The Luria-Delbrück Fluctuation Test as a Classroom Investigation in Darwinian Evolution

RECOMMENDED FOR ~~AP Biology~~ College freshman biology

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ABSTRACT

Microbial cultures swiftly adapt to lethal agents such as antibiotics or viruses by acquiring resistance mutations. Does this remarkable adaptability require a Lamarckian explanation, whereby the agent specifically directs resistance mutations? Soon after the question arose, Luria and Delbrück devised a clever experiment, the fluctuation test, that answered this question in the negative: microbial adaptation, they showed, is entirely consistent with a Darwinian explanation. Their 1943 article is a classic of biology literature, with practical and theoretical implications that continue to expand today. Implementing an updated fluctuation test in a college teaching lab provides a simple experimental setting in which beginning students learn to apply basic principles of evolutionary biology and scientific reasoning, while gaining hands-on experience in core technical advances of contemporary life science.

Key Words: *Darwin; Lamarck; mutation; yeast; stress; cancer; epigenetic modification.*

○ Introduction

Breast cancer patients whose tumors are responsive to estrogen are typically treated with anti-estrogen drugs. In the course of therapy, tumors often become resistant to treatment. Were resistant mutations lurking in a small subpopulation of cancer cells before chemotherapy, or did they arise during treatment? Next-generation sequencing reveals that both can be true (Robinson et al., 2013); the ongoing clinical relevance of this question is part of the 72-year legacy of the Luria-Delbrück experiment.

Luria and Delbrück (1943) sought to explain a striking characteristic of microbial cultures, which seemed to challenge Darwinian orthodoxy: when such cultures are exposed to a lethal agent (e.g., an antibiotic or virus), variant cells that are resistant to the agent

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emerge with remarkable speed and regularity. The cultures appear to "adapt" rapidly to the lethal challenge. The survivors' resistance is heritable, in that their progeny remain resistant after the agent is withdrawn. In the language of modern molecular genetics, the resistant variants have DNA mutations that confer heritable resistance.

Luria and Delbrück considered two opposing explanations for the origin of microbial resistance mutations, which we will call the *post-exposure* versus *pre-exposure* hypotheses. Post-exposure mutations arise only *after* the microorganisms encounter the agent. Such mutations were envisaged by Luria and Delbrück's contemporaries as "Lamarckian," in that heritable resistance to the agent would be directed by the microorganisms' struggle to survive the agent. Pre-exposure mutations, in contrast, arise *before* contact with the agent, and thus independently of the selective advantage they confer. This hypothesis can be said to be "Darwinian," in that resistance to a particular agent emerges because that agent *selects* for resistant mutants, not because it specifically directs mutations that confer resistance to itself.

To distinguish these two hypotheses, Luria and Delbrück inoculated several dozen culture tubes containing a small volume of *nonselective* medium with a few nonresistant bacteria each; the nonselective medium lacked the lethal agent. After incubating the cultures to growth saturation, they spread each culture on *selective* agar medium in a Petri dish. The selective medium contained the lethal agent, so that only resistant mutants could grow; the dishes were incubated to allow resistant colonies to form. The results were striking: colony counts fluctuated widely from dish to dish, a result that overwhelmingly favors the pre-exposure over the post-exposure hypothesis (see "Performance & Results of the Fluctuation Test" below).

The fluctuation test has since been replicated countless times with a variety of microbes and lethal agents. In each case, great

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fluctuation in dish-to-dish colony counts has been observed, thoroughly corroborating the pre-exposure hypothesis. Meanwhile, it has become clear that post-exposure mutations do occur under some conditions of stress, and that stress-induced mutability is an important aspect of microbial physiology and cancer biology. Nevertheless, there is no definitive evidence that these post-exposure mutations are Lamarckian in the sense of being specifically directed at genes that can relieve the stress (see "Further Class Discussions" below).

