Chapter 10

Multi-species Interactions: Indirect Effects

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Introduction

This laboratory exercise was developed for use in an introductory ecology and evolution course required of undergraduate biology majors at the University of Chicago. This course is part of a sequence that essentially takes the place of introductory biology courses found at many colleges and universities, and this lab could be adapted for use in such traditional courses. It could also form the foundation for a series of more advanced exercises in ecology, behavior, or experimental design and analysis.

This lab requires students to observe guppies (*Poecilia reticulata*) feeding on *Daphnia pulex* in the presence and absence of aquatic plants and then to test for differences in foraging rates between guppies in the two treatments. The main objective of the exercise is to provide a clear example of an indirect effect (an effect of one species on a second species that arises only in the presence of one or more other species), and to emphasize the potential importance of indirect effects in ecology. Students should understand that the dynamics of populations in multi-species communities are not necessarily predictable from the study of pairwise interspecific interactions if indirect effects are present. The lab is designed for a lab period of three hours and twenty minutes. Details and timing of set-up are included in the Materials section below.

At the beginning of the lab period, it is a good idea to review what we mean by indirect effects and why they are important (discussed in the Student Outline). Depending on the statistical background of your students, you might also need to introduce or review *t*-tests (Appendix B), which will be used to test for differences in consumption rates between the "plant" and "no-plant" treatments. It is also useful to demonstrate feeding strikes using the guppies in the stock tank and extra *Daphnia*. Show the students that the fish generally need to handle the prey, which may involve spitting out and sucking in the prey several times before actually swallowing it. Tell the students not

to count each "sucking in" during handling of a single prey item as a separate prey strike. If the fish ends up swallowing the item, score the strike as "successful." If the fish eventually rejects it, count the strike as "unsuccessful."

Materials

Equipment:

- 8 10-gallon aquaria
- 1 20-gallon aquarium with hoods,
- Whisper filters,
- fluorescent lights;
- opaque tank dividers for the 10-gallon tanks;
- 1 fish bowl or 5-gallon aquarium for *Daphnia*;
- aquarium nets;
- 8 computers with StatView® software (www.statview.com) and Microsoft Excel

Supplies:

dark brown or black aquarium gravel;

- Tetra Min or other good tropical fish food;
- NovAqua chlorine remover/stress-coat;
- black tissue paper and tape;
- 72 test-tube caps;
- 24 small Nalgene beakers;
- 8 plastic dropping pipets with the tips cut off;
- plastic sandwich bags and rubber bands for adopting out fish

Live organisms:

- at least 48 feeder guppies (purchased from a pet store or ordered from a supplier like RMS Aquaculture *(www.rmsaquaculture.com);* if your vendor is willing to pick out females for you, you should order only 2- or 3-dozen fish);
- 3 *Daphnia magna* cultures (500-student size, ordered from Wards Biology; *www.wardsci.com*);
- 12 bunches of a feathery-leaved aquarium plant like *Myriophyllum* (ordered from Carolina Biological *(www.carolina.com)* or purchased at a good aquarium store).

Tanks:

Start setting up for this lab about two weeks before the scheduled lab date. You will need eight 10-gallon tanks for the experiments. You'll also need one 20-gallon tank to use as a guppy stock tank. Each tank should be equipped with a Whisper filter system, hood, and fluorescent light, and the bottoms of the tanks should be covered with a thin (about 0.5-inch) layer of gravel. The gravel should be rinsed very carefully to make sure no fine dust is present. Each of the 10-gallon tanks should also have an opaque, plastic tank divider inserted across the center. Make sure that the sides of each divider fit snugly up against the tank walls and that the bottom is buried in the gravel so the guppies cannot cross from one side to the other. Cover the back and sides of each tank with black tissue paper. This helps reduce stress on the fish associated with people moving around the room, and it helps make the *Daphnia* more visible for the students.

Let the water sit in the tanks, with filters running, for at least three days before you add fish or plants. Allowing the water to age a bit will help to eliminate chlorine and other toxins; you may also wish to use a chlorine remover and anti-stress treatment (purchased at a pet shop) to give these already stressed fish a fighting chance.

Fish:

The guppies for this lab can be purchased from a pet shop or ordered from a supplier like RMS Aquaculture (www.rmsaquaculture.com). Buy at least 4-dozen feeder guppies (fewer if you are able to limit your order to females) about a week before the lab date. If ordering guppies to be shipped, you are probably better off getting more, since many die in transit. You can generally expect about 25–30% die off over the

first few days in the lab, even if you purchase the fish in a pet shop. Order enough guppies so you will have at least 16 large female guppies available for the labs.

When you get the guppies to the lab, float the sealed bag in the 20-gallon stock tank for about 15 minutes. Open the bag and add approximately 0.5 cup of aquarium water; do this three or four times at 5-minute intervals. Gently net the guppies out of the bag and place them in the tank, and dispose of the bag and its water. Allow the fish to acclimate and feed for several days.

