors; it also might reduce photochemical reactions (e.g., vitamin D synthesis), leading to physiological pathologies.

Finally, many *Drosophila* labs routinely maintain their stocks on constant twenty-four-hour light regimes (to eliminate time-of-day cues; M. R. Rose, personal communication); but a constant photoperiod will disrupt circadian patterns of behavior (Markow 1975, 1979; Paranjpe et al. 2004) and physiology (Pittendrigh 1960). In all these examples, LNS experiments will be selecting on lines that at least initially suffer laboratory induced (if inadvertently so) anomalies or even pathologies, such that the resulting

evolutionary trajectories might differ from those of healthy lines. To be sure, lines evolving under constant photoperiod may adapt to such conditions (Sheeba et al. 1999), but whether those lines can serve as reliable models for "natural" organisms is uncertain.

One solution is to try to make laboratory environments more natural. Bradshaw and Holzapfel (2001) have done just that with pitcher plant mosquitoes. They use real pitcher plants as microhabitats, and they use natural photoperiods (including twilight) and thermoperiods. Unfortunately, simulating natural environments will not always be desirable in LNS experiments—consider the natural habitat of *E. coli*.

SIMPLICITY CAN BE DECEIVING

LNS experiments are designed with a view toward simplicity: selection is reduced to one or few variables, selection is chronic, and selection is uniform across replicates (Cohan and Hoffmann 1986). Simplicity is desirable not just because it makes LNS logistically tractable or useful for model building and model testing, but also because simplicity has been a distinctive feature of the experimental method since Francis Bacon. Nonetheless, simplicity can spawn several problems that relate to the intensity of selection, as well as the temporal and spatial dimensions over which it is applied.

Acute Shifts in Selection At the initiation of an LNS experiment, lines are usually transferred suddenly to different environments and maintained there for generations (figure 22.3a). The rationale for "steplike" shifts is compelling: the experiment is logistically simpler and is likely to foster a response to selection prior to the next grant cycle. Even so, steplike changes

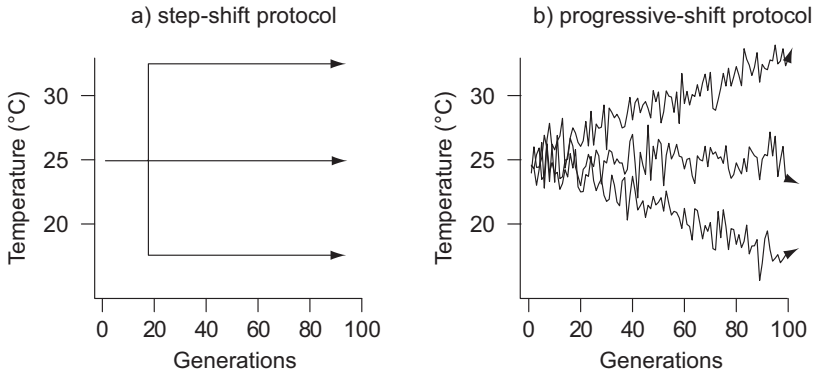

FIGURE 22.3
a, The standard protocol for an LNS experiment involves sudden and chronic shifts in the environmental conditions. b, An alternative protocol suggested by Brakefield (2003) would be to simulate a more gradual (and fluctuating) shift in conditions.

hardly mirror natural environmental changes, which are generally episodic (see also Garland and Kelly 2006 regarding the opportunity for plasticity to evolve). Brakefield (2003) argued that steplike protocols might yield misleading evolutionary trajectories and proposed LNS experiments in which environments are shifted "in a more gradual and realistic manner over generations" (figure 22.3b). Although obviously time-consuming for long-lived organisms, this approach is practical with microorganisms, as was demonstrated (see the box) over a century ago by Dallinger (1887). In any case, whether this issue is a real concern remains to be determined and is in fact an interesting opportunity for investigation.

Chronic Selection A related simplicity concern is that environmental shifts in LNS experiments are typically chronic and sustained (figure 22.3a). And even if selective environments are eventually reversed (Estes and Teotónio this volume), they are still chronic. Do organisms in nature generally experience chronic and sustained environmental shifts? In some cases (e.g., if an organism emigrates to a cold environment), selection could indeed be somewhat chronic in nature. But at single localities, environmental factors such as temperature are highly variable, even in the face of a sustained environmental trend. After all, selection is anything but chronic in *Geospiza* finches on the Galapagos (Grant and Grant 2003).