A Classroom Yeast Fluctuation Test

We have developed a yeast fluctuation test that has been successfully performed over the past 5 years by 20 freshmen per year. It is a component module of a lab that substitutes for the regular lab part of our beginning biology majors course. Each investigational module is intended to engage students actively in learning, and to connect multiple threads of biology within a coherent intellectual framework. The fluctuation-test module affords students who have no prior lab experience an opportunity to test a key prediction of evolutionary biology by simple means. Their investigation integrates scientific reasoning with basic microbiology and molecular genetics. It counters common student misconceptions that are shared by some high school biology teachers (Mead & Scott, 2010; Yates & Marek, 2014): that evolution can't be tested; that microbes evolve resistance "on purpose."

The entire module consists of 11 interconnected lab experiments, computer labs, demonstrations, lectures, and discussions (a detailed instructors' manual and teaching materials is available at mls@missouri.edu). Here, we describe only the two core experiments, which can be taught as a stand-alone module. They span five lab sessions as summarized in Table 1 (session numbers refer to this table).

Our fluctuation-test module is based on Sue Jinks-Robertson's undergraduate genetics lab at Emory University and uses a parent yeast strain, YFT1, that is a direct descendant of strain SJR1921 from her lab at Duke University. The lethal agent is the antibiotic canavanine. Two types of spontaneous mutation result in canavanine-resistant colonies: mutations in the "red" gene (as we call it) give rise to red colonies, while mutations in the "white" gene give rise to white colonies. The experiment focuses on mutations in the red gene. In the full 11-session module, students identify the

red gene bioinformatically as an ochre suppressor tRNA gene (Mudrak et al., 2009) and learn how nonsense suppression fits into the genetic design of the parent strain; these considerations are not essential to the logic of the fluctuation test, however, and are not described here.

Microbiological Preparations

Nonselective Media

These media are made ≥2 weeks before Session 1. They have no canavanine and thus allow both canavanine-sensitive parental cells and canavanine-resistant mutant cells to grow. Liquid medium is made by dissolving 2.5 g yeast extract, 5 g peptone, 7.5 g glycerol, and 62.5 mg adenine in 250 mL water; adjusting the pH to 5.6 with HCl; and autoclaving. Agar medium is made in the same way, except that 2.75 g bacto agar is added before autoclaving; autoclaved medium is allowed to cool to ~60°C and poured into five disposable 100-mm Petri dishes, which are dried in an incubator or at room temperature for ≥4 hours before use.

Selective Agar Medium

This medium is made ≥2 weeks before Session 2. It has canavanine and thus allows only canavanine-resistant mutant cells to grow into colonies. Dissolve 106.8 g DOB (dropout base; e.g., MP Biochemicals 4025-022) and 2.96 g CSM-Arg (complete supplements mixture minus arginine; e.g., MP Biochemicals 4510-112) in 4000 mL water; adjust pH to 5.6 with HCl; dispense 1000 mL into each of four 2-liter polypropylene Erlenmeyer flasks containing 11 g bacto agar; autoclave; after cooling to ~60°C, add 1 mL of 60-mg/mL L-canavanine sulfate (Sigma C9758; filter-sterilized and stored frozen in 1-mL aliquots) to each flask and mix thoroughly while minimizing bubbles; pour ~25 mL into each of ~160 disposable 100-mm Petri dishes; allow the dishes to dry for 2 weeks at room temperature (dry dishes give compact, easily scored colonies; some dishes are likely to be lost to mold during drying, but only 120 are needed for the lab).

Performance & Results of the Fluctuation Test

About 2 weeks before Session 1, streak for single colonies from a 25% glycerol stock of YFT1 (stored at –80°C in single-use 100-µL

Table 1. Elements of the yeast fluctuation test module. Each session lasts 100 minutes.