About 4 days before the lab starts, transfer two large females to each observation tank (one on each side of the divider). Females are drab, while males are generally colorful and have tubular anal fins (they use the anal fin as an intromittent organ, since guppies are live-bearing fish). Try to avoid selecting heavily pregnant females, since baby guppies are quite difficult to remove from the experimental tanks.

Feed the fish any good-quality tropical fish food flakes. You will probably have to crumble the flakes slightly. The fish can be fed twice a day on weekdays and once a day on weekends. Do not feed the fish in the morning before an experiment; feed them once after each lab is finished (in the afternoon or evening), so they will be hungry for the next lab period. Starting a day or two before the first lab period, feed the fish a little *Daphnia* to get them used to hunting live prey. Make sure to use relatively small *Daphnia*.

According to most animal-use regulations, you must keep a log sheet of feedings, dead fish removals, and any tank maintenance (there should not be any cleaning since the fish will only be in the tanks for about two weeks). At the end of the labs, you can adopt out the fish to students.

Plants:

Buy about 12 bunches of *Myriophyllum*; you can substitute *Cambomba* if *Myriophyllum* is unavailable. You need something feathery and full that will substantially obstruct the fishes' view of the *Daphnia*. Only a good aquarium store will have plants when you need them; Carolina *(www.carolina.com)* also sells plants, but they are sometimes in poor condition upon arrival, and they are somewhat expensive.

Use plastic test-tube caps as planting cups; you'll need eight or nine caps per aquarium. Strip the leaves from the bottom inch of the plant stems and insert four of them into each cap. Fill the caps with gravel and gently place them in the tanks. Depending on the fullness of the plant stems, place the caps in a 3 -3 -3 or a 3-2-3 pattern across one half of the tank. Try to use plants that are tall enough to reach the surface of the water so that the plant density is roughly even from the bottom of the tank to the surface. You might need to adjust the water level in the tanks to achieve this consistent plant density.

***Daphnia*:**

For a week of four lab periods, you will need three of the 500-student cultures of mixed-size *Daphnia magna* (Wards Biology; *www.wardsci.com*). Stagger the shipment dates so that fresh *Daphnia* are available throughout the lab week. Transfer the animals immediately upon receipt into a large fish bowl or small aquarium. Do not add any water, since the change in ion concentration can kill them. Keep the culture cool and out of direct sunlight. When transferring *Daphnia* with a dropping pipet, always transfer them directly into the water rather than through the air. If air gets trapped under the carapace, the *Daphnia* will float to the surface and die. You might want to have students practice this maneuver before starting observations, since they will need to transfer individual prey to the experimental tanks during the lab.

Lab Supplies:

Per team of four students: aquarium with fish, plants, *etc.,* as described above; two Nalgene beakers containing 30 small *Daphnia* each (the lab will go a lot faster if you count them out beforehand, but if you have time to spare, you can have the students count them out.); one Nalgene beaker containing approximately 40 small *Daphnia* (counted out before lab) to replace those eaten by the fish; two plastic dropping pipets with the tips cut off; two data sheets per replicate (one for "plants" treatment, one for "no-plants" treatment; see sample in Appendix A)

Computers:

In this lab, we use Microsoft Excel for data entry and StatView for statistical analysis (see *www.statview.com* for software information). Set up an Excel spreadsheet into which the data for each replicate can be entered. The spreadsheet should calculate the total number of prey consumed and attacked

over each 60-minute trial as well as the average number consumed and attacked within each time interval for the plant and non-plant treatments (Appendix D). The data entry generally goes most smoothly if the lab instructor enters the data and then distributes it to the class (*e.g.,* by using a floppy disk to copy the file to each lab computer). Don't save the file as "text (tab delimited)" for import into StatView until all the data are entered, since this will not preserve the formulas that calculate the totals and averages. It is also possible to perform *t*-tests and create graphs in recent versions of Excel. Instructions for doing so are found in Microsoft Excel Help.

Student Outline

Introduction

Ecologists have devoted much of their research to describing and understanding the dynamics of single-species populations or the interactions between two species. Such simple situations lead to repeatable observations and predictable theoretical outcomes, thereby producing a high degree of understanding about populations in near-isolation. One can think of these results as giving an "ideal" behavior of populations against which we can contrast the behavior of real populations, in much the same way that physicists explore worlds without friction or gravitational interactions in a solar system with only two objects (a sun and a planet or a planet and a moon). In reality, of course, ecological systems contain many more than two species. It is important to ask whether this complexity can produce novel effects that would not be observed or predicted from simple one- or two-species systems, and whether such effects are significant. Unique effects arising from the complexity of having more than two species present are termed *indirect effects*: effects of one species on a second species that arise only in the presence of one or more other species. Recently, ecologists have become very interested in the importance of, and mechanisms behind, indirect effects.

