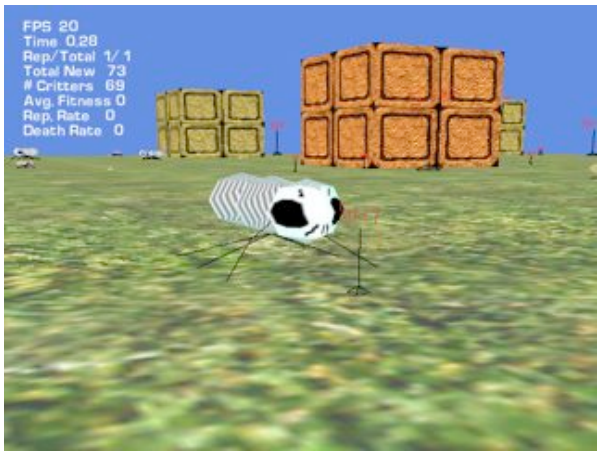
CRITTERS!

An Inquiry-based Laboratory Simulation for Teaching Evolutionary Biology



Student Guide

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Introduction

Welcome to the Student Guide for the Critters! simulation environment! Our goal is to provide information to assist you in the use of the Critters! model to learn about evolutionary biology. Be sure to consult the ReadMe! file (an Appendix to this document) for details on the model, its interface, and keyboard controls. We hope you have fun with this learning tool. Best wishes with your research!

How Evolution Occurs

Organisms express physical traits based upon their genetic complement. We refer to units of genetic information as *genes*. Genes code for polypeptides, which result in proteins that are either part of the physical structure of a living organism or participate in the organism's metabolism. Genes are passed from parent to offspring at the time of reproduction. We call the genetic makeup of an organism its *genotype*, while the physical and physiological traits are called the *phenotype*.

Genes exist in multiple versions, called *alleles*. Populations usually contain more than one allele for each gene, each of which exists at a given frequency. An example will illustrate the concept of an allele frequency: Assume that two alleles exist for flower color in a population of plants (dark blue and light blue). These plants are diploid and one gene codes for flower color, so each plant has two alleles for this trait, 500 plants = 1000 alleles. Of 1000 alleles in the population, 800 of the alleles are for expressing the dark blue flower, while 200 are for expressing the light blue color. We would say that the frequency of the dark blue allele is 0.80 (i.e., 800/1000 = 0.80 or 80%). The frequency for the light blue allele is 0.20. Evolution occurs when these allele frequencies change for one or more of several different reasons. Allele frequencies are known to change due to: (1) mutation, (2) gene flow, (3) genetic drift, (4) non-random mating, and (5) natural selection.

Mutation

Alleles are fundamentally different nucleotide sequences in DNA. Structural changes in DNA may lead to information changes that produce new alleles in a population. [Note that changes in DNA don't always produce a different allele, because some nucleic acid substitutions do not result in a different amino acid in the final polypeptide product.] The changes in DNA that produce new or different alleles are known to occur by two mechanisms: mistakes in copying DNA and chemical changes associated with radiation and compounds that interact with DNA.

The mechanism of copying DNA relies upon a class of enzymes called transcriptases. These enzymes are not perfectly reliable copiers of DNA. Although many of them have proofreading capabilities to correct copying errors and other enzymes that can interact with DNA to repair a bad copy of an allele, mistakes slip past these systems occasionally and remain part of the organism's DNA. Mistakes in copying DNA that will become the DNA of gametes (i.e., sperm or eggs) or DNA in cells destined to become daughter cells by simple division (e.g., bacterial reproduction) are the source of variation that can produce evolutionary change.

The other mechanism is well known to anyone with a few freckles on skin that has been exposed to the ultraviolet radiation of the Sun. Ultraviolet radiation can cause mutations in DNA. When skin is exposed to the Sun, some cells' pigment alleles are damaged. These cells may otherwise be quite normal and proliferate to produce a freckle on the skin surface that is visible to the naked eye. If alleles are damaged that involve the normal cell cycle such that the cells divide out of control, the person will suffer from a

melanoma (skin cancer). Besides UV radiation, many chemicals are DNA mutagens (e.g., asbestos, benzene, DDT, many others).

All populations examined by biologists are subject to constant mutation. Mutation is usually neutral or bad for an organism because it usually has no influence on protein structure and function or it interferes with the normal structure and function of proteins. Very occasionally, the mutation produces a new allele that is beneficial. When such alleles are present in the sex cells (i.e., sperm or eggs), they may be transmitted to offspring. With mutation constantly changing populations' allele frequencies, mutation is an important evolutionary mechanism that generates variation for natural selection (described below) to act upon.

Gene flow

Gene flow is the term given to changes that result in the input of new alleles or the loss of alleles due to migration. Returning to our dark and light blue flower allele frequencies from above, what would happen to such a system if 100 plants arrived into the population that only had dark blue flower alleles? Each plant would have two alleles for the trait, so the 100 individuals would arrive with 200 dark blue alleles. Because these migrants are the same species as the initial population, they can interbreed with the existing population – we can take the initial population and the migrants as one new larger population. If we assume that all of the migrants mate evenly into the existing population and we recalculate the allele frequencies, we find that they have changed simply due to the input of the new alleles: 1000 dark blue alleles/1200 total alleles = 0.83. The allele frequency changed from 0.80 to 0.83 – this is evolution by gene flow.

Genetic drift

If we start with a population of 500 plants having an allele frequency of 0.80 dark blue flower alleles and a hurricane hits and kills 300 plants, the remaining 200 plants will almost certainly have a slightly different allele frequency by chance alone. If the allele frequency changes in a random way, we called this type of evolution *genetic drift*. Drift can completely eliminate rare alleles in populations because a handful of individuals with a rare allele could die in any generation and not pass the allele on to the next generation.

Genetic drift is often associated with the *bottleneck effect*, where large numbers of individuals die in some catastrophe leaving a handful of individuals with a different genetic makeup than the original population. Bottleneck events can drastically reduce the genetic variability of a population. A second type of genetic drift occurs with the *founder effect*: When a few individuals of a large population colonize a new area. The new population will likely only have a fraction of the genetic variation of the original gene pool. Among other interesting facts, this has contributed to our current understanding that humans evolved in Africa and moved out of Africa to colonize Europe and Asia – the genetic variation of African populations is the highest in the world.

Non-random mating

Non-random mating occurs when either gender of a sexually-reproducing species chooses a mate based upon the presence or absence of an inherited trait or when individuals mate due to circumstances of their location in the environment. Imagine a situation where the females of a population selectively mate with certain males. Such mating choices will change the allele frequencies of a population. Usually, the traits that the females are using to select mates will become more prevalent in the population over time. This mechanism is at play in peafowl populations, where sexual selection by females has resulted in large fancy male tail feathers with many eyespots.

Natural selection

Natural selection is the differential survival and reproduction among organisms that have heritable traits that confer an advantage in a given environment. Natural selection is the only mechanism that directly leads to adaptation. Those organisms that have the best set of traits for survival and reproduction in a given environment produce more offspring on average than organisms with traits less appropriate for survival and reproduction (Darwin, 1859). On average, 'fitter' organisms contribute more alleles than less fit organisms. The consequence of changes in allele frequencies due to natural selection is *adaptation* to the environment. An adaptation is a phenotype that increases the survival or reproduction of an individual compared to other individuals without the trait.

Natural selection cannot act upon traits that (1) have no basis in genetics (are not heritable), (2) have no variability within the population, or (3) do not confer a benefit (or a reduction) to survival and/or reproduction. Because Darwin's postulates generate testable predictions, each can be studied in the field and laboratory using the scientific method.

The Genetics of Critters!

Except for gender, the genetic model implemented in Critters! is polygenic; that is, each of the phenotypes expressed by the individual organisms (e.g., speed, adult size, resource attractiveness) are the product of several genes.

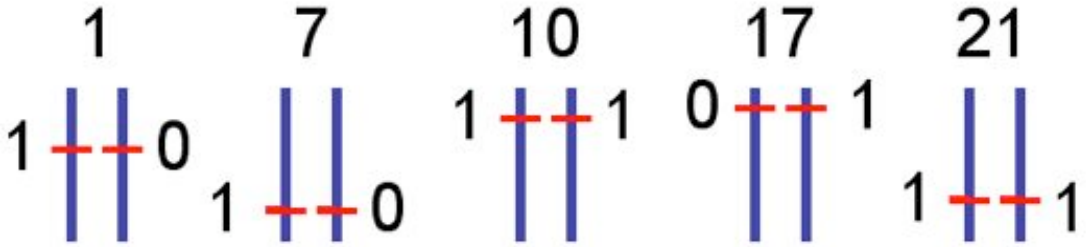

Figure 1. Speed alleles for a hypothetical critter

Take the speed of a critter for example. There are 5 genes that code for the speed of a critter. At the beginning of a simulation, the positions of these genes (called 'loci') are randomly determined. In Figure 1, we see that speed allele loci are on homologous chromosome pairs 1, 7, 10, 17, and 21 (one chromosome of each pair came from the mother and one from the father). The speed allele values are either a 1 or a 0 (zero). To get the speed of this critter, we simply add up the allele values: 1+0+1+0+1+1+0+1+1+1 = 7. This critter expresses a phenotype speed of 7 steps per turn in the model. Notice that polygenic inheritance as illustrated here is sometimes called 'additive inheritance' for this reason: The sum of the influences of individual alleles produces the phenotype. All of the traits in Critters! are determined in this way except for the gender of a critter. For gender, one gene with two possible alleles determines the gender. Homozygotes are female while heterozygotes are male.