Will chronic selection alter evolutionary trajectories relative to episodic selection? We suspect so. Although this question has not to our knowledge been systematically approached in any theoretical model, a useful comparison can be made between microbial populations undergoing clonal evolution in continuous serial dilution culture (SDC) versus those undergoing clonal evolution in continuous nutrient-limited chemostat culture (CC). These two selection regimes are commonly used, but few appreciate that selection in SDC is continuously varying, whereas selection in CC is constant. Nutrient limitation—and nutrient excess—are imposed regularly and episodically in the former, whereas nutrient limitation is imposed chronically in the latter (Kubitschek 1970). In the absence of antagonistic pleiotropy, fitness advantages can be secured in SDC culture by multiple adaptive mechanisms that include a decrease in lag time, an increase in maximum specific growth rate, and an increase in yield and/or survivorship in stationary phase. By contrast, fitness gains in CC are generally restricted to improvements in the capacity to scavenge limiting nutrient and/or to convert that limiting nutrient into cell mass (Brown et al. 1998). The different opportunities for evolutionary adaptation that exist under these different selection regimes may in part account for the differences observed between them in the pace and tempo of evolutionary change (Helling et al. 1987; Lenski and Travisano 1994), as well as the relative advantages that accrue to haploids versus diploids (Paquin and Adams 1983; Zeyl et al. 2003). Relative to episodic selection, chronic selection may increase the rate of response, and it may even alter the evolutionary trajectory.

A direct approach to the issue of evolutionary trajectories from chronic versus episodic selection could be achieved, at least in principle, by conducting a parallel selection experiment: in one treatment, selection would be applied chronically over hundreds

of generations, while in another would be applied episodically every other generation, or even according to a randomized schedule. This would be practical only with a few organisms (e.g., bacteria and yeast), and the experimental design and analysis should take into account the fact that conditions defined as "relaxed selection" with respect to one agent, might constitute yet another type of selection.

Duration of Selection The time scale of selection may also alter conclusions. Faced with pressures from funding agencies (or dissertation advisers), researchers will generally start to monitor responses immediately after beginning selection and will be tempted to publish once a trend becomes apparent. For a variety of reasons, however, evolutionary trajectories can shift and even reverse over time (Archer et al. 2003; Rose et al. 2005; Santos et al. 2006): (1) some adaptive responses may require novel genetic mutations, which take time to appear (Knies et al. 2006), and (2) trajectories may be modified by novel epistatic interactions.

Behavioral Compensation Is Impossible In pursuit of simplicity, typical selection protocols prevent organisms from using behavior to help compensate for the imposed selection and thus may lead to aberrant evolutionary trajectories (see also Rhodes and Kawecki this volume). Consider the options available to ectotherms facing climate change (or other environmental challenges) in nature. Recall what might be called "Bartholomew's First Law of Physiological Ecology": namely, the first response of any animal will be to use behavioral adjustments to try to evade or at least ameliorate those changes (table 22.1; Bartholomew 1958, 1964; Slatkin and Kirkpatrick 1983). For example, many ectotherms shift habitat and time of activity along an altitudinal or latitudinal gradient (Hertz and Huey 1981; Clarke 1987; Pascual et al. 1993). As a result, their (activity) body temperatures are often remarkably similar across altitudes or latitudes (Jones et al. 1987; Huey et al. 2003). In fact, if behavioral thermoregulation is fully compensatory, then ectotherms facing climate change in nature might experience selection only on traits involved with the behavioral shifts, and not on thermal sensitivity per se (Bogert 1949; Huey et al. 2003).

Now consider an LNS experiment specifically designed to elucidate evolutionary responses to climate change. Typically, replicate lines would be maintained for many generations at different fixed temperatures in environmental chambers, where thermal heterogeneity is essentially nil. In such environments, the study subjects would have little or no opportunity to use behavioral thermoregulation to modify their temperatures from that of their resident thermal regimes (Gibbs 1999). As a result, LNS must act directly on their thermal sensitivity. In a very real sense then, LNS experiments transform mobile animals into "plants," organisms with relatively limited ability to use behavior to evade environmental challenges (Bartholomew 1958; Bradshaw 1972; Huey et al. 2002). Consequently, the evolutionary trajectories organisms follow in response to climate change in nature will likely differ from those evolving in response to fixed and forced temperatures in an LNS experiment.

