RESEARCH ON LEARNING

Investigating Dispersion of Leaf Symbionts: Exploring Specific Symbioses

NABT BioClub RECOMMENDATION

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Abstract

Symbioses can range from mutualisms to parasitisms; the latter are the foci of this exercise. The way in which parasites are distributed among hosts (their dispersion) can have profound importance for how they and their hosts coevolve, and for many other facets of their biology. Accordingly, many researchers, including ecologists and medical practitioners, study dispersion of parasites in detail. Fungi are commonly observed parasites on leaves of trees. I describe one way to randomly sample leaves to quantify dispersion of such parasites and test whether dispersion is related to a variety of explanatory variables. Significant quantities of data can be generated in relatively short order and pooled for a class; many patterns can emerge that challenge students to find logical interpretations. Relatively sophisticated students could test whether parasites have a random dispersion pattern by comparing the histogram they generate to that of a Poisson distribution. Data can be analyzed in a simple fashion or via advanced mixed models.

Key Words: *Dispersion patterns; fungi; leaves; parasite; Poisson distribution;* Rhytisma *spp.; symbionts; wasp galls.*

○ Symbionts

Symbiosis means living together, and the term is applied to non-conspecifics with persistent, intimate associations. The nature of a symbiotic relationship can vary; both participants may benefit (a *mutualism*), only one may benefit while the other is unharmed (a *commensalism*), or one may take advantage of the other (a *parasitism*). In many symbioses, one organism is larger than the other and is called a *host*. Some symbionts live on the outside of their hosts (called *ectosymbionts*), such as ectomycorrhizal fungi on tree roots, whereas others live inside their hosts (*endosymbionts*), such as endomycorrhizal fungi that grow within plant roots and virtually all other tissues. Anderson and May (1979) also distinguished *microsymbionts*, including viruses, bacteria, some fungi, and protozoans; and *multicellular* macrosymbionts, such as gall wasps. Both participants in a symbiosis are selected to maximize their benefits while minimizing their costs. Thus, there are ongoing evolutionary arms races between pairs of participant species, and a mutualistic symbiosis can quickly become a parasitic symbiosis.

"Understanding dispersion patterns of parasites is essential to understanding many aspects of host–parasite coevolution."

○ Dispersion & Parasites

For a variety of reasons, ecologists and epidemiologists expend significant effort to document dispersion patterns of organisms, including parasites (Clark & Evans, 1954; Hubbell, 1979; Ramón et al., 2016). For ecologists, there are three extreme dispersion patterns that are conventionally used as yardsticks: uniform (even), clumped (patchy), and random (Figure 1). There are both abiotic and biotic influences on how these dispersion patterns arise; these influences are usually identified after an organism's dispersion pattern has been documented.

Parasites rarely have uniform dispersion among hosts; usually, most hosts have no or few parasites, whereas only a few have high-intensity *infestations* (a term reserved for ectoparasites) or *infections* (a term reserved for endoparasites) (Goater & Holmes, 1997). A frequency distribution (a histogram) for parasite intensities (number of parasites per host) typically has a distribution similar to that in Figure 2. In some cases, if a population is experimentally treated to remove parasites, the parasites will become reestablished on the same host individuals that were previously affected (Goater & Holmes, 1997). Similarly, you may have observed that some students or employees miss many days of class or work each year because of illness, whereas others rarely become ill; and a few individuals may be chronically ill. Some variation in susceptibility arises because of

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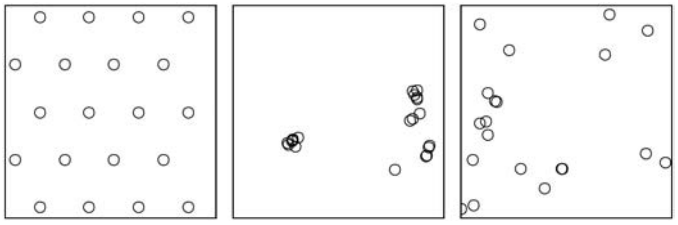

Figure 1. Left to right: uniform (even), clumped (patchy), and random dispersion patterns.