The chromosomal-based inheritance in Critters! allows them to make gametes via meiosis with genetic recombination. Further, when mutation strikes one of these loci, it simply converts the allele value at that position to the other value (e.g., for speed alleles 1 and 0, a 1 is converted into a 0 or a 0 is converted into a 1). Because the total number of alleles in the population at a given time is easy to determine and the actual number of 'high speed' alleles is also easy to add up, we can calculate the allele frequency for the 'high speed' allele easily. For example 100 critters would have 1000 alleles for speed (100 critters × 5 genes/critter × 2 alleles/gene = 1000 alleles). If the total number of 'high speed' alleles in the population were 650, then the allele frequency for the 'high speed' allele would be 0.65 for the population (650 'high speed' alleles/1000 total alleles = 0.65). A change in this frequency would represent evolution by one or more of the mechanisms outlined above. Critters! gives students an opportunity to study evolution because the important factors in the evolutionary biology of real populations are part of the model.

Research Basics & Using the Model

The Scientific Method

Experimentation with deduction followed by inference is critical to scientific progress. The scientific method is an orderly process of deductive reasoning, where the observer concisely states at least two hypotheses and puts those hypotheses in direct contact with nature by designing and conducting one or more experiments that will rule out hypotheses that are not supported. Following the application of deductive reasoning in the rejection of unsupported hypotheses, we reason with inference that our supported hypotheses will also hold for other similar situations in nature.

The steps of the scientific method include: (1) formally stating a question about nature, (2) formally listing mutually exclusive explanations for the phenomenon at issue, including one statement that reflects a lack of knowledge about what will occur (the null hypothesis), (3) a carefully designed experiment (or set of experiments) to rule out hypotheses that are incorrect, (4) the collection of data (results) of experimentation, and (5) a conclusion on which hypothesis seems to garner the most support and an answer to the original equation if the null hypothesis is rejected.

When we follow these steps, a great wealth of understanding is produced. Scientific investigation is a *process* to obtain knowledge. Secondary school students and undergraduates should explicitly practice using the scientific method in preparation for active research careers.

Controls & Treatments

In experimental design, there are two classifications of groups to make an experiment: the control groups and the treatment groups. We do not manipulate the control groups. We manipulate or influence the treatment groups. By comparing the data collected from the two types of groups at the end of the experiment, we learn if the treatment made a difference.

Hypotheses

In the traditional, statistical approach to stating hypotheses, the first hypothesis (the *null hypothesis*) states that the treatment has no effect on the outcome (no difference will be seen between the control and treatment groups). In addition to the null hypothesis, there will be one or more mutually exclusive *alternate hypotheses*. The alternates express the notion that a change will occur in response to a treatment.

For example, we could ask, “What is the influence of high mutation on natural selection?” The null hypothesis is “There is no relationship between high mutation and natural selection.” We can state two alternate hypotheses: (1) “High mutation increases the strength of natural selection,” and (2) “High mutation decreases the strength of natural selection.” Notice that all three statements are mutually exclusive. Our control groups are populations exposed to a normal rate of mutation. Our treatment groups are

populations exposed to higher than normal rate of mutation. If we find that the response of natural selection is stronger in the treatment group than in the control group, we can reject the null hypothesis and alternate hypothesis #2. If weaker, we reject the null hypothesis and alternate hypothesis #1. If we do not find a difference between control and treatment groups, we say that *we cannot reject the null hypothesis*.

When the treatment and control groups are not different, it is easy to make the mistake of thinking that the null hypothesis is somehow correct. This is NOT the case! *A failure to reject the null hypothesis is not support for the null hypothesis*. We merely have the finding that our alternates are not correct – there might exist another explanation that we did not test that could be correct, our statistical test may not have been powerful enough to rule out chance as an explanation for a small difference, or there is even the possibility of a poor experimental design, bias, or measurement error; therefore, we never know from experiments that do not rule out the null hypothesis if the null hypothesis is correct. This is a general property of scientific inquiry: Scientists do not prove hypotheses are correct and especially never find support for the null hypothesis. Scientists find support for hypotheses and sometimes fail to reject a null hypothesis. The old expression "scientific proof" is meaningless in science, and these points are very important for young researchers to learn.

Experimental Design

Experimental design often involves creatively producing methods and tools to assess the states of living things in the control and treatment groups. Because it is fundamentally a creative process, it is very difficult to come up with hard and fast rules. There are a few broad generalizations that can be made: (1) attempt to control for variability in nature, (2) attempt to remove bias in sampling, and (3) attempt to control measurement error.

Variability is a serious problem. Organisms are highly variable in their anatomy, physiology, and behavior. Populations of organisms are not stable assemblages. Communities of populations are even more chaotic than populations. We seek to remove the wild fluctuations in parameters that we measure to see the differences. This is where replication and statistics enter. To control for variation, we set up multiple control groups and multiple treatment groups and compare them with a special type of mathematics called *statistics*, which helps us to determine if a difference is likely or unlikely given the data and replication we have in our study. Secondary school students cannot be expected to learn detailed statistics, but they can be expected to understand that using an average and a measure of variation (e.g., standard error, confidence interval) is required to make a determination if two groups are different.

In Figure 2, we see that the average weights of two groups of crickets are different. Students might mistakenly say that the treatment had a clear influence on cricket weight. Not so! Examine Figure 3, which shows the averages and the data collected to make those averages. Notice that the points of both groups largely overlap. If only the students take the additional step of computing a simple standard error (a measure of confidence about the average), as seen in Figure 3, they would not likely make the mistake of thinking that the treatment made a difference. The data here reveals that the

difference in the averages is not *significant*. The word "significant" has a special meaning in science to denote that statistics have provided some measure of confidence that two groups are different. When scientists report that a treatment had an effect, they implicitly mean a "statistically significant" effect.