Sometimes LNS experiments can be redesigned to solve (or at least help solve) this problem of behavioral imprisonment (Gibbs 1999, 2714). Davis et al. (1998) developed an ingenious experiment, which shows that ecological realism is indeed possible. They were interested in studying ecological responses of *Drosophila* species to climate change, but their methodology could easily be applied to evaluate evolutionary responses in an LNS experiment. One of their experiments involved sets of eight cages distributed among four incubators (thus two cages/incubator) that differed in temperature. To simulate climate warming, they set some incubators at 15°, 20°, 25°, or 30°C; and to simulate climate cooling, they set other incubators at 10°, 15°, 20°, and 25°C. In some experiments, the eight cages were connected in series via tubing; and flies could thus move among cages and chambers (e.g., between 20° and 25°C). In other sets, the tubing was blocked, so flies were held at fixed temperatures, as in a typical LNS experiment.

Davis et al. (1998) introduced three species of *Drosophila* either individually or simultaneously into the cages, and they even added parasitoids in some experiments. Consequently, these complex experiments enabled this team to monitor the ecological consequences of interactions involving behavior, temperature, interspecific competition, and parasitism.

To study the impact of climate warming, one could maintain this laboratory scenario (Davis et al. 1998) across many generations. We expect that flies would preferentially spend most of their time in the cages with the favored temperatures. However, competition for those favored thermal cages might force part of the population to occupy suboptimal thermal environments ("ideal free distribution" of Fretwell and Lucas 1970), perhaps thus modifying selection on thermal sensitivity itself (Levins 1968).

Spatial Variation Is Eliminated Most LNS experiments use simple environments that attempt to eliminate any spatial variation in LNS. However, consideration of the Davis et al. (1998) study (discussed earlier) suggests that the presence or absence of spatial heterogeneity in LNS may sometimes influence results. A remarkable example is seen in a study of the bacterium *Pseudomonas fluorescens*. Rainey and Travisano (1998) studied how these bacteria evolved by LNS in unstirred (spatially heterogeneous) versus stirred (well-mixed, spatially homogeneous) microcosms. In the spatially heterogeneous microcosms, the bacteria underwent a rapid adaptive radiation, evolving visibly distinct morphs with marked niche preferences; but in the homogeneous microcosms, morphs stayed uniform. Obviously, anyone contemplating an LNS experiment on such microorganisms must decide in advance whether their stocks will be unshaken or stirred.

A CASE STUDY: A SELECTION EXPERIMENT AT ODDS WITH FIELD STUDIES

So far our chapter has focused on problems that LNS studies face in testing hypotheses derived from comparative studies in nature. Although we have enumerated a variety of problems, we cannot be sure when these are trivial and when they are significant. This

"academic" problem becomes very real when the results of an LNS experiment contradict a comparative hypothesis. Does such a lack of concordance mean that our hypothesis was flawed or that key aspects of our selection experiment were flawed?

Let's take a close-up look at an ambitious and excellent LNS experiment specifically designed to test a comparative hypothesis. The fly *D. subobscura* is native to a broad range of latitudes in the Old World from North Africa to Scandinavia, and so its populations experience a strong climatic gradient (Krimbas 1993). In the late 1970s, *D. subobscura* was accidentally introduced into South America (Brncic and Budnik 1980) and then into North America (Beckenbach and Prevosti 1986). It spread rapidly on both continents, where it now occurs over a broad latitudinal (climatic) range.

Evolutionary biologists soon recognized that the Old and New World flies provided an ideal opportunity for studying the evolution of geographic variation (Brncic et al. 1981; Prevosti et al. 1988; Ayala et al. 1989). The Old World flies provide a convenient evolutionary baseline, as these flies have had thousands of years to evolve clinal patterns. The New World flies serve as a "grand experiment in nature" (Ayala et al. 1989). Studies of the magnitude and patterns of geographic variation in these flies provides insight into the rates and predictability of evolution on a geographic scale (Prevosti et al. 1988; Ayala et al. 1989).

Two traits show pronounced latitudinal clines in the baseline Old World populations and have also been intensively studied in the New World. About eighty chromosomal inversions have been described in the Old World (Krimbas 1993), and the frequency of many show strong latitudinal patterns (Menozzi and Krimbas 1992). For example, the "standard" inversions of the various chromosomes are common in northern Europe, but rare to the south. Wing size also changes clinally (Prevosti 1955; Misra and Reeve 1964; Pegueroles et al. 1995; Huey et al. 2000; Gilchrist et al. 2004), as it does in many other *Drosophila* (Coyne and Beecham 1987; James et al. 1995; van't Land et al. 1999), and it is positively related to latitude.