Session	Timing	Activities
1		Introduce the fluctuation test; start yeast cultures in nonselective liquid medium
2	At least 3 days after Session 1	Spread yeast cultures on selective agar Petri dishes; discuss anticipated results according to pre-exposure versus post-exposure hypotheses
3	At least 7 days after Session 2	Count Petri dishes; extract colony DNA; polymerase chain reaction (PCR); discuss implications of colony counts
4	After Session 3	Purify PCR product; submit samples for sequencing
5	Enough time after Session 4 for sequence data to be returned and edited	Align sequences; identify the mutation in each mutant colony analyzed; discuss implications of sequence data

aliquots) onto a nonselective agar Petri dish and incubate 7 days at 30°C; about 1 week before Session 1, restreak a single, well-separated white colony onto a second nonselective agar Petri dish and incubate at 30° until Session 1; the second streak dish is the source of colonies for Session 1 (next section).

Just before Session 1, five 22.5-mL nonselective liquid medium cultures (the "bulk" cultures) are inoculated with five well-separated colonies on nonselective agar medium (previous section) as follows. Use a separate sterile inoculating loop to suspend each colony in 100 μL water in a microtube; vortex vigorously and allow aggregates to settle for 5 minutes; pipette 35 μL of supernatant into the liquid medium; the final concentration is ~10^5 cells/mL (exact concentration not important). Very few if any of the cells at the outset are canavanine-resistant mutants.

In Session 1 itself, students pipette 60-μL portions from the bulk cultures into seventy 13-mL culture tubes (Sarstedt 60.541.021 or equivalent; must be tightly sealed to prevent evaporation; 14 tubes per bulk culture). Those tubes, and the remainders of the bulk cultures, are shaken at 30°C until Session 2 (3–5 days later), by which time cell density has increased ~1000-fold to 10^8 cells/mL.

In Session 2, students first pipette 50 replicate 60-μL samples from one of the bulk cultures into 50 additional culture tubes. They then spread the contents of all 120 culture tubes – the seventy individual 60-μL cultures and the fifty 60-μL bulk-culture samples – onto Petri dishes with selective agar medium (previous section), each Petri dish thus receiving 6 million cells. The Petri dishes are incubated at least a week at 30°C until Session 3, allowing canavanine-resistant colonies to develop fully.

In Session 3, students record the red and white colony counts on all 120 Petri dishes (they also begin the sequence analysis described in the next section). The red colony counts from a recent year are graphed in Figure 1. They are typical not only of 5 years' results in the lab course, but also of fluctuation tests in general, including the original Luria-Delbrück experiment. Bulk-culture sample counts cluster about their mean, as predicted for random sampling from a single cell population. Individual-culture counts, in contrast, fluctuate widely; 43 of the 70 dishes have no red colonies at all, while two "jackpot" dishes have 99 and 175. White colony counts had the same striking pattern (not shown).

These results differ sharply from the expectations of the post-exposure hypothesis, according to which resistance mutations don't arise until *after* cells first encounter canavanine on the selective agar

medium. In that scenario, each dish, whether from an individual culture or a bulk-culture sample, receives 6 million cells, none of which is already resistant. Once they land on the selective medium, all cells have the same low probability of successfully mutating to resistance and thus founding a colony. Each cell mutates independently of all other cells, regardless of its clonal history. Under the post-exposure hypothesis, therefore, colony counts should be clustered about their mean on *both* bulk-culture sample and individual-culture dishes, in stark contrast to the actual results in Figure 1.

On the other hand, the results are fully consistent with the pre-exposure hypothesis, according to which resistance mutations occur randomly during growth in nonselective medium. In this scenario, resistant mutants are already present in the cultures before spreading on selective medium; each mutant gives rise to a colony, while unmutated parental cells – the overwhelming majority – are killed by canavanine. The number of resistance *mutation events* during nonselective growth in individual cultures will be clustered about their mean. By contrast, the number of resistant *mutants* following nonselective growth (and thus the number of colonies on the selective Petri dishes) will fluctuate widely from culture to culture. That's because mutation events during nonselective growth are randomly distributed not only with regard to *number*, but also with regard to *time*. Mutation events that occur early during nonselective growth give rise to exponentially larger clones of resistant mutants than mutation events that occur late, as suggested in Figure 2 (upper half). The extreme fluctuation evident in the individual-culture colony counts in Figure 1, with many dishes having zero colonies and a few jackpot dishes having many colonies, is therefore expected. Unlike the individual-culture dishes, bulk-culture sample dishes represent random samples from a single population of fully grown cells; the only source of fluctuation is therefore random sampling error, and the colony counts cluster about their mean (Figure 2, lower half). The low fluctuation in colony counts observed for these control dishes in Figure 1 is thus anticipated in both post-exposure and pre-exposure scenarios.