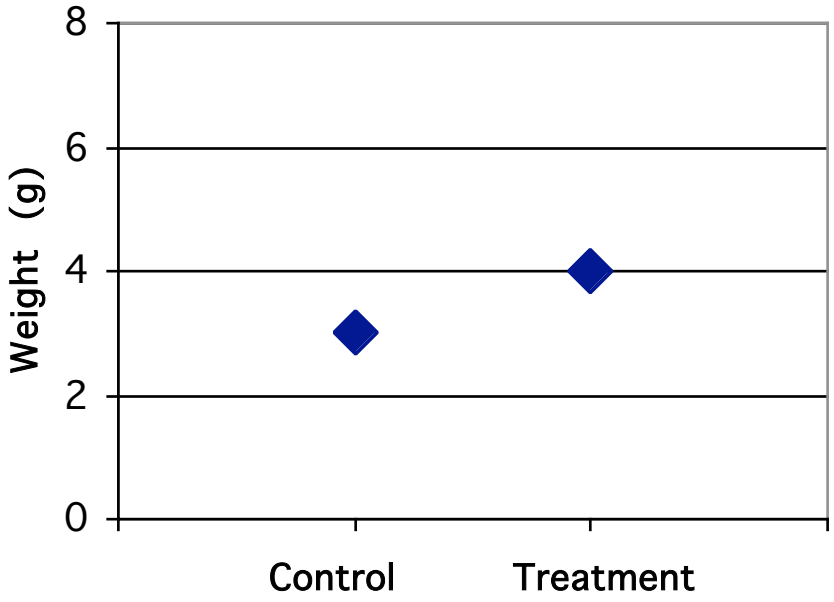

Figure 2. Average size of control and treatment crickets after a feeding study

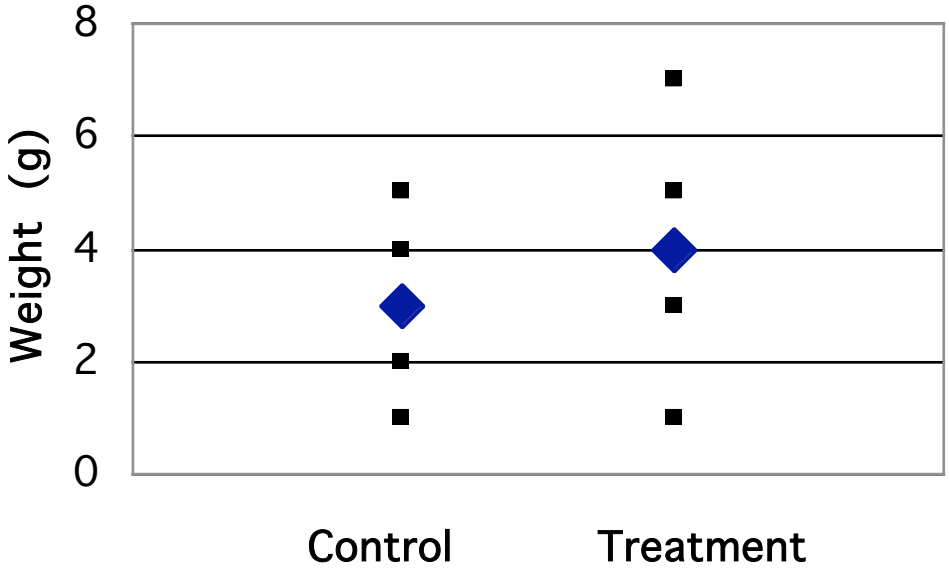

Figure 3. Averages shown with the data that generated the averages

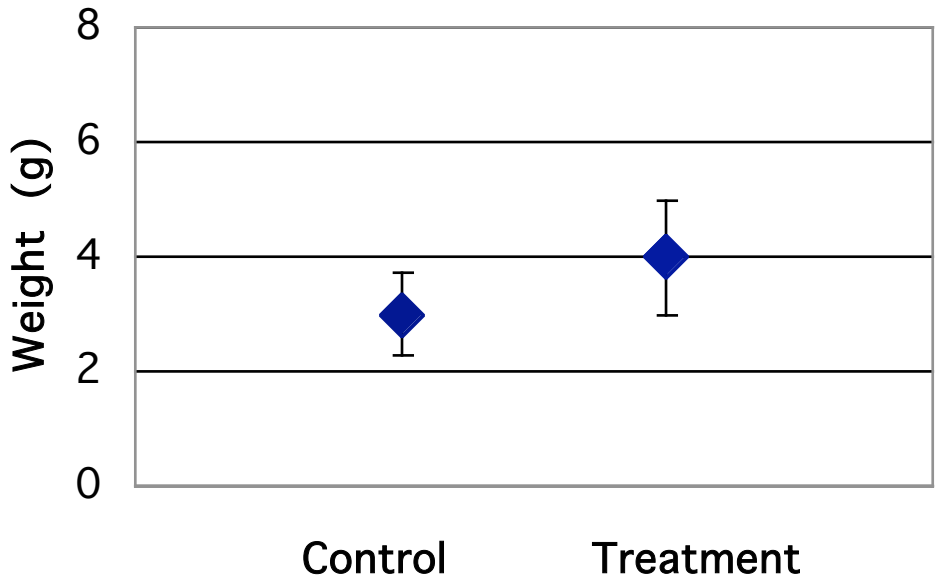

Figure 4. Averages with a measure of variation
(one standard error above and below the averages)

Even if a significant difference is found, we still might be wrong. Statistics is not about proving a statement correct – that cannot be done. If we say that we are 95% sure that a difference is present, then by default there is a 5% chance that we are wrong. The cutoff of 95% (1 chance in 20 that we are wrong) is considered good enough in science to reject the null hypothesis and state that a difference was found. It is also interesting to note that the 5% chance of making a mistake and rejecting a null hypothesis when we shouldn't also gives us a fair amount of *power* in our test. That is to say, if we were to set the bar as high as say a 1% chance of making a mistake, we may rarely find differences in nature because our test loses power to separate groups when they are really different.

Confidence Interval Calculations

A simple way to infer that two groups are probably different is to compare their 95% confidence intervals. A 95% confidence interval is a range of values were you can be 95% sure that that true average is in the interval. The confidence interval takes into account ordinary sampling error. When the intervals of two groups overlap, you can infer that any difference between the averages of the two groups could be explained by chance alone.

Here is an example of how to calculate a confidence interval. You will need 4 pieces of information to calculate the interval: (1) The mean of the group ($\bar{y}$), (2) The standard deviation (s), (3) The number of observations (n), and (4) the critical value of 95% confidence for the degrees of freedom ($df = n-1$).
Observations (y): 10, 19, 13, 15, 14, 19, 11, 21, 13

(1) The mean: $\bar{y} = \frac{\sum y}{n}$; $\frac{10+19+13+15+14+19+11+21+13}{9} = 15$

(2) The standard deviation: $s = \sqrt{\frac{\sum (y-\bar{y})^2}{n-1}}$ = 3.8 (see calculation table below)

Observation	**Mean**	**Difference** ($y - \bar{y}$)	**Squared Difference** $(y-\bar{y})^2$
10	15	-5	25
19	15	4	16
13	15	-2	4
15	15	0	0
14	15	-1	1
19	15	4	16
11	15	-4	16
21	15	6	36
13	15	-2	4
		Total [$\sum(y-\bar{y})^2$]	118
		Divide by n-1 $\frac{\sum (y-\bar{y})^2}{n-1}$	14.8
		Take square root $\sqrt{\frac{\sum (y-\bar{y})^2}{n-1}}$	**3.8**

(3) Number of observations: $n = 9$

(4) Critical value (95% c.i.; $df = n-1 = 8$): critical value = 2.306 (see table below)

df	Crit. Val.	df	Crit. Val.	df	Crit. Val.	df	Crit. Val.	df	Crit. Val.
1	12.706	5	2.571	9	2.262	13	2.160	17	2.110
2	4.303	6	2.447	10	2.228	14	2.145	18	2.101
3	3.182	7	2.365	11	2.201	15	2.131	19	2.093
4	2.776	**8**	**2.306**	12	2.179	16	2.120	20	2.086

Calculation of the confidence interval:

$$\bar{y} \pm \text{Critical Value} \bullet \frac{s}{\sqrt{n}}$$

$$15 \pm 2.306 \bullet \frac{3.8}{\sqrt{9}} = 15 \pm 2.9 = [12,18]$$

If the confidence intervals of two groups overlap, then we may infer that any difference in the group averages could be explained by chance (i.e., sampling error); therefore, we will not reject the null hypothesis. If the confidence intervals of two groups do not overlap, then we may infer that chance alone probably does not explain the difference; therefore, reject the null hypothesis.

Two other important issues are sampling bias and measurement error. It is obvious that we should not allow our own bias to interfere with collecting a random

sample. Our bias can easily change the outcome. When we take measurements, we will always find some error in our estimates. We should always seek to control for measurement error with carefully designed and properly used tools. You should get experience in confronting your bias in sampling and the potential for measurement error to obscure results in your projects. Statistical tools, such as forming confidence intervals, do not take into account error outside of sampling error. Statistical methods cannot assist you when you make poor assumptions, state flawed hypotheses, use uncalibrated equipment, make mathematical calculation errors, or record your data incorrectly. These topics are excellent discussion points in your group work as you work out your experimental design.

Controlling Mutation

Mutation in Critters! is the average number of mutations (allele conversions) per gamete produced. A value of 0.1 is interpreted as 1 mutation in 10 gametes, and a value of 1 would be 1 mutation on average per gamete. The Critters! defaults file has an entry for setting the default value of mutation in the model. The value may also be changed in the interface (Figure 5).

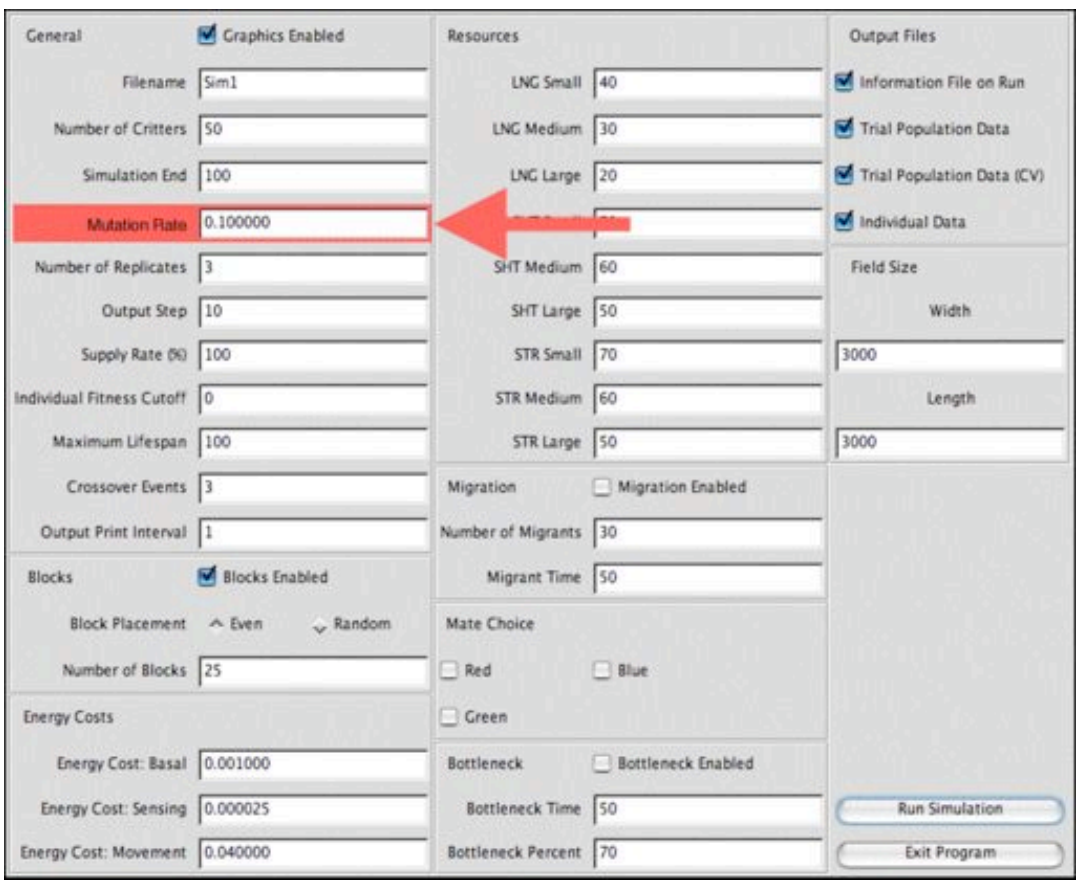

Figure 5. The control of mutation rate is in the interface

Influencing Gene Flow

Introducing a given number of randomly generated critters into the model at a given time during the simulation produces gene flow. The number of critters to introduce and the time to introduce them is in the defaults file, and a switch in the interface will allow you to change these parameters in the interface (Figure 6).