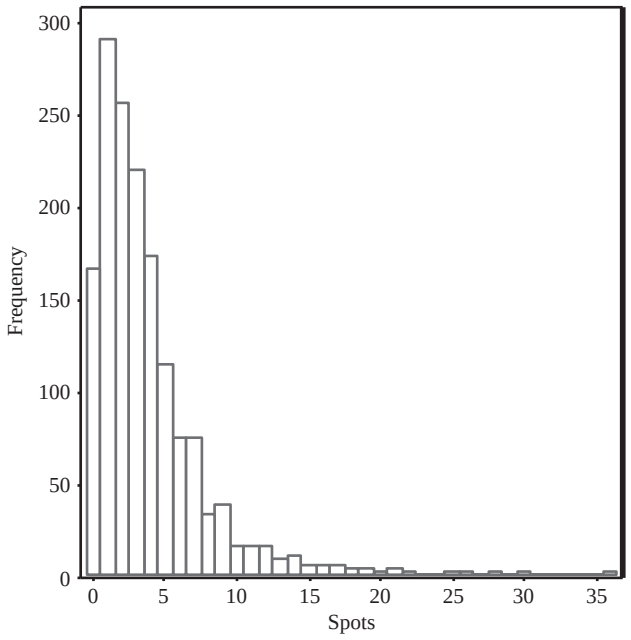

Figure 2. Histogram of 1550 leaves for which numbers of tar spot fungus spots were quantified by a parasitology class in 2009. The histogram was generated in Minitab, but it could also be generated in Excel or using a web application (e.g., http://www.socscistatistics.com/descriptive/histograms/Default.aspx).

environmental variation in encounters with parasites; some is attributable to genetic influences on host susceptibility; and some is due to interactions between environments and genes. Regardless, understanding dispersion patterns of parasites is essential to understanding how host and parasite populations may coevolve, how much hosts should invest in combating parasites, transmission dynamics and epidemiology, and many other aspects of host–parasite coevolution. Fungi and wasp galls on leaves or other plant locations provide many different host–symbiont systems that can be used to study some of these issues. Here, I describe an exercise involving tar spot fungi (often *Rhytisma acerinum* and *R. punctatum*; Figure 3), but the basic approach I describe could be applied to wasp galls or other leaf symbionts (e.g., those described in Russo, 2007; Alford, 2012). Instructors interested in running a lab such as this should venture into the field and look around for conspicuous blemishes on leaves of a plant species they can identify. They could collect some preliminary data to test for patterns, although the exercise could also be carried out blind to the outcome.

Figure 3. A Norway maple leaf with tar spot fungi (photo by author). The red circle indicates where two spots have fused. The author scored this as seven spots.

○ Tar Spot Fungi

Tar spot fungi survive winter in leaf litter; as the weather warms, spores are carried by air currents to emerging leaves, where they establish mycelia (branching fungal growths) that gradually begin forming the characteristic ~15-mm black spots that give the fungus its name, often ringed by yellow (Figure 3; Weber & Webster, 2002; Lapointe & Brisson, 2011). Tar spot becomes very conspicuous on leaves of maples (*Acer* spp.) in September through October. It has been reported from Northern and Central Europe (Leith & Fowler, 1988; Adams & Volk, 2007; Kosiba, 2007), Iowa and Wisconsin (Adams & Volk, 2007; Hudelson, 2012), Ohio and New York (Hudler et al., 1987), Quebec (Lapointe & Brisson, 2011), and Nova Scotia (present study) and is likely found in intervening and more distant jurisdictions. One of the most heavily affected host species is Norway maple (*Acer platanoides*; Lapointe & Brisson, 2011), an introduced and widely planted ornamental species (in 26 states and six provinces). For simplicity in this exercise, assume that there is a single fungal species affecting the trees that are sampled. Some authorities have determined that these fungi are parasitic (Lapointe & Brisson, 2011), although that relationship may depend on factors such as the health of the tree and the *intensity* (number of symbionts per host) of symbiosis (Adams & Volk, 2007). Depending on the kind of symbiosis, one may predict different responses by the participants (e.g., host defenses); this may affect dispersion patterns of fungi within and among trees. Regardless, one can test for dispersion patterns in relation to several easily measured variables.

○ Sampling to Avoid Bias

A key element of sampling is to avoid obtaining biased results. This requires taking as much subjectivity as possible out of the process, and the overarching principle is to sample randomly. Random sampling can be achieved in numerous ways and can be tailored to the particular question one is addressing. Basically, one should never know what data lie on the horizon; one should be completely blind to results. Below, I describe one procedure for randomizing the choice of leaves for collecting data on tar spot (and these procedures

can be easily modified), but there are general principles that apply to any sampling regime. The first is being able to generate a random starting point, and the number of potential starting points should coincide with how many random options one needs. If only two are needed, flipping a coin works. If six are needed, a die; if 12, two dice, etc. Random number tables can be found in ecology and statistics books, or one could close one's eyes and point to a phone number in a phone book, choosing however many digits one wants to use. One can also type in "random number generator" on the web and find numerous options. Another option is spreadsheets. If you have access to Excel, type "=rand()" into a cell (leaving out the quotations) and a random number between 0 and 1 will be generated. Multiplying this value by a fixed value and using the "Number" pull-down menu to control the number of decimals can generate a range of random numbers suited to any exercise one chooses (e.g., "=rand()*100" with no decimal places will give random numbers between 0 and 100). Many stats packages also have random number generators. The worst way to sample in this exercise would be to just approach a tree and gravitate, for example, to leaves that are eye-catching.