Two basic mechanisms can cause indirect effects. First, indirect effects can be caused by *chains of interactions* linked by shared species. These indirect effects can arise if we imagine two sets of interactions between species pairs, in which one species participates in both interactions. In this case, one species (A) interacts with another species (B), thereby changing its abundance, and species (B) also interacts with a third species (C), changing its abundance. Consequently, A causes a change in the abundance of B, which in turn causes a change in C, so A has an indirect effect on C. If B were absent, this effect of A on C would be absent, so the effect arises only in the presence of another species. Although this type of indirect effect arises only in the face of ecological complexity, it is readily predicted if we know how multiple pairs of species interact with each other. Therefore, we can potentially predict these indirect effects through our reductionist understanding of how pairs of species interact, and studying these simple interactions continues to have great value.

Figure 1. Basic mechanisms causing indirect effects.

The second type of mechanism causing indirect effects occurs when one species *modifies the interaction* between two other species. In this case, two species (B and C) might interact with each other in isolation in a well-characterized manner. When another species (A) is added however, the

interaction between B and C changes in some way. Again, this represents an indirect effect of A on C: The effect of A on C would be absent if B were not present to interact with C. If interaction modifications are important in real ecological communities, they raise some profound issues regarding the study of natural systems. Specifically, they raise the specter that reductionist studies between pairs of species may give us limited insight into the organization of natural communities, because the interactions in nature may be completely different. In reference to ecological systems, people often proclaim, "the whole is more than the sum of its parts." Interaction modifications represent a reason why this statement could be true.

(As an aside, the issue of indirect effects lurks in the background in other biological fields, where reductionism proliferates. Will the conclusions from studies of single genes depend on what other genes are present in a cell? Can the development and physiology of an organism be understood from the study of one or two cells in isolation? As studies in other fields progress, they, too, are likely to face the difficult issues and uncertainty that ecologists face when studying complex communities).

So is there reason to think that interaction modifications can occur? The answer is likely to be "yes." One species can change the behavior of another species, which in turn affects interactions with other species. For example, the presence of a predator species may make a prey species less active to avoid detection, but lower activity can reduce encounter rates (and hence feeding) with the prey of the prey species. Therefore, the prey of the prey indirectly benefits from the predator. Also, if predators become satiated eating one type of prey, their feeding rate on other species will also be reduced, so shared prey species might indirectly benefit each other. Finally, for visual predators such as birds or fish, the presence of one species may make other species more difficult to detect by causing visual interference. For example, katydids (relatives of grasshoppers) often look similar to tree leaves, so when trees are present, predators have a harder time locating katydids.

Today you will look for evidence of indirect effects caused by interaction modification by studying the interactions between fish and their zooplankton prey. In particular, you will explore whether the presence of aquatic plants makes it harder for fish to find and eat zooplankton.

Methods:

The predators in this exercise will be female guppies, *Poecilia reticulata*, a small poeciliid fish native to streams in Central America and islands of the Caribbean. The prey will be a common species of zooplankton in lakes and ponds, known as *Daphnia* (or water fleas). Each group will use a fish tank that has been divided into two compartments with a plastic screen that permits water flow between sections but does not allow the *Daphnia* to pass through. One side of the tank will contain aquatic plants (genus *Myriophyllum*), and the other side will have no plants. A single fish will be placed in each side of the tank. You will then add 30 *Daphnia pulex* to each side of the tank. You will observe fish feeding rates over the course of 60 minutes in the presence and absence of plants. Your results will then be pooled with those from the rest of the class for analysis to determine whether fish feeding rates are lowered in the presence of aquatic plants. You will also plot fish feeding rates during 5-minute intervals over the course of the 60-minute experiment to explore how the feeding response of the fish changes as the number of prey consumed increases.

You should distinguish between successful strikes and unsuccessful strikes in your observations and record the two separately. In a successful strike, the fish notices the prey, approaches it, strikes at it, handles it, and consumes it. The most common way this fish handles the prey is by sucking it into its mouth and then spitting it back out, which it may do repeatedly. You should not record each individual sucking-in/spitting-out event as a separate strike. If the fish ends up eating the prey after handling it this way a few times, that counts as a single successful prey strike. In an unsuccessful strike, the fish notices, approaches, and strikes at the prey, but the prey

either escapes during handling, or the fish loses interest and swims away. Your laboratory instructor will have you watch some demonstration guppies feeding on *Daphnia* so you know what to look for during your observations.