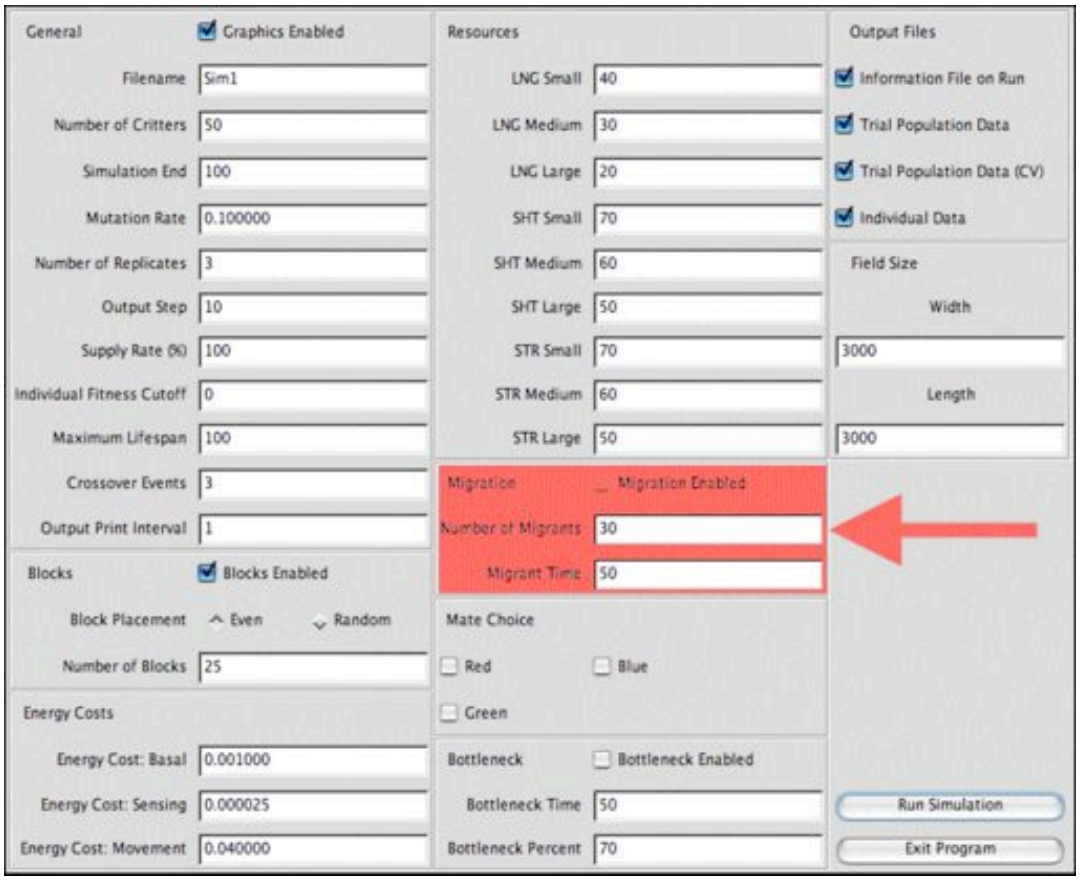

Figure 6. Clicking the switch activates the control of migrants

Genetic Drift

Drift is a natural phenomenon for allele frequencies in the model under most conditions. Critters! contains a switch to turn on a bottleneck event during the run. You can specify when you would like the bottleneck event to occur and what fraction of the population you would like to kill (Figure 7).

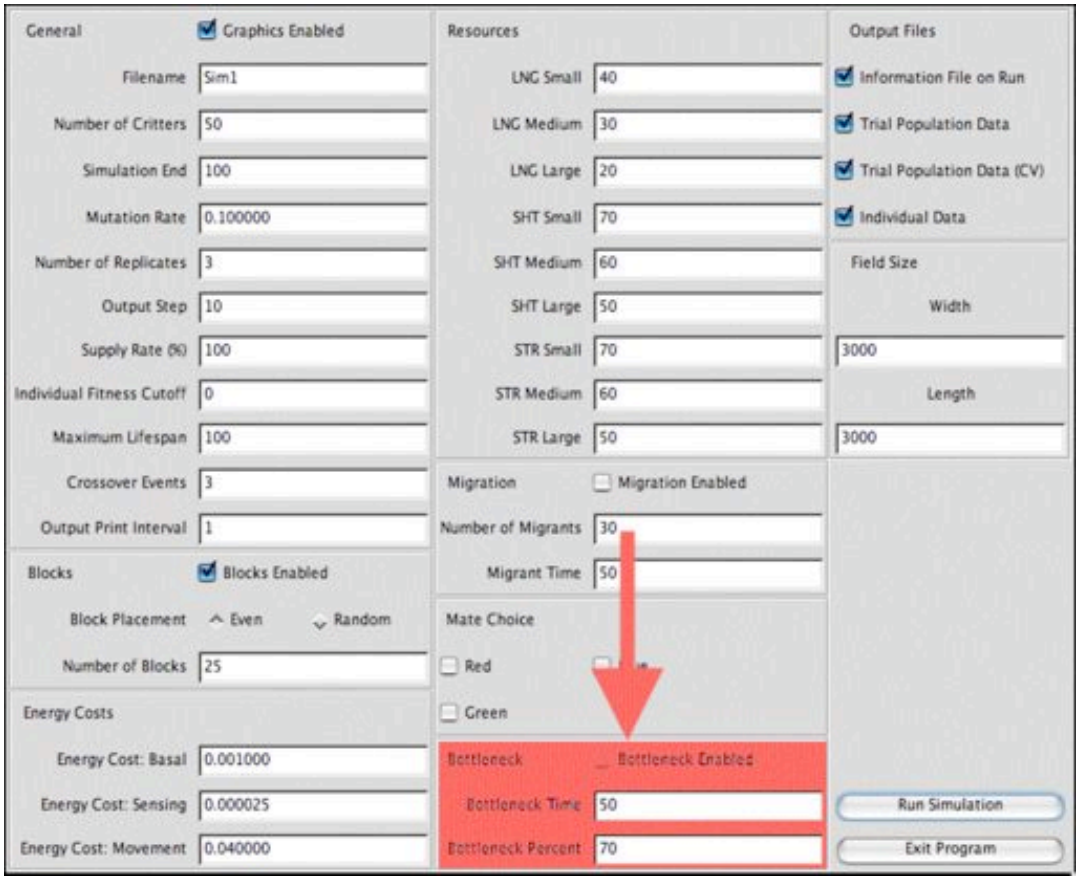

Figure 7. Switch to activate a bottleneck event and entries to control the time and size of the event

Activating Non-random Mating

Non-random mating in critters is determined by sexual selection by male critters. Ordinarily, males will be attracted to any fertile female in their vicinity. With one or more of the mate choice switches turned on (in the defaults file or in the interface, Figure 8), males will preferentially mate with females that have a color value higher than the average color value for that color in the population. For example, if the red switch is turned on, males will only mate with females that are redder than average. Note that it might be difficult to get a surviving population with all three switches turned on because males might be so picky that mating rarely occurs and the population crashes.