○ Sequence Analysis of Resistant Mutants

Many distinct mutations in the red gene result in red canavanine-resistant colonies (Giroux et al., 1988; Mudrak et al., 2009). Only
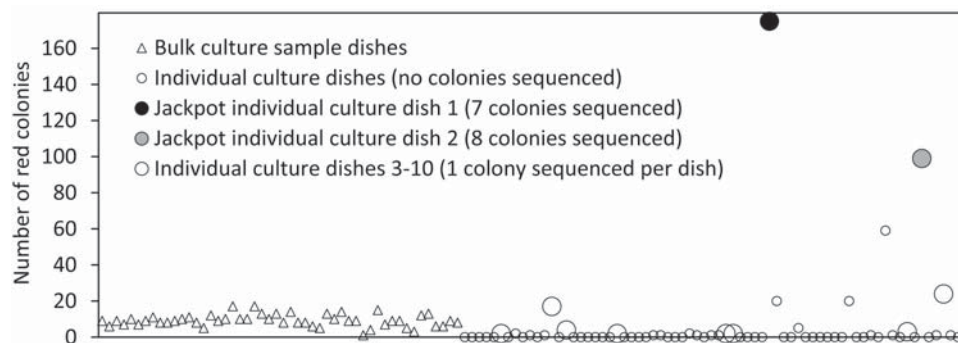

Figure 1. Scattergram of red colony counts from fall 2013. Triangles correspond to the 50 bulk-culture sample dishes; circles correspond to the 70 individual culture dishes. The large black, gray, and open circles correspond to the 10 individual culture dishes whose colonies were sequenced.
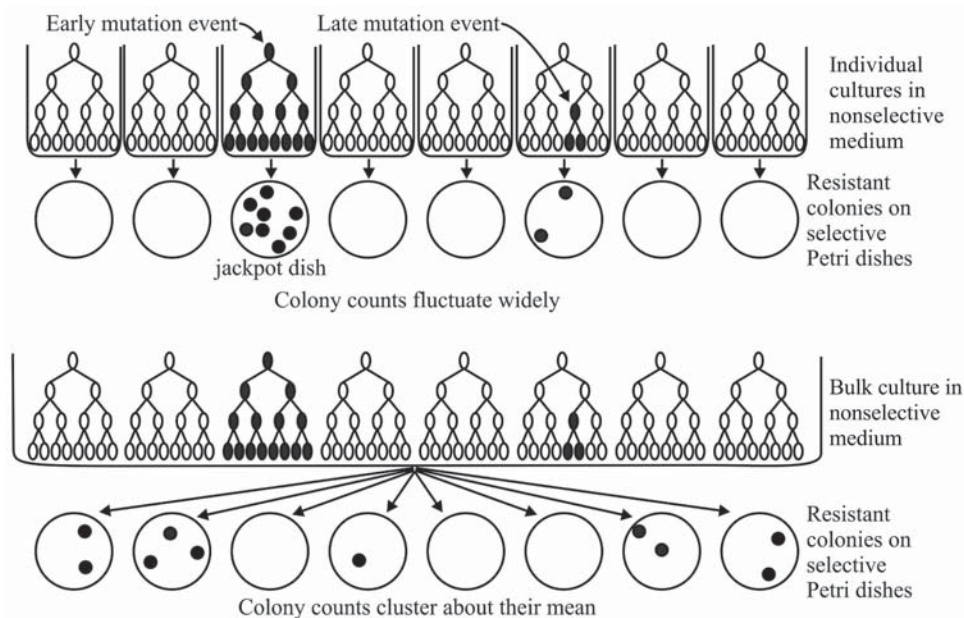
Figure 2. Expectations of the pre-exposure hypothesis. Upper half: individual 60-µL cultures and corresponding individual culture dishes. Lower half: control bulk culture and corresponding 60-µL bulk-culture sample dishes; a bulk culture is equivalent to hundreds of individual cultures mixed together in a single culture vessel. Bifurcating lineages represent exponential growth in nonselective medium; black ovals in these lineages represent canavanine-resistant mutant cells. Black circles in the Petri dishes represent canavanine-resistant colonies. Early mutation events in individual cultures give rise to exponentially larger mutant clones than late mutation events. The fluctuation in colony counts on the corresponding individual culture dishes is therefore much higher than expected for random sampling error. The same large disparity in mutant clone size occurs in the bulk culture, but the mutant and parent cells from all lineages are mixed together in a single population, from which random samples are spread on selective dishes. The only source of fluctuation in colony counts, therefore, is random sampling error, as suggested in the lower half of the figure.