Experimental Protocol:

1. Turn off the room lights at the start of the experiment to help reduce your visibility to the fish. Also, be sure to unplug the filter so that the *Daphnia* won't be sucked into it.
2. Work in groups of four: two observers, a *Daphnia* stocker, and a timer. (Note: for a small lab section, it is possible to work in groups of three and have the stocker also be the timer, or to have the instructor call out the 5-minute intervals. It may also be necessary to have each group do two replicates, *i.e.,* two 1-hour observations in different tanks).
3. You will be given two plastic beakers containing 30 *Daphnia* each. Since you will keep the prey density constant through the duration of the experiment, you will also be given a reserve container containing approximately 40 *Daphnia* (in aquarium water) that can be added to the aquarium to replace those eaten by the fish.
4. When the observers are ready, the stocker will gently pour a beaker of 30 *Daphnia* prey into each side of the tank. Be sure to pour the prey away from the predator, rather than right in front of it. Begin timing the experiment. As prey are consumed, they should be immediately replaced with *Daphnia* from the reserve beaker to keep the overall prey density constant. Carefully transfer the *Daphnia* from the reserve beaker using a plastic pipet that has been cut to provide a large opening. Again, replacement should be away from the fish. *Always transfer the animals gently from water to water, i.e., don't squirt them through the air.* If air gets trapped under the carapace, the *Daphnia* will float to the surface and die. The timer should call out 5-minute time intervals. During each 5-minute interval, the observers should record each attack on a *Daphnia* prey item. Tally the predation events in the appropriate column on the data sheet according to whether the capture attempt was successful (prey eaten) or unsuccessful (prey missed or spit out). Observation will end after 60 minutes.
5. Submit your data sheet to your instructor, who will enter the data in an Excel spreadsheet that will be distributed to each group's computer. Each lab group will analyze the data for the entire lab section.

Data Analysis:

1. Use the class data file for all your analyses.
2. Using StatView, plot the consumption rate (number of successful prey strikes per 5-minute interval) and the attack rate (number of successful and unsuccessful prey strikes per 5-minute interval) vs. time (in 5-minute intervals) for the "no plants" treatment and the "plants" treatment:
    a) Make sure the Excel spreadsheet is saved as "text tab-delimited" for import into StatView.
    b) Launch StatView on your computer.
    c) Under the "File" menu in StatView, select "Open." Find the class data file, select the filename, and click on the "Open" button. Click on the "Import" button in the box that appears.
    d) Under the "Analyze" menu, select "New View."
    e) Click on the triangle next to "Bivariate Plots." Choose "Line Chart" from the list that appears below "Bivariate Plots."
    f) In the "Variables" window that should be on the right side of the computer screen, you will see a list of the variables in your data set. Select "avg # consumed/interval" and click on "*Y*-variable." Select "time interval.2" and click on "*X*-variable." Select

"treatment.2" and click on "Split By." Click on the "Create Analysis" button at the top of the analysis window. A plot of consumption rate over time should appear in the analysis window.

g) Click outside the graph in the analysis window to deselect it. In the "Variables" window, select "avg # attacked/interval" and click on "*Y*-variable." Select "time interval.2" and click on "*X*-variable." Select "treatment.2" and click on "Split By." Click on the "Create Analysis" button at the top of the analysis window. A plot of attack rate over time should appear below your first graph.

h) So you can distinguish between the two treatments in each plot, you should change the appearance of the points for the "NP" ("no plants") treatment. To do this, click on the symbol next to "NP" in the legend of the first graph. Under the "Draw" menu, drag the arrow down to "Point and select a shape other than the open circle. The points corresponding to the "no plants" treatment in your first graph should all change. Do the same thing for your plot of attack rate over time.

i) Save your results, but do not close the data set or the analysis window — you will use them in the rest of the analysis described below.

3. You will use a one-tailed *t*-test to determine whether the number of prey consumed in absence of plants was significantly higher than the number consumed when plants were present. If this is true, it is evidence for an indirect, protective effect of plants on the zooplankton prey.

Instructions for running t- tests in StatView

1. Use the same data set and analysis window you left open from the plots of consumption and attack rate.
2. In the analysis window, click on "Unpaired Comparisons."
3. In the variable browser, select "treatment" and click on "Add." Do the same for the "prey consumed in 60 minutes" variable.
4. Click on the "Create Analysis" button in the analysis window. In the box that appears, select "Upper" under "Tail," and click "O.K." This will test the alternative hypothesis that the mean number of prey consumed in 60 minutes is greater in the "no plants" treatment than in the "plants" treatment.
5. The analysis should now run, and you will see two tables. The upper table gives you the results of the *t*-test. The lower table gives you the means and standard deviations for the two groups of data.
6. Make sure the results of the first *t*-test are deselected, and repeat the steps above using "prey attacked in 60 minutes" and "treatment" as your variables.