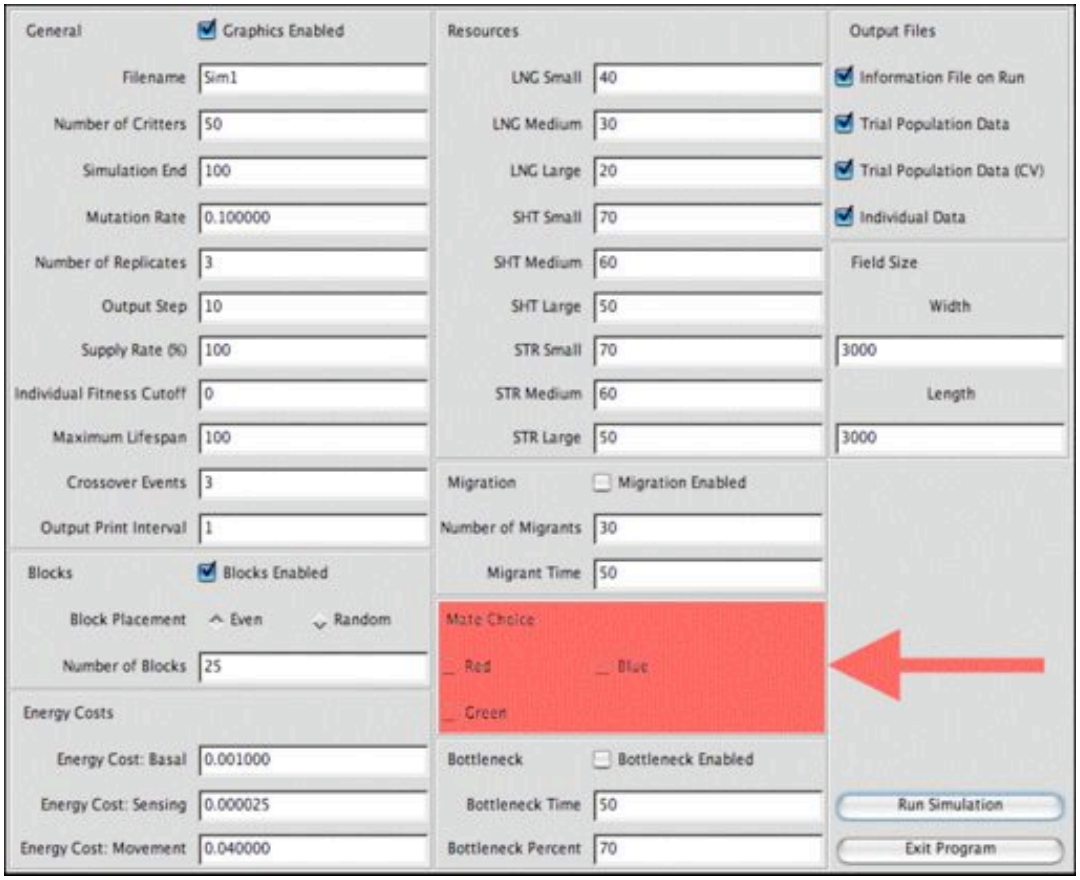

Figure 8. Clicking the switches activates the control of male color choice in mating

Natural Selection

Natural selection is the granddaddy of evolutionary change. Mutation, genetic drift, gene flow, and non-random mating were unknown before Darwin's work because biologists of the day had little understanding of genetics until Mendel's work was rediscovered in the early 20th century. Natural selection is a process that produces adaptation. An adaptation is a feature of an organism that confers higher fitness (reproductive capacity) over other organisms without the feature. These features are produced in a process involving variability, heritability, biotic potential, and differential survival and/or reproduction.

First, a trait such as size, speed, or color must be variable. If there is no variation in the trait, then the process of natural selection cannot occur. Critter variation is easy to show by graphing the phenotypes of individual critters. You can see the common bell-shaped curve – many critters have a value on or close to the average while fewer critters have more extreme values.

Next, the trait must be heritable; that is, it must be a trait that is controlled to some degree by genetics such that it can be passed from parent to offspring. Many of the traits in critters are explicitly produced by genetics. For example, color is explicitly produced by a set of genes for each of the color values red, green, and blue. The color of a parent contributes directly to the color of an offspring with no influence of the environment. Other traits are not solely determined by genetics. For example, age at time of death is set

to a maximum of 100 clock units. When critters do not live this long, this trait is determined by a complex combination of how many times they could mate (genetically controlled) and their ability to feed (a complex interaction of their phenotype for speed, adult size, and attractiveness to resources, etc.). Age at the time of death is a complex trait determined by both genes and the environment. To ensure that a trait is heritable, we graph the mid-parent value of a trait (the average phenotype of the parents) against the mid-offspring value of the trait (the average phenotype of the offspring). If a regression line on these data points has a significant positive slope, the trait is heritable. If the slope is close to 1, then the trait is determined entirely by inheritance (any deviation due to mutation and genetic drift).

Third, resources must be limiting in such a way that more offspring are produced than survive to reproduce. This sets up a competition among the critters for survival and reproduction.

The three factors above lead directly to non-random survival and reproduction: Those organisms with the best average set of traits produce more offspring (and contribute more alleles) to the next generation than less fit organisms. For important traits, the allele frequencies change in favor of the allele that confers a higher (or lower) value of the trait that produces the higher fitness.

Differential reproductive output is also easily studied with the Critters! model. You can look at the fitness (number of eggs laid) for groups of critters expressing different phenotypes at the same time in the model by pulling data from the Individual data files produced, which detail every critter that lived during the replicate model runs. If natural selection is at work, fitness will be significantly higher for one group over another group (e.g., red critters over green critters, small critters over large critters).

Frequency-dependant Selection

Frequency-dependant selection occurs when an individual with a rare trait has a higher fitness than the bulk of the individuals in the population that do not have the trait. The trait is usually something that interacts with mate choice, predator-prey relationships, or competition. For example, color is not explicitly tied to fitness in the Critters! model (when the 'Mate Choice' switches are all 'OFF'). If a predator arrives and has an easy time killing light colored critters just because they are common and the predator develops a search model for prey that is based on the most common color, then the darker color critters will be the subject of frequency-dependant selection. They will have a higher fitness than their lighter-colored brethren simply due to the fact that they are rare. In the future, if they become very common, frequency-dependant selection will end when the predators change their search strategy and look for darker critters.

To act as a predator in the model, keyboard commands allow a target to appear on the field (Figure 9). You can determine the prey search strategy and move through the environment killing critters that match the prey model (Figures 10, 11). You can also go into 'resource target mode' to go into competition with groups of critters by removing specific resources from the field as the model runs (Figures 12, 13). These acts can be performed while the model is paused giving you plenty of time to make for very strong selection (details of the keyboard commands are in the Read Me! file).

Figure 9. Activation of 'target mode – critter' reveals
a crosshair on the screen; pressing 'i' provides
an information readout on the screen

Figure 10. Moving the crosshair under a critter reveals
its particulars in the information readout

Figure 11. Tapping the spacebar kills the critter;
an entry in the individual data file notes that
this critter was killed by 'predation'

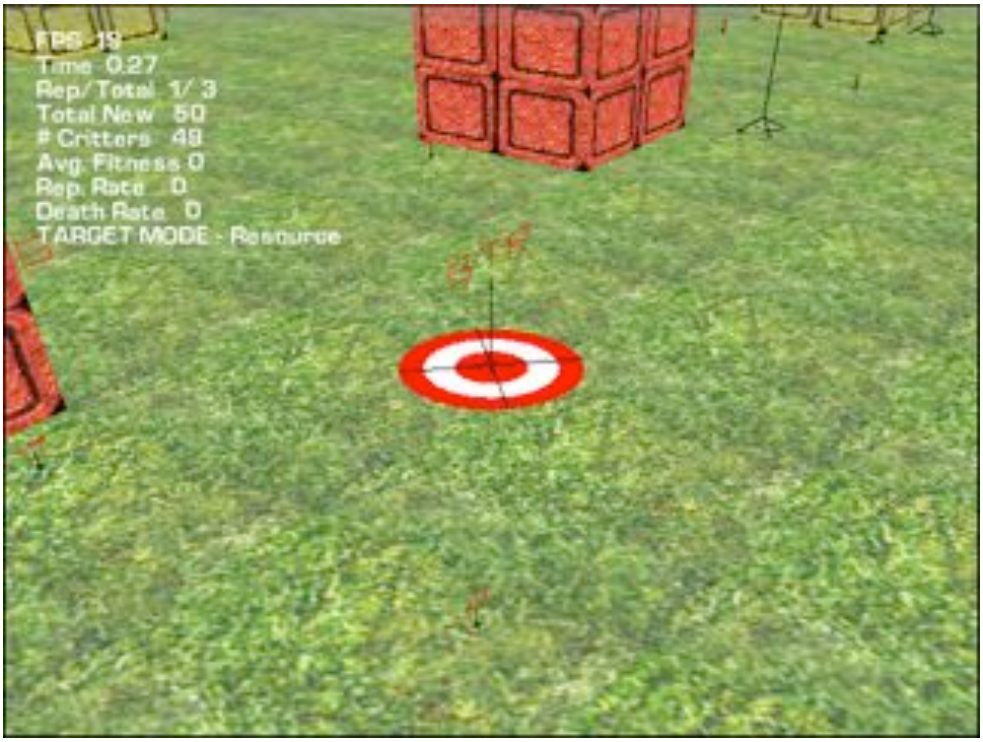

Figure 12. While in 'target mode – critter,' tapping
the 'r' key puts you into 'target – resource' mode

Figure 13. The crosshair may be moved under
a resource; tapping the spacebar removes the resource

Notes and Suggestions

Your instructor will not help you during your inquiry-based project. You will only take full ownership of your project if you are given plenty of flexibility. You can study any of the evolutionary mechanisms using whatever experimental design you wish. It might mean that every group in the class decides to study the same aspect of evolution. Research has shown that this form of education will enable you to learn more about evolutionary biology than listen-and-learn lectures and short-term memory-taxing exams.