by rare coincidence will two independently arising mutant colonies harbor identical mutations. Conversely, if multiple red canavanine-resistant colonies from a single Petri dish harbor identical red gene mutations, it's likely that they're identical by descent rather than by coincidence (i.e., that they're members of a single mutant clone, arising from a single mutation event in a single ancestral cell). That's an expected occurrence in the pre-exposure hypothesis, according to which jackpot dishes represent early resistance-mutation events that establish large clones of identical resistant mutants. The post-exposure hypothesis, in contrast, predicts that no two mutants in different colonies should be identical by descent, even if they're on the same dish. That's because post-exposure mutation events occur in each cell independently after it has landed on the selective agar medium. Sequencing the red gene from multiple colonies on a single jackpot dish thus provides an additional critical test of the post-exposure versus pre-exposure hypotheses. The test is cheap and easy using polymerase chain reaction (PCR) and DNA sequencing technology. Students sequence the red gene from multiple red colonies on two jackpot dishes, and from single red colonies on eight additional individual-culture dishes as controls.

In Session 3, students use disposable plastic inoculating loops to scoop up well-separated red colonies and suspend them in 30 µL lysis solution (0.2% sodium dodecyl sulfate) in 200-µL PCR tubes. The 24 tubes are heated to 90°C for 4 minutes in a thermocycler in order to lyse the yeast cells and release their genomic DNA. The tubes are centrifuged briefly in a microfuge to pellet cell debris, and 10 µL of each supernatant is pipetted into 90 µL water

in a 500-µL microtube. A 5-µL portion from each microtube is pipetted into a fresh PCR tube, to which is added 95 µL of a PCR pre-mix (1.8 mL water, 500 µL 5× Phusion HF reaction buffer from Fisher; 50 µL 10-mM dNTP mix; 1.25 µL 1-mM forward primer; 1.25 µL 1-mM reverse primer; 25 µL Phusion Hot Start II high-fidelity DNA polymerase from Fisher, added just before use). The forward and reverse primers (5′-gatgtaggtcttttcacctggagg-3′ and 5′-gtctgctgttgcaacttatttg-3′, respectively) are chosen to amplify a 763-base pair (bp) segment of yeast chromosome III containing the 89-bp red gene in the middle (GenBank accession no. KP210072). The PCR tubes are vortexed and loaded into a thermocycler, which executes the amplification program (30 seconds at 98°C; 35 cycles of 10 seconds at 98°C, 30 seconds at 61°C, and 20 seconds at 72°C; 5 minutes at 72°C; and an indefinite "soak" at 4°C). The PCR products are stored in the freezer until Session 4.