We will use $P = 0.05$ as the cutoff for a significant test. If we are able to reject the null hypothesis (*i.e.,* if $P < 0.05$), then there is only a 5% probability that the difference we see between the two means is due to chance rather than to some "real" difference (*i.e.,* that we are wrong in rejecting the null hypothesis). Look at the *P*-value in the last column of the upper table for each *t*-test. Were the two means significantly different, or did the presence of plants have no effects on fish feeding rates? You should also look at the actual means and standard deviations in your table of results, as well as at the plots of feeding rate over time, since these will give you biologically interesting information that you can't get from a *P*-value alone.

Questions: Please attach your StatView output and answer the following questions.

1. Do you see any evidence for an indirect effect in this experiment? What type: interaction chain or interaction modification? Explain your answer, and be sure to discuss your response in terms of the outcome of the *t*-test.
2. Examine your plots of feeding rate vs. time. Does predation rate change over time, and if so, how does it change?
3. What type of predator functional response (Type I, Type II, or Type III) do your data suggest? Explain what features of your data led you to this conclusion.
4. Do you think the presence of plants affects the functional response of the fish? If so, how?
5. If the predators exhibit a type II functional response, do you think this could be a mechanism that counteracts the protection from predation afforded the *Daphnia* by the aquatic plants? Explain.
6. Design an experiment using these organisms that will allow you to test for changes in guppy diet breadth (with respect to prey size) as a function of changes in *Daphnia* density. Explain how you would set up your experiment and how you would collect and interpret the data. What statistical test(s) might you use to analyze the data? You might find it useful to employ diagrams in describing your experimental design and data analysis.

Notes for the Instructor

As the lab is presented above, each observation period covers 1 hour divided into 5-minute intervals. For small laboratory sections, each lab group might need to do two replicates (*i.e.,* two 1-hour trials) in two different tanks to obtain the recommended eight replicates. One problem with this arrangement is that the guppies tend to forage actively at the beginning of the observation period and then do almost nothing for the rest of the time, which leads to a lot of idle time during the lab period. The drastic decline in feeding toward the end of the observation period also tends to obscure the real differences between the "plant" and "no plant" treatments, making it harder to get a significant *t*-test result. Workshop participants at the 2001 ABLE conference concurred that 30 minutes of observation would be preferable to a full hour. This new arrangement would also allow for an increase in the number of replicate observations during a lab period. For example, one could set up 12 tanks, divide the class into six teams, and have each team do two sets of observations. This scenario would liberate lab time for data analysis and discussion and would prevent the boredom that arises when students are forced to stare at an inert guppy for two hours. It is important to note that each individual guppy can be used over several days of observation (we have used them for up to four consecutive days at the University of Chicago). However, no guppy should be used for more than one lab period on a given day, since the fish should be hungry at the beginning of the observation period.

Another problem with the lab as presented in the Student Outline concerns the nature of the refuges for *Daphnia* in the experiment. The goal of the lab is to see whether the refuges provided by plants influence the predator-prey interactions between guppies and *Daphnia*. The gravel on the bottom of the tank, however, provides an additional and very effective prey refuge in both the "plant" and "no plant" treatments. The *Daphnia* head for the gravel soon after they are added to the tank, which may be one reason that guppy feeding decreases so drastically after the first few minutes of observation. Workshop participants suggested that the observations would be more useful, and that the prey might remain in the water column, if there were no gravel in the tanks. If you choose to set up the tanks without gravel, you should seal the junction between the divider and the bottom of

the tank, since one effect of the gravel is to prevent experimental guppies from moving between the halves of the tank.

The questions at the end of the Student Outline are used in an ecology and evolution course in which students have learned about predator-prey interactions and functional responses. Other types of questions might be more appropriate for a general introductory biology course or for a more advanced ecology or behavior course. In particular, it could be useful for students to think about why they measured the variables they did in this exercise and to consider other ecological questions they could try to answer using the guppy/*Daphnia*/plant system.

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Appendix A
Data Sheets

For 1-hour observations:
Treatment: (circle one) PLANTS NO PLANTS
Team Number:_____
Replicate Number:_____

	Time Interval	Successful Attacks	Unsuccessful Attacks
1	0–5 min.		
2	5–10 min.		
3	10–15 min.		
4	15–20 min.		
5	20–25 min.		
6	25–30 min.		
7	30–35 min.		
8	35–40 min.		
9	40–45 min.		
10	45–50 min.		
11	50–55 min.		
12	55–60 min.		

For half-hour observations:
Treatment: (circle one) PLANTS NO PLANTS
Team Number:_____
Replicate Number:_____

	Time Interval	Successful Attacks	Unsuccessful Attacks
1	0–3 min.		
2	3–6 min.		
3	6–9 min.		
4	9–12 min.		
5	12–15 min.		
6	15–18 min.		
7	18–21 min.		
8	21–24 min.		
9	24–27 min.		
10	27–30 min.		