You might consider that your project has ended in a disaster where your group is unable to reject the null hypothesis for the simple reason that you botched the experimental design. No, it is not a disaster! You can learn a great deal from failure (as we all do). Your project will mostly be judged on what you say about what you did …not what you actually did.

Critters! has even more to offer outside of the items listed above: initial population size, block number and placement, resource distribution and supply rate, energetics, and maximum lifespan are all aspects of critter lives that you may address in experiments by running the model under different scenarios. You can even go so far as to modify the core genetics values (i.e., values of the alleles) in the Critters! defaults file. You are strongly encouraged to have fun with it!

Exercise 1: Working with Hypothesis Statements

1. Mark each of these as either a null hypothesis or an alternate hypothesis:

A. There is no relationship between fur thickness and body temperature in seals.
☐ Null ☐ Alternate
B. Predators find it more difficult to spot green tree frogs on green surfaces.
☐ Null ☐ Alternate
C. Tree growth rate is not related the annual rainfall amounts.
☐ Null ☐ Alternate
D. Maximum tree growth rate is seen where the annual rainfall is greater than 100 cm.
☐ Null ☐ Alternate
E. A zero tree growth rate is seen where the annual rainfall is less than 5 cm.
☐ Null ☐ Alternate

2. State a null and alternative hypothesis for each of the following questions:

A. Is the rate of digestion in garter snakes a function of body temperature?

Null:

Alternate:

B. Does a marsh have greater species richness (number of species) than a forest of the same area?

Null:

Alternate:

3. Can you state two mutually exclusive alternate hypotheses for the following?

Question: What is the relationship between sunlight intensity and plant growth?
Null: There is no relationship between sunlight intensity and plant growth.

Alternate #1:

Alternate #2:

4. What is wrong with the following null and alternate hypothesis statements?
[Note: There are two things wrong here.]

Question: What is the relationship between water temperature and fish schooling?
Null: Water temperature and fish schooling are related.
Alternate #1: The higher the water temperature, the greater the fish schooling.
Alternate #2: The lower the water temperature, the lower the fish schooling.

Exercise 2: Experiments, Bias, and Error

1. Label the following variables as independent or dependent:

A. In a study on the effectiveness of rat poison, researchers fed rats food laced with poison and food laced with a placebo. They counted the number of surviving rats after 5 days.

The quantity of poison:	☐ Independent	☐ Dependent
The number of surviving rats:	☐ Independent	☐ Dependent

B. In a study on the influence of lunar cycles on frog calls, researchers constructed a simulated field in the laboratory where they could control the appearance of an artificial moon. They counted the frequency of calls in response to the artificial lunar cycle.

The artificial lunar cycle:	☐ Independent	☐ Dependent
The frequency of frog calls:	☐ Independent	☐ Dependent

2. In the frog example above, name eight things that the researchers should attempt to control in their experimental design:

A. E.

B. F.

C. G.

D. H.

3. In the frog example above, the researchers use a bullfrog on 10 nights with short lunar cycles and 10 nights with long lunar cycles. They tabulate the frequency of calls for short and long lunar cycles. They find that under the long lunar cycle, the frequency of calls from the frog was higher. They send this result to a journal for publication, but the editor rejects the paper immediately. Why?

4. If we were studying the effect of rat poison on rat memory, we could give rats small doses of rat poison (treatment group) and placebo (control group). We could compare the performance of control and treatment rats in mazes. If we incorrectly measured the time it took rats to reach the food in the maze with an uncalibrated or broken stopwatch, what type of error would this generate? Can statistics overcome the error and report a robust result?

5. Name something in the rat study that cannot be controlled that could lead to an incorrect conclusion:

Exercise 3: Analyzing Data with Statistics

1. Why can't researchers just use averages to tell if two groups of measurements are similar or different?

2. An entomologist [a biologist who studies arthropods (insects)] wished to learn if the respiration rate of earthworms is influenced by the chemical cues left by earthworm-eating garter snakes. A group of 20 worms was randomly divided into two groups: 10 control worms and 10 treatment worms. The researcher exposed each of the 10 control worms to ordinary newspaper shreds for ten minutes and found their respiration rates with a calibrated gas analyzer before and after exposure. The differences in their respiration rates were recorded. The researcher exposed each of the 10 treatment worms to newspaper shreds for 10 minutes that had contact with garter snakes for 24 hours, and the differences in their respiration rates were recorded. The following data (change in respiration) was obtained:

Control (n=10; μmol CO_2/h, 20°C)	Treatment (n=10; μmol CO_2/h, 20°C)
–10	20
–18	19
0	–2
0	13
–6	–8
6	16
12	18
–5	12
–15	–3
–7	14

Find the 95% confidence interval for each set of measurements. Can you reject the null hypothesis (earthworm respiration rate is not related to snake chemical cues) in this case?

Exercise 4: Proof, Error, and Nulls

1. If you create 90% confidence intervals for your data, what is the chance that in each case the true mean lies outside of the interval?

2. There is no such thing as "scientific proof." Why not? If you do a carefully controlled experiment, why can't you prove that something is true?

3. If I incorrectly report that incubation temperature controls the gender of bluebird offspring from a carefully controlled set of observations and with the proper use of a statistical test, what type of error have I committed? Is that the more serious or less serious type of statistical error? Why?

4. After a study on bat guano in South American caves, an investigator can find no relationship between the amounts of guano deposited per year and the frequency of tourist visits to five frequently visited caves. The researcher reports that cave visitation does not interfere with bats in these caves based upon this result. What is wrong with the researchers conclusion?
[Note: There are two possible answers here.]

Appendix: Critters! Read Me! File

Critters! Version 2.0 ©2006 Luke G. Latham II
Read Me! File - Updated 2/3/06 ©2006 Luke G. Latham II

I. SYSTEM REQUIREMENTS (Apple Macintosh)
A. Apple PowerMac (400 MHz G4 or better recommended)
B. Mac OS 10.x.x (OS 10.4.4 recommended)
C. 50 MB free Hard Drive space
D. 100 MB free RAM

I. SYSTEM REQUIREMENTS (Microsoft Windows)
A. Pentium-class PC (400 MHz P4 or better recommended)
B. Microsoft Windows XP (with latest Service Packs)
C. 50 MB free Hard Drive space
D. 100 MB free RAM

II. PROGRAM DESCRIPTION

Critters! is a 2D simulation of virtual organisms for the study of mutation, selection, resource supply, and fitness. Critters are born, acquire resources, grow, mate, reproduce, and die in a virtual world. Students of evolutionary biology can study allele frequencies and/or individual fitness by analyzing the results of simulations.

III. MODEL PARAMETERS/DESIGN

A. General

1. A clock runs continuously in the model. There are two time units: clock ticks and clock units. A clock tick is the smallest unit of time; each critter can commit one action in a clock tick (e.g., birth, take a step, turn, mate, consume a resource, or die). A clock unit is 100 clock ticks. In the overlay display, whole numbers in the clock readout are clock units, while the decimal portion of the number is clock ticks.

2. Individuals move on a 2D field.

3. Spatial structure (blocks) are on the field that critters can't see through or move through.

4. Three types of resources are on the field to support critters' growth and metabolism (STR, structural resource, markers on the field are 5, 10, and 15 units; SHT, short-term energy resource, markers on the field are 5, 10, and 15 units; LNG, long-term energy resource, markers on the field are 1, 2, and 3 units). The required amount of SHT to reach the next size class when growing is double the amount required for the previous size class. The required amount of STR is the cube of the size class.

Growth Requirements for Each Size Class

Size	STR	SHT	LNG
1	1	1	0
2	8	2	0
3	27	4	0
4	64	8	0
5	125	16	0
6	216	32	0
7	343	64	0
8	512	128	0
9	729	256	0
10	1000	512	0

5. The capacity for STR and SHT is the total of (1) the amount needed to reach the next size class, and (2) the amount needed to lay 10 eggs at the maximum investment level times the size class (i.e., the capacity of egg laying climbs by a factor of 10 for each size class). The capacity for LNG is the amount needed to lay 10 eggs at the maximum investment level times the size class plus 1 unit.

Limit on Capacity for Each Size Class

Size	STR	SHT	LNG
1	278	278	61
2	567	567	121
3	874	874	181
4	1205	1205	241
5	1566	1566	301
6	1963	1963	361
7	2402	2402	421
8	2889	2889	481
9	3430	3430	541
10	2700	2700	601

6. Consumed resources are resupplied at each clock unit (100 clock ticks). The resupplied resources are randomly placed on the field. The user can change the frequency of resource resupply in the interface.
7. Traits: Egg hatching period (0-10 clock units+1 clock unit per egg investment of STR resource), sensing range (10-200 pixels for resource sensing, double that distance for mate sensing in males), adult size (1-10), speed limit (1-10), color (red, green, and blue genes), gender (male or female), egg investment, fertility trigger (females only; 5-20 clock units after reaching adult size), turning trigger (0-10% chance when no attractive resource is in the area), resource attractiveness alleles for all nine resources (0-10 for each resource type/size; note that the size (small=3, medium=6, large=9) and distance (scaled 0-10 value based upon the distance within the range of view) are equally considered in choosing the most attractive local resource), number of matings (1-3). All traits are additive except for gender, which is controlled by a single gene (heterozygotes are male).