In Session 4, students use a QIAquick PCR purification kit (Qiagen no. 28104) to purify their PCR products, yielding 100 µL elution buffer containing 1–2.5 µg DNA free of interfering molecules such as excess PCR primers. An 8-µL portion of each purified PCR product is pipetted into two 1.5-mL microtubes containing 8 µL forward or reverse sequencing primer (5′-gggaatgcagctgcgtacgc-3′ and 5′-ggctatagaaagccctgccgg-3′, respectively) at a concentration of 3 µM. The primers prime from opposite sides of the red gene, ~200 bp from the ends of the 763-bp PCR product. The primer-PCR product mixtures (2 per colony, 48 altogether) are submitted to the university's DNA core facility for sequencing using an Applied Biosystems 3730 × l 96-capillary DNA Analyzer with Big Dye Terminator

Figure 3. Sequence alignment of the parental red gene with the mutant red gene observed in 23 red canavanine-resistant colonies. The Dish 1 and Dish 2 colonies are from jackpot individual culture dishes 1 and 2 in Figure 1; the Dish 3–10 colonies are single colonies from individual culture dishes 3–10 in Figure 1. Bold letters in the colony sequences indicate nucleotides that differ from the corresponding nucleotides in the parental gene; dots indicate positions where the colony sequence and the parental red gene are identical.

cycle sequencing chemistry. The instructor edits the data in preparation for Session 5.

In Session 5, an online program such as Clustal-omega is used to align the edited DNA sequences in order to identify the red gene mutation in each colony. First, the information from the two complementary sequences from each colony is merged to create a single consensus sequence; since the sequences overlap throughout the 89-bp red gene, there is almost never any ambiguity in that gene's sequence. Next, the consensus sequences are aligned with one another and with the sequence of the nonmutant parent strain in order to identify the mutation in each red colony. Typical results are shown in Figure 3. All clones from a single jackpot dish have identical mutations, whereas clones from different dishes have different mutations. This fully corroborates the pre-exposure hypothesis, which explains jackpot mutants on a single dish as identical by descent; but argues against the post-exposure hypothesis, which can only explain the outcome as multiple improbable coincidences.

Colony sequencing acquaints students with two foundational technologies of modern biology, PCR and DNA sequence analysis. Learning how they work reinforces an understanding of the biology of chromosome replication. Aligning the resulting sequences in order to identify canavanine resistance mutations (Session 5) highlights the indispensable role of bioinformatics in life sciences.

○ Further Class Discussions

While fluctuation tests provide definitive evidence for pre-exposure mutations, they don't rule out the possibility of post-exposure mutations. That's because the lethal agent (canavanine in our case) kills parent cells before they have a chance to adapt. Modified fluctuation tests in which the selective medium allows parent cells to survive, but not to form colonies unless they acquire a new growth-permitting mutation, have in some cases revealed mutations that are post-exposure by stringent criteria (reviewed by Rosenberg, 2001). Post-exposure mutations are generally *induced* by the stress imposed by the selective medium. For instance, bacteria under stress may adopt a hypermutable state by expressing error-prone DNA polymerases and DNA repair systems (Rosenberg, 2001). Breast cancer cells with pre-exposure mutations conferring genetic instability may, under the stress of estrogen deprivation, acquire new mutations facilitating growth in low estrogen (Robinson et al., 2013). Although stress-induced mutations may preferentially target a subset of genes, there is no convincing evidence that they are *directed* (i.e., specifically targeted to a subset of genes that can mutate to permit growth). Stress-induced mutations are an adaptive response to the stress, but as far as we know they are still "Darwinian" rather than "Lamarckian," in that they are not specifically directed to genes that can relieve the stress.

Environmental stress and developmental signals can induce another kind of heritable change: epigenetic modifications, which are DNA or chromatin marks that alter gene expression without changing the DNA sequence. The modifications are readily reversed, a process that is itself an epigenetic modification. These modifications (including their reversal) are often both adaptive and directed to specific genes. Do they therefore qualify as Lamarckian "mutations"? To regard either stress-induced mutations (previous paragraph) or epigenetic modifications (this paragraph) as "evolution" is to stretch the meaning of the term. In each case, what have evolved are the cellular pathways that mediate the responses, not the individual responses themselves. The mediating pathways have evolved over time by Darwinian variation and natural selection, the same process explored in miniature by the students' fluctuation-test investigation.

○ Acknowledgments

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