○ Exploring Dispersion in Leaf Symbionts

Tell students to bring a ruler, pad and pencil, and suitable clothing for spending two or more hours outdoors. To get students calibrated on procedure (below), take the whole class to an infected tree that is at least 150 cm tall, with branches that are within arm's length. Run through the procedure below, ensuring that everyone knows what to do. Once everyone is confident they know what to do, have them break into pairs and disperse in different directions so that everyone collects independent data. Each pair of students should be able to sample three or more trees per hour.

Procedure Using Random Sampling

1. When the presence of a symbiont on a tree is confirmed (do not work with trees that have no symbionts, and work only with a single species of *Acer*), toss a pencil or other linear object in the air. Use the orientation of the linear object to decide which side of the tree to start sampling. Identify a point approximately halfway between the trunk and the farthest reach of the lower branches. Close your eyes, and reach up and choose a leaf (don't pull it off).
2. Record the data indicated in Table 1: habitat, location, leaf color, and number of spots. Depending on what is available, find a contrast in sampling habitats; this could be "urban," "forest," "residential," etc. Try to divide sampling effort roughly equally among habitats. For location, choose either "tree" (= still on the tree regardless of color) or "ground," and for color choose either "green" or "yellow." Alternatively, one could quantify the proportion of each leaf that is green (excluding areas occupied by the fungus).
3. After collecting data for the first leaf, close your eyes and take one step in a clockwise direction in the circle that you could draw around the tree between the trunk and halfway to the farthest reach of the lower branches.
4. Repeat until you have collected data for 20 leaves on each tree.
5. Next, search the ground under the tree for fallen leaves. If there are many more than 20 fallen leaves, toss your pencil in the air and record the same data for the leaf closest to the pencil. Use the same procedure as above to find the next fallen leaf to sample. If there are fewer than 20 leaves in sight, sample as many as you can find that are likely to have come from the focal tree.
6. Do not preferentially sample leaves that have spots; once you've confirmed that a tree is infected, zero values for a leaf are an important part of the data.
7. For columns with numeric entries, write only numbers (i.e., never write ">10" or "~15"), and do not leave empty cells in the data sheet.
8. For columns with text entries (other than student names), write only lowercase.
9. Continue to collect data until a predetermined time; then return to the lab. Data can be pooled for the class prior to analysis.

Questions to Ponder

1. Ask the students how they think the fungi get on maple leaves. Because the fungi arrive as tiny airborne spores shortly after leaves open, this will affect answers to subsequent questions.

Table 1. Sample data. Enter all text variables except student names in lowercase.

Students	Habitat	Tree	Leaf	Location	Color	Spots
A & B	urban	1	1	tree	green	4
A & B	urban	1	2	tree	green	0
. . .	. . .	. . .	. . .	. . .	. . .	. . .
A & B	urban	1	20	tree	green	12
A & B	urban	2	1	tree	green	0
A & B	urban	2	1	ground	yellow	1
C & D	forest	1	1	tree	yellow	17
C & D	forest	2	1	tree	green	8
C & D	forest	2	2	ground	green	2

2. Ask the class what their expectations are for how tar spots will be distributed among leaves. Given that the fungus is at the whim of air currents to get dispersed, the class may expect spores to be randomly distributed. However, spores are likely released in pulses and probably have patchy survival because of variation in habitat. Moreover, susceptible hosts are probably not randomly distributed. This may lead to clumped distributions of symbionts. The procedure for testing for a random distribution is described below.
3. Should leaves on the tree have more spots than those on the ground? If the tree tries to rid itself of diseased leaves, the prediction would be fewer spots on leaves still on the tree. If the fungus–tree symbiosis is a mutualism, the opposite prediction would be made.
4. Should yellow leaves have more tar spot than green leaves? If the fungus is harming the leaves, the tree may respond by resorbing green pigments, or the fungus may tap into those pigments for nutrition, potentially leaving the leaves yellow. Harm is predicted to be greater for leaves with more fungus spots. Another reason that yellow leaves might have more tar spots is that leaves are simply older and have therefore been exposed to more spores and, on top of this, are senescing. If the symbiosis is a mutualism, the opposite pattern would be predicted.
5. Does the class predict more tar spots in a particular habitat? Depending on the habitats being compared, several factors may be important. In the case of residential versus forest habitats, the former may receive more fertilizers and so the trees there may be better at resisting fungi. On the other hand, raking of leaves may facilitate the spread of spores. Differences in humidity between habitats may affect spore dispersal, and different conditions in soil where fallen leaves lie may affect overwinter survival. In addition, there may be differences in the density and diversity of suitable hosts in the two habitats. As another consideration, competition may differ among habitats; trees may have more resources to devote to fighting off fungi where competition is lower. There are many other possible influences.

Data Analysis

To start, calculate *prevalence* (proportion of infected hosts), mean intensity (mean number of spots per leaf), and standard deviation (SD) in intensity. If using Excel, to obtain means, type "=average ()" and then put the cursor inside parentheses, and drag the cursor from the top of the column with data entries to the bottom. For the SDs, use the same procedure with "stdev()". Plot a