Appendix B
t-*tests*

The *t*-test is used to test the statistical significance of the difference between two means, or arithmetic averages (i.e., the sum of the observations in a sample divided by the number of observations in the sample). The statistical null hypothesis is H_O: $\bar{x}_1 = \bar{x}_2$, where $\bar{x}_1$ is the mean of one sample and $\bar{x}_2$ is the mean of the second sample. In words, therefore, the null hypothesis is that the means of the two samples are equal. Two sample means are said to be statistically different if there is a low probability that the samples were drawn from the same population. We reject the null hypothesis of no difference between means if this probability is larger than a conventional cut-off, and we fail to reject the null hypothesis if the probability is smaller than this cut-off. The probability cut-off is known as the level of statistical significance; if we reject the null hypothesis, we say that the means are significantly different from each other. The most commonly used cut-off probability (or α-level) is 0.05. This number tells us the probability that the two samples appear to be from different populations but are actually from the same population (i.e., they look different, but the difference is due to chance). The α-level is a measure of type I error, which is rejection of the null hypothesis when it is actually true. In the case of an α-level of 0.05, there is a 1 in 20 chance that we will say two samples are different when in fact the differences are due to chance.

The *t* statistic is calculated as:

$$t = \frac{\left|\bar{x}_1 - \bar{x}_2\right|}{S_d}.$$

S_d is the standard error of the difference between two means. A standard error of a statistic provides information about how variable that statistic would be if it were calculated for multiple samples from the same population. A large standard error suggests that the statistic (in the case of S_d, the difference between two means) is a poor estimate of the population parameter (in this case, of the difference between the two population means). S_d can most easily be calculated in two steps as follows. First we calculate a value called the *pooled variance*:

$$S_p{}^2 = \frac{\left[\sum\left(x_1{}^2\right) - \frac{\left(\sum x_1\right)^2}{N_1}\right] + \left[\sum\left(x_2{}^2\right) - \frac{\left(\sum x_2\right)^2}{N_2}\right]}{\left(N_1 - 1\right) + \left(N_2 - 1\right)},$$

where the first term in the numerator is the "calculator formula" for the sum of squared deviations of each observation in sample 1 from the mean of sample 1. Likewise, the second term in the numerator is the sum of squared deviations of each observation in sample 2 from the mean of sample 2. N_1 is the number of observations in sample 1, and N_2 is the number of observations in sample 2. This pooled variance is basically an average variance within each of the groups, i.e., it tells us how much "scatter" there is among observations within each of the samples. From $S_p{}^2$ we can calculate S_d as:

$$S_d = \sqrt{S_{p^2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}.$$

We can now plug this into our original formula to calculate *t*.
Let's go through a *t* -test example. We have two samples such that $N_1 = 10$, $N_2 = 15$, $\bar{x}_1 = 3.5$, and $\bar{x}_2 = 4.5$.
The pooled variance, $S_p{}^2$, has already been calculated to be 20.
State the null hypothesis that we wish to test. H_O: $\bar{x}_1 = \bar{x}_2$.

Select the significance level (α) for rejecting the null hypothesis. In this case we will use $\alpha = 0.05$.

Compute the test statistic. In this case $S_d = \sqrt{20\left(1/10 + 1/15\right)} = 1.826.$

and $t = \dfrac{(4.5 - 3.5)}{1.826} = 0.548.$

Once we have calculated the *t*-statistic, we must compare it to the critical values in a *t*-table. You need three pieces of information to use a *t*-table:

(1) The significance level you wish to use. The maximum conventional α-level is 0.05, but critical values of *t* for α = 0.01 are also provided in the table below. Statistical significance at an α-level of 0.01 means that there is only a 1% probability of rejecting the null hypothesis when it is actually true.
(2) The calculated value of the *t* statistic.
(3) The number of degrees of freedom (df) associated with your test statistic. You don't need to worry too much about the theory behind degrees of freedom. The number of degrees of freedom associated with a test statistic is related to the sample size and to the number of parameters that were estimated in the calculation of that test statistic. In the case of a *t*-test for the difference between two sample means, the number of degrees of freedom is $n_1 + n_2 - 2$.

Once you know these three pieces of information, you can look up the critical value in the *t*-table corresponding to the appropriate significance level and number of degrees of freedom. If the *t*-statistic that you calculated is larger than the critical value in the table, then there is a significant difference between the two sample means at the designated α-level (i.e., you reject the null hypothesis). If the *t*-statistic that you calculated is less than the critical value in the table, then there is no significant difference between the two sample means at the designated α-level (i.e., you fail to reject the null hypothesis).

The degrees of freedom in our example are equal to $(N_1 - 1) + (N_2 - 1)$, and in this case df = 23. We now look up the critical value of *t* in a table of critical values for df = 23 and $\alpha = 0.05$. In this case, the value is 2.069. If the calculated value of *t* is greater than the critical value of *t* from the table, we can reject the null hypothesis. In our example, 0.548 is less than the critical value of 2.069, so we do not reject the null hypothesis. For these two samples, there is no statistically significant difference between the means.