The observed latitudinal patterns in the Old World suggest that inversions and wing size might be subject to selection from temperature or related climate factors. This hypothesis is reinforced by the discovery that similar latitudinal clines in inversion frequencies (Prevosti et al. 1988; Balanyà et al. 2003; Balanyà et al. 2004) and in wing size (Huey et al. 2000; Gilchrist et al. 2004) had evolved rapidly in both North and South America. More important, the frequency of "low-latitude" inversions at particular localities have increased over time, seemingly in accord with recent climate warming (Orengo and Prevosti 1996; Rodríguez-Trelles et al. 1996; Rodríguez-Trelles et al. 1998). For example, in twenty-two of twenty-six populations spread over three continents, climates have warmed over sample intervals; and low-latitude inversions have increased in frequency (Balanyà et al. 2006). All this comparative evidence strongly suggests that inversion and wing size clines are adaptive and that temperature (climate) is a key selective agent.

To test putative role of temperature in the evolution of these clines, evolutionary geneticists (Santos et al., 2004, 2005, 2006) in Barcelona developed an exemplary experiment in

perhaps the experimental conditions don't adequately mimic natural ones. As Santos et al. (2005) noted, their laboratory environments had fixed temperatures, whereas natural ones have daily and seasonal variation in temperature. Moreover, their flies had ample food resources, which sometimes will not be the case in nature; their flies were not exposed to interspecific interactions, which might co-vary with latitude; and their flies experienced constant densities, which might not reflect patterns in nature. Finally, flies in a population cage are probably not challenged physiologically in the same ways as flies in nature. For example, individual differences in flight performance may have little impact on fitness to fly in a small population cage, and thus laboratory selection on wing size might be weak or nonexistent.

At present, perhaps the safest conclusion is that evolutionary trajectories resulting from this experimental manipulation of temperature are inconsistent with the hypothesis that temperature drives clines in inversion frequency and in wing size. Whether this is the "fault" of temperature or of LNS is currently and frustratingly unclear.

ON MODIFYING LNS EXPERIMENTS

Given that we need LNS to test comparative hypotheses, how can we improve LNS? Are there midflight corrections that solve (or at least reduce) some of the concerns? We think so, and we offer a few suggestions (see also Rose et al. 1996, 232–236).

At the risk of sounding professorial, we do advocate learning from others. There is a lot of accumulated wisdom in experimental evolution, and we can all learn from the mistakes of the past. Moreover, we would add that our ecological colleagues have gained extraordinary experience over the decades in experimental approaches, and we evolutionary biologists would do well to learn from their experiences.

Knowing the natural history of one's study organism is essential to the design of meaningful experiments, whether they be focused on ecology, or on evolution (Hairston 1989). Unfortunately, embarrassingly little is known about the natural history of the very organisms most suitable (Feder 1996) for experimental evolutionary studies (e.g., *Drosophila*, *E. coli*, *S. cerevisae*, *C. elegans*, *Mus*).

Earlier we addressed the catch-22 concerning selection on field-fresh versus laboratory-adapted stocks. Roff and Fairbairn (2006) have recently found a clever way to turn this disadvantage to an advantage. Their goal was to study evolutionary changes in the sand cricket (*Gryllus firmus*) during adaptation to the laboratory. This cricket has a striking wing dimorphism: long-winged morphs are migratory, but the short-winged ones are not. Given the known trade-off between migratory capability and fecundity in these morphs, Roff and Fairbairn predicted that domestication should result in a reduced frequency, an increased fecundity, and a decreased mass of flight muscles of long-winged females, but in little change in these traits of short-winged females. Importantly, they used quantitative genetic theory and measurements to predict evolutionary trajectory of these traits during domestication. Their predictions were verified.

An advantage of laboratory evolution is that one can control most variables and manipulate only one or a few. But probably most of the patterns that we seek to test are likely the result of selection involving many interacting processes (Quinn and Dunham 1983). Dunham and Beaupre (1998) argued that "the potential for multiple casual mechanisms must be incorporated into the construction of ecological theory and into the design of ecological experiments." The same should hold for the design of evolutionary experiments. Similarly, the possibility of responses with "multiple solutions" can be a key reason of including replicate lines in selection experiments (Garland 2003).

Consider the LNS experiment with *D. subobscura* described earlier. Perhaps humidity as well as temperature should have been manipulated, such that the conditions would range from cool and high relative humidity to warm and low relative humidity. In a humid environment, higher temperature will increase metabolism but won't increase evaporative water loss; but in a dry environment, higher temperatures will increase metabolism and water loss.