List of Traits, Number of Genes for Each Trait, and Allele Values for Traits

	Trait	number haploid genes	number diploid genes	allele1	allele2	possible phenotypic range
1.	Egg Hatching Period	3	6	1.0	0.0	0-6
2.	Sensing Range	5	10	20.0	1.0	10-20
3.	Adult Size	5	10	1.0	0.0	1*-10
4.	Speed Limit	5	10	1.0	0.0	1*-10
5.	Color (red)	5	10	0.1	0.0	0-1†
6.	Color (green)	5	10	0.1	0.0	0-1†
7.	Color (blue)	5	10	0.1	0.0	0-1†
8.	Gender	1	2	1.0	2.0	m/f
9.	STR egg investment	3	6	5.0	1.0	6-30
10.	SHT egg investment	3	6	5.0	1.0	6-30
11.	LNG egg investment	3	6	1.0	0.0	0-6
12.	Fertility Period	5	10	2.0	0.5	5-20
13.	LNG-small attractiveness	5	10	1.0	0.0	0-10
14.	LNG-med attractiveness	5	10	1.0	0.0	0-10
15.	LNG-large attractiveness	5	10	1.0	0.0	0-10
16.	SHT-small attractiveness	5	10	1.0	0.0	0-10
17.	SHT-med attractiveness	5	10	1.0	0.0	0-10
18.	SHT-large attractiveness	5	10	1.0	0.0	0-10
19.	STR-small attractiveness	5	10	1.0	0.0	0-10
20.	STR-med attractiveness	5	10	1.0	0.0	0-10
21.	STR-large attractiveness	5	10	1.0	0.0	0-10
22.	Turning Genes	5	10	1.0	0.0	0-10
23.	# matings	3	6	0.5	0.0	1*-3

*Note: One (1) is the minimum value of this trait. Even if all alleles are zero (0), the phenotype is set to a value of one (1). !Note: Allele frequencies shown in the 'Population Data' file is for the 'high value' allele, which is shown in this column. †Note: Color values are always adjusted to percentages of maximum (0-1); therefore, changing the value of the alleles only changes the variation associated with color and/or the relative contributions of each color.

B. Life History
1. Critters are obligate sexual reproducing organisms.
2. Females become fertile at a genetically-predisposed period (Fertility Period) following growth to adult size.
3. Females lay as many eggs as they can at their genetically-predisposed investment levels (STR investment, SHT investment, LNG investment). The range of investment levels are: STR: 6-30 units/offspring; SHT: 6-30 units/offspring; LNG: 0-6 units/offspring.
4. An egg will hatch after a period of time determined by its genetic-predisposition (Egg Hatching Period) and investment of STR resource (STR investmentl; i.e., larger eggs take longer to hatch).
5. After laying eggs, a female's fertility timer starts again. However, females are genetically-predisposed to lay eggs 1-3 times, and males may mate 1-3 times (# matings). Death immediately follows the final mating event.
6. Critters live for a maximum of 100 clock units, unless the Maximum Lifespan is changed by the user in the interface (a special feature of Critters!+).
7. Mating conforms to scramble competition polygynandry.

C. Genetics
1. Critters carry genes on 27 haploid chromosomes (100 gene positions per chromosome).
2. The phenotypic values of traits are determined by alleles, which come in two forms for each trait and have a numerical value [see allele1 and allele2 columns in Trait chart (above)]. These allele values are added to determine the phenotype for that trait.
3. Meiosis occurs at gamete formation with recombination (random; 1-3 crossover events per homologous chromosome pair).
4. Critters' genomes mutate at gamete formation; the frequency depends on the user specified mutation rate, which is the approximate number of mutations per gamete.
5. The mutations are simple allele conversion mutations.
6. The alleles and gene positions are determined randomly at the start of an experiment. For each replicate, the gene positions are redistributed on the chromosomes. Therefore, for each population generated, the linkage groups will change.

D. Physiology
1. There exists a dynamic equilibrium between LNG and SHT resources. One SHT = 100 LNG, but there is a 10% loss in converting between the two types of resources.
2. Resources are depleted proportional to size [basal cost (energy cost x critter size)], speed [movement (energy cost x critter speed)], and sensing range [distance (energy cost x sensing range)].
3. Critters die when short-term energy (SHT) equals zero.

E. Behavior
1. Critters seek out and consume resources based upon the size of the resource, its distance, and the genetic predisposition for that resource type. There exist nine attractiveness traits, one for each type and size of resource on the field. Size, distance, and attractiveness are weighted about equal in determining which resource to approach. While moving toward an attractive resource, an even more attractive resource may come

into view. The critter will alter its course to move toward the new, more attractive resource.

2. If no resource is in the vicinity, critters usually take a step forward, but occasionally they turn – the frequency of these turns are based upon behavior turning genes (Turning Genes, 0-10% chance of turning per step).

F. Mechanics

1. User input to set up the simulation is recorded from the graphical user interface (GUI).
2. The program outputs the results of simulations over time (allele frequencies, population sizes, reproductive rates, death rates, average fitness, average speed, average sensing range, average size, proportion of males in populations, death rates, and intrinsic replication rates).
3. The program outputs the characteristics of individuals. The user can specify a fitness cutoff level for writing out critters (e.g., 0 = write out all critters, 1 = write out critters that laid at least 1 egg, 2 = 2 eggs laid, etc.)
4. The model will run in graphics ON and graphics OFF modes. Graphics-off mode is much faster for obtaining results for analysis. Graphics-on mode is useful for visualization, conceptualization, and education.
5. Non-random mating may be studied by activating a color switch in the interface.
6. Migration may be studied by setting up the model to introduce a randomly-generated group of migrants at a specified time.
7. Frequency-dependant selection or predator schemes may be studied by targeting and killing critters during the model runs using keyboard commands (a special feature of Critters!+).
8. Competition may be studied by targeting and killing resources on the field during the model runs using keyboard commands.
9. Bottleneck events may be simulated by specifying a time and percentage of the population to kill.

IV. DATA FILES

The model will write out an "Info" file, which contains information on the user-specified parameters. Three other data files are available at the conclusion of a simulation. One file contains the population parameters for each replicate. Another file contains the coefficient's of variation for the population parameters. The final file contains the individual parameters of critters at the time of death.

In addition to the ‘allele1’ frequency codes listed above, the following labels are used in data files generated by the program. The metrics are calculated on the user specified Output Step (there is more information on setting the Output Step in the 'Program Operation' section below).

```
N - Number of critters at the time of output.
rR - Replication (birth) rate; the number of eggs laid per clock unit
over the Output Step time interval.
avFit - Average fitness; the average number of eggs laid per living
individual (includes males and females) at the time of output.
avSp - Average speed; the average speed of critters at the time of
output.
avSR - Average sensing range; the average sensing range of critters at
the time of output.
avSz - Average size; the average size of critters at the time of
output.
pMale - Proportion males; the proportion of males in the population at
the time of output.
dR - Death rate; the number of critters that die per clock unit over
the Output Step time interval.
r - Intrinsic rate of increase (the instantaneous rate of increase);
birth rate minus death rate at the time of output.
```

V. PROGRAM OPERATION

Double-click the application's icon to run the software.

A. The user must accept the License Agreement for the program to run.

B. The user selects the field size. The sizes available are 1000x1000, 2000x2000, and 3000x3000. Larger field sizes will slow the model with graphics turned on due to the higher graphic demands.

C. A control screen allows the user to set up the simulation(s). Note that an asterisk ("*") below indicates the default value.

1. Filename - ("Sim 1"*) If you run additional simulations in the same folder, you can use a different name in this field. If you leave "Sim 1" in this field, the program will overwrite the results of the previous simulation(s).
2. Graphics - (OFF, ON*) The simulation uses OpenGL graphics to display the critters and their virtual world. Running with graphics on will enable you to see what is occurring in real time. If you are running a longer simulation, the program runs much faster with graphics turned off. When running with the graphics off, the Escape (esc) key will terminate the simulations.
3. Number of Critters (200*) This is the initial population size of critters that will be generated for populations.
4. Blocks? (OFF, ON*) Critters cannot see through or move through blocks on the field.
5. Block Placement (Even*, Random) Only available if blocks are turned on. This option allows the user to request an even distribution of blocks on the field or random placement of blocks. If 'Even' is chosen, blocks are placed evenly on the field.
6. Number of Blocks (25*) Only used if blocks are turned on and Block Placement is set to Random. The user can click in this edit field and type in the number of blocks they would like to appear on the field.
7. Sim End (100*) This is the end time for simulations.
8. Mutation Rate (0.1*) This is the mutation rate to be applied. The units are 'average number of mutations per gamete.' The value is an approximation because random number generators determine when to apply a mutation to a given loci and recombination tends to scramble the alleles on homologous chromosomes; therefore, the value entered here will reflect the average number of mutations in a gamete.
9. Number of Replicates (1*) This is the number of simulations to be run. The program can handle up to 8 replicates.
10. Energy Cost: Basal (0.001*) This is the basal energy cost that all critters pay each clock tick in the model. It is multiplied by the critter size to determine the loss of short-term energy units on that time step.
11. Energy Cost: Sensing (0.000025*) This is the energy cost applied to the sensing range of the critter. It is multiplied by the distance that the critter can see and subtracted from their short-term energy supply.