Table 1. *t*-table.

df	***0.05***	***0.01***		***df***	***0.05***	***0.01***
1	12.708	63.657		19	2.093	2.861
2	4.303	9.925		20	2.086	2.845
3	3.182	5.841		21	2.080	2.831
4	2.776	4.604		22	2.074	2.819
5	2.571	4.032		23	2.069	2.807
6	2.447	3.707		24	2.064	2.797
7	2.365	3.499		25	2.060	2.787
8	2.306	3.355		26	2.056	2.779
9	2.262	3.250		27	2.052	2.771
10	2.228	3.169		28	2.048	2.763
11	2.201	3.106		29	2.045	2.756
12	2.179	3.055		30	2.042	2.750
13	2.160	3.012		40	2.021	2.704
14	2.145	2.977		60	2.000	2.660
17	2.110	2.898		120	1.980	2.617
18	2.101	2.878		∞	1.96	2.576

Another way of presenting these results is to give the probability value (or *P*-value) that corresponds to your *t*-statistic for the appropriate number of degrees of freedom. If your test statistic is significant at α = 0.05, then the *P*-value is less than 0.05 (i.e., $P < 0.05$). In this example, there is a less than 5% chance that you are rejecting the null hypothesis when it is actually true. When you perform a *t*-test using a computer, the results usually include an exact *P*-value. There is also an applet at *http://www.stat.sc.edu/~ogden/javahtml/pvalcalc.html* that will calculate exact *P*-values for *t*-tests.

You might have noted that we did not explicitly state the alternative hypothesis for the *t*-test in the example above. This was because there are several possible alternative hypotheses depending on whether or not there is an a priori reason to expect a difference between the means in a certain direction. The test as we performed it above was a "two-tailed test," which implies an alternative hypothesis in which the two means are different, but we don't know beforehand which one we expect to be larger. It is also possible to perform a "one-tailed test," in which the alternative hypothesis is that one mean in particular is expected to be larger than the other.

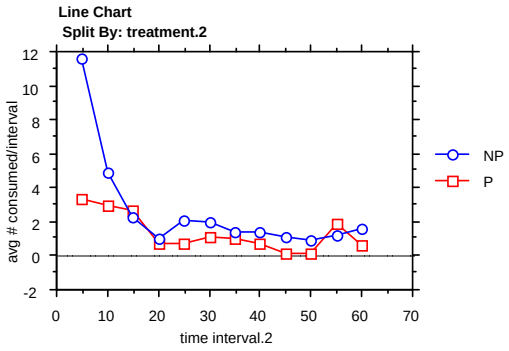
Imagine, for example, that you want to test for differences in SAT scores between a sample of second graders and a sample of college freshmen. Your null hypothesis is that average score is the same between the two groups. Before the students even sit down to take the test, you would expect that a difference in mean score, if it were to exist, would be such that college freshmen have a higher average score than second graders. You would base this on the amount of cognitive development and level of education in the two groups; your decision would not be based on a difference you saw after the tests were completed and scored. When the alternative hypothesis specifies a particular direction for the difference between two means, a one-tailed test is more powerful than a two-tailed test (*i.e.,* you are more likely to get a significant result if there really is a difference).

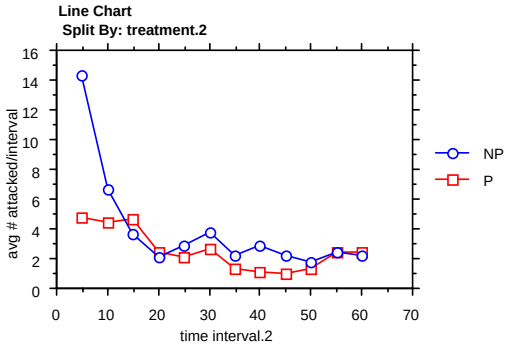
Now imagine that you want to test for differences in SAT scores between a sample of college freshmen with blond hair and a sample of college freshmen with brown hair. Stereotypes about blondes notwithstanding, you would not necessarily expect the difference in mean SAT score to be in one direction or the other — you would just be interested to see if there is a difference. In this case, you should do a two-tailed test. If you have the two groups of students take the exam and then notice that the blondes have a higher mean score, you can't decide at that point to do a one-tailed test, because you implicitly had to do a little two-tailed test in your head to even notice that difference. In this case, a two-tailed test is warranted.

If you wish to look up a critical value in a *t*-table for a one-tailed test, you must look under an α-level twice that of the significance level you want to use (*e.g.,* if you want to test for significance at $\alpha = 0.05$, you must compare your *t*-statistic to the critical value corresponding to $\alpha = 0.10$, which is available in more extensive versions of the *t*-table than the one included in this appendix). When you perform *t*-tests using statistical software, you can generally choose whether you want to perform a one-tailed test or a two-tailed test. If you are ever unsure whether you are justified in using a one-tailed test, you should use the more-conservative two-tailed test.