A call for greater ecological realism is not without precedent. Ecologists have developed sophisticated laboratory facilities that can mimic simple terrestrial ecosystems. At Silwood Park, for instance, the Ecotron consists of fifteen environmental chambers able to control and manipulate photoperiod, illumination (balanced spectrum, dawn/dusk simulation), temperature, humidity, rainfall, and even CO_2 (Lawton 1996). The chambers house multispecies ecosystems, allowing for complex ecological interactions of plants and animals. Using such a facility for experimental evolution would be expensive, but feasible. If ecologists can build and run an Ecotron, surely evolutionary biologists can build and run an Evotron!

Another option is to borrow from another experimental technique in ecology—the cattle tank. These are typically used for aquatic systems, but they are large enough to house salamanders, frogs, and fish. Potentially one could manipulate tanks and look at evolutionary shifts over time.

Of course, one might attempt LNS-type experiments in the field (Bennett and Lenski 1999). This has been done successfully in a few cases (Losos et al. 2004; Irschick and Reznick this volume). Such studies will be logistically challenging (and sometimes unfeasible). Moreover, their design may not enable genetic and environmental effects to be easily discriminated (Bennett and Lenski 1999).

An informative variant is to use field releases of LNS-engineered phenotypes. Kristensen et al. (2007) released *D. melanogaster* that had been selected for increased heat or cold resistance, and then they measured relative ability of the lines to reach baits under hot or cold field conditions. Such releases of experimental lines provide interesting tests of whether phenotypic shifts produced by laboratory selection result in enhanced fitness in nature.

Finally, experiments must be designed so that presumed causal mechanisms can in fact play a role (Dunham and Beaupre 1998). For example, if we wish to test experimentally the hypothesis that temperature is the selective agent, then we need to design an

experiment that allows temperature to have a mechanistic impact. In the case of the clinal increase in wing size with latitude in *Drosophila*, we might assume that temperature exerts its force in nature via its mechanistic effect on flight dynamics. Therefore, to test whether temperature might drive the wing size cline, we need to design an experiment in which relative flight ability might influence fitness (Weber 1996; Marden et al. 1997). But would flight ability influence fitness in a small population cage? Probably not.

In some cases, mechanism may not be obvious a priori. For example, inversion frequencies change with latitude and temperature, but the mechanism (if in fact temperature is a causal agent) for a causal relationship is presently mysterious to us. For that reason, we cannot see how to design an experiment that realistically allows mechanism.

CONCLUSION

Although we have focused on problems that can plague LNS experiments as emulations of natural selection in the wild, we do not mean to imply that LNS experiments are without utility. Quite the contrary. There are many ways to study evolution, some descriptive, some experimental. As has been noted repeatedly (Huey et al. 1991; Huey and Kingsolver 1993; Rose et al. 1996; Gibbs 1999; Garland 2003; Swallow and Garland 2005; Futuyma and Bennett this volume; Rose and Garland this volume), each method has its advantages, and each has its limitations. Moreover, an awareness of limitations can open opportunities for novel studies (e.g., chronic vs. nonchronic selection). In any case, a complete understanding of evolution will require the application of multiple integrated approaches. We see LNS as an essential tool for testing field-derived hypotheses, but one that must be handled thoughtfully, used along with other tools, and interpreted with care.

No matter how hard we work, no experiment or study will ever be perfect. We need to do away with the "Myth of Definitive Results" (Underwood 1998) and recognize that our view of evolution is deeper if we look at it through different and complementary glasses, not just though LNS ones. And we should try to improve the validity of each approach, learning as we go. As Underwood (1998, 345) noted, "The hallmark of progressive ideas is that they progress. Given that there is a good chance we are wrong quite often, we should be prepared to discover how wrong as fast as possible."

SUMMARY

Experiments using laboratory natural selection (LNS) can illuminate the genetic architecture underlying complex traits, reveal evolutionary trajectories associated with different population structures and modes of selection, and provide derived lines with "exaggerated" or "novel" phenotypes. But LNS experiments have inherent problems and limitations, especially when used as to simulate natural selection in the wild. Certain problems can be so severe that they compromise or confound evolutionary and functional interpretations. Of these, some can be circumvented by modifying traditional experimental

designs, but others cannot. Consequently, researchers contemplating LSN experiments face a classic catch-22 or double-bind. They know in advance that LNS may be an effective way to test a given evolutionary hypothesis, but they also recognize that the resulting conclusions may be of uncertain validity.

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