12. Energy Cost: Movement (0.04*) This is the energy cost of movement. It is multiplied by the speed of the critter and deduced from the short-term energy supply.
13. Output Step (10*) The program calculates metrics and writes out data files as the simulation proceeds. This edit field controls the frequency of output. It is recommended that you start by determining the number of data points that you would like (number of columns that your spreadsheet data will occupy). Divide the length of simulation by the number of data points you would like, and that will provide you with the proper entry for the Output Step. For example, if you want 50 data points (a 50 column spreadsheet of data over time) and selected a Simulation End of 500, then you would set the Output Step to 10 (500/50 = 10). The program would write output every 10 clock units over the simulation period, which would provide you with a 50 column spreadsheet. Note that most spreadsheet applications will choke on datasets larger than 250 columns, so plan accordingly.
14. Resource Supply Rate (100*) This is the percent chance that resources will be resupplied on a given clock unit during the run. The value may range from 1 to 100. If set to 100, resources will be resupplied every clock unit during simulation. If set to 10, there is a 10% chance on each clock unit that the resources will be resupplied.
15. Reports - These checkboxes allow the user to control which data files are produced during simulation.
16. Fitness Cutoff for Individual Critter Output (0*) - Use this field to specify a cutoff value for writing out individual critter data during the simulations. For example, a value of 0 here will result in writing all of the critters' individual parameters. A value of 2 will result in writing only those critters that laid at least 2 eggs. WARNING: Critters! will generate THOUSANDS of individuals under long runs. If you set this value to '0' and write out all critters to the data files, you will end up with huge data files. Unless you need to know critter parameters for critters that laid no eggs (zero-fitness critters), then it is suggested that you use a value of at least 1 here (i.e., only record critter data if the critter laid at least one egg). Otherwise, you may be limited to shorter runs or resource-starved critters who do not lay too many eggs. If your experimental design calls for use of the individuals' data files, a few test runs should help you determine proper settings for this parameter.
17. Resources (the defaults for 9 resource types/sizes will appear when program is run) These edit fields allow the user to specify precisely the type and number of resources in the simulations. The total number of resources should not exceed 1000.
18. Migration Switch (OFF*, ON) – Turns on a migrant introduction feature. The user specifies the number of randomly generated migrants to introduce and the time of their introduction.
19. Mate Choice (Red: OFF*/ON; Green: OFF*/ON; Blue: OFF*/ON) – Turns on color preferences by males for females. When set to on, males only mate with females that have a higher than average color value for that color. Only one of these switches can be set.
20. Maximum Lifespan (100*) – Critters die when they reach this age.
21. Bottleneck event switch (OFF*, ON) – Turns on the bottleneck even feature.
22. Bottleneck time (50*) – Specifies when the bottleneck event is to occur.

23. Bottleneck percentage of population to kill (70*) – The fraction of the population to kill during the bottleneck event.
24. Migration event switch (OFF*, ON) – Turns on the migration even feature.
25. Migration time (50*) – Specifies when the migration event is to occur.
26. Number of migrants (30*) – The number of randomly generated migrants that will enter the population at the migration time.
27. Run Simulation - Will start the simulation(s).
28. Exit Program - Will exit the program.

D. Keyboard controls [only available during graphics-on simulations, except for Escape (esc)]
1. Arrow keys - move forward, move back, turn left, turn right
2. G - toggle the graphics on and off
3. K - elevate the camera
4. , (comma) - lower the camera
5. L - tilt camera up
6. . (period) - tilt camera down
7. Escape (esc) - exit simulation
8. P - pause the simulation [Note: Camera controls work when the simulation is paused (i.e., the user can move about the field during a paused simulation)]
9. A - toggle the allele frequency monitor on/off
10. W – OpenGL wire frame mode (shows the polygon nature of 3D graphics)
11. O – toggles the overlay on and off
12. I – provides a target cross-hair to get information on a specific critter (run the cross-hair under a critter to see its characteristics)
13. T – enter target mode to kill critters or resources
14. R – toggle between 'TARGET: Critter" and "TARGET: Resource" modes
15. (spacebar) – kill critter/resource in the cross-hair
16. C – toggle fast and slow camera speed modes

E. Screen Abbreviations

FPS – Frames per second
The following data is shown as the model runs:
Time – Current time
Rep/Total – Replicate # underway/Total number of replicates requested
Total New – Total critters generated so far
#Critters – Number of organisms in the current population
Avg. Fitness – The average number of eggs laid per living individual
Rep Rate – The number of eggs laid over the output step time interval
Death Rate – The number of dead critters over the output step time interval

The following data is shown when looking at individual critters:
Code – The unique code number of the critter marked on the screen
Gender – Male/female
Age – Current age
Adult Size – The adult size for this critter
Size – The current size of this critter
Speed – The speed of this critter (pixels/step)
Color1(red) – The red value for this critter (range: 0-1)
Color2(green) – The green value (0-1)
Color3(blue) – The blue value (0-1)
SensRange – The sensing (vision) range for this critter (pixels)
FertFlag – For females: 0=egg, 1=juvenile, 2=adult, non-fertile, 3 = adult, fertile
FertTimePer – Period of time between reaching adult size and becoming fertile
FertTime – The actual time that an adult female will become fertile
X – The x coordinate of this critter
Y – The y coordinate
Dir – The direction that this critter is moving
NumEggsLaid – Number of eggs laid by this critter
LNGunit – Current number of long energy units held by this critter
SHTunit – Number of short energy units
STRunit – Number of structural units
EggHatchPer – The length of time for this critter to hatch
EggHatchTime – The actual time of egg hatching for this critter
RepEvntsRem – Number of mating attempts remaining for this critter
ResAtt_ – Resource attractiveness figures (0-10) for each size/type of resource
Parent1 – The critter's father code number
Parent2 – The critter's mother code number

VI. VERSION HISTORY

11/23/02 – v.1.0 – Final Release
02/20/03 – v1.1 – Interface provided in one screen; numerous bug fixes; OpenGL enhancements
06/15/03 – v1.2 – New feature: An individual's parents' code numbers are recorded in the individual data file, which makes it possible to lookup the parents' phenotypes
01/19/04 – v1.3 – New features: Predator/competitor and critter information features added with keyboard controls; added 'cause of death' information to the individual data files; added migration tool; added mate choice by color tool; added ability to change default maximum lifespan, added bottleneck event feature.
03/08/04 – v1.4 – Changed camera controls to allow for z-axis elevations as high as 2000 pixels. New feature: Ability to run model on multiple field sizes.
06/25/04 – v1.5 – Updated genetics model using floats and longs over strings for genetic trait codes.
02/3/06 – v2.0 PC version complete.

Acknowledgements

Critters! was programmed on an Apple® 400MHz G4 PowerMac®, an 800MHz iBook®, and a dual-2GHz G5 using FutureBASIC® by STAZ Software, Inc. (7582 Alakoko Drive, Diamondhead, MS, 39525, 800–348–2623, http://www.stazsoftware.com). The C++ version was ported in Apple's Xcode® IDE. The OpenGL® game engine was originally based upon a Lost Minds game engine by Lars Gafvert (with Chris Stasny, aka. Staz, and Andy Gariepy of Staz Software, Inc.). The paper *Evolution of digital organisms at high mutation rates leads to survival of the flattest* published in *Nature* (412: 331-333) was inspirational for our Critters! project. Thanks to Claus O. Wilke, Ph.D. and his colleagues for conducting important research in this area and writing so eloquently on the topic in *Nature*. NSF Grant OOP-00-83381 supported the development of this manual. The Sandra S. Pieper Foundation provided financial support for this project. Thanks to the good people at this foundation for continued support. We thank the Department of Biological Sciences at Towson University (Chair: Richard Seigel, Ph.D. Larry Wimmers, Ph.D. instructed one of us (L.L.) in the science and art of inquiry-based teaching. Thanks to Ken Karakotsios and Michael Bremer (the developers of SimLife®). Ken Karakotsios provided helpful advice. Thanks to Karl Sims and Marshall Mathers for inspiration. Farooq Mela, Craig Plunkett, Tom Bartolucci, and programmers at the CodeGuru forums (Paul McKenzie and Abhishek Padmanabh) helped with the C++ version of the software. The following corporations have been helpful by providing hardware, software, and support: Staz Software, Inc.; Apple Computer, Inc.; Silicon Graphics, Inc. (OpenGL); and Aladdin Systems, Inc. (Stuffit Expander).

Legal Information

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