Appendix C

Sample Results





Unpaired t-test for prey consumed in 60 min
Grouping Variable: treatment
Hypothesized Difference Š 0

	Mean Diff.	DF	t-Value	P-Value
NP, P	15.375	14	1.916	.0380

Group Info for prey consumed in 60 min
Grouping Variable: treatment

	Count	Mean	Variance	Std. Dev.	Std. Err
NP	8	31.500	382.571	19.559	6.915
P	8	16.125	132.411	11.507	4.068

Unpaired t-test for prey attacked in 60 min
Grouping Variable: treatment
Hypothesized Difference Š 0

	Mean Diff.	DF	t-Value	P-Value
NP, P	16.250	14	1.451	.0845

Group Info for prey attacked in 60 min
Grouping Variable: treatment

	Count	Mean	Variance	Std. Dev.	Std. Err
NP	8	47.250	777.643	27.886	9.859
P	8	31.000	226.286	15.043	5.318

Figure 2. Sample results for Indirect Effects laboratory exercise.

Appendix D

Sample Excel Spreadsheet with Formulas

	A	B	C	D	E	F	G
1	lab group	replicate	time interval	treatment	successful	unsuccessful	total strikes/interval
2	1	1	5	P	enter data	enter data	"=SUM(E2:F2)"
3	1	1	10	P	enter data	enter data	"=SUM(E3:F3)"
4	1	1	15	P	enter data	enter data	"=SUM(E4:F4)"
5	1	1	20	P	enter data	enter data	"=SUM(E5:F5)"
6	1	1	25	P	enter data	enter data	"=SUM(E6:F6)"
7	1	1	30	P	enter data	enter data	"=SUM(E7:F7)"
8	1	1	35	P	enter data	enter data	"=SUM(E8:F8)"
9	1	1	40	P	enter data	enter data	"=SUM(E9:F9)"
10	1	1	45	P	enter data	enter data	"=SUM(E10:F10)"
11	1	1	50	P	enter data	enter data	"=SUM(E11:F11)"
12	1	1	55	P	enter data	enter data	"=SUM(E12:F12)"
13	1	1	60	P	enter data	enter data	"=SUM(E13:F13)"

	H	I
1	prey consumed in 60 min	prey attacked in 60 min
2	"=SUM(E2:E13)"	"=SUM(G2:G13)"
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		

	J	K	L	M
1	time interval	treatment	avg # consumed/interval	avg # attacked/interval
2	5	P	"=AVERAGE(E2,E14,E26,E38,E50,E62,E74,E86)"	"=AVERAGE(G2,G14,G26,G38,G50,G62,G74,G86)"
3	10	P	"=AVERAGE(E3,E15,E27,E39,E51,E63,E75,E87)"	"=AVERAGE(G3,G15,G27,G39,G51,G63,G75,G87)"
4	15	P	"=AVERAGE(E4,E16,E28,E40,E52,E64,E76,E88)"	"=AVERAGE(G4,G16,G28,G40,G52,G64,G76,G88)"
5	20	P	"=AVERAGE(E5,E17,E29,E41,E53,E65,E77,E89)"	"=AVERAGE(G5,G17,G29,G41,G53,G65,G77,G89)"
6	25	P	"=AVERAGE(E6,E18,E30,E42,E54,E66,E78,E90)"	"=AVERAGE(G6,G18,G30,G42,G54,G66,G78,G90)"
7	30	P	"=AVERAGE(E7,E19,E31,E43,E55,E67,E79,E91)"	"=AVERAGE(G7,G19,G31,G43,G55,G67,G79,G91)"
8	35	P	"=AVERAGE(E8,E20,E32,E44,E56,E68,E80,E92)"	"=AVERAGE(G8,G20,G32,G44,G56,G68,G80,G92)"
9	40	P	"=AVERAGE(E9,E21,E33,E45,E57,E69,E81,E93)"	"=AVERAGE(G9,G21,G33,G45,G57,G69,G81,G93)"
10	45	P	"=AVERAGE(E10,E22,E34,E46,E58,E70,E82,E94)"	"=AVERAGE(G10,G22,G34,G46,G58,G70,G82,G94)"
11	50	P	"=AVERAGE(E11,E23,E35,E47,E59,E71,E83,E95)"	"=AVERAGE(G11,G23,G35,G47,G59,G71,G83,G95)"
12	55	P	"=AVERAGE(E12,E24,E36,E48,E60,E72,E84,E96)"	"=AVERAGE(G12,G24,G36,G48,G60,G72,G84,G96)"
13	60	P	"=AVERAGE(E13,E25,E37,E49,E61,E73,E85,E97)"	"=AVERAGE(G13,G25,G37,G49,G61,G73,G85,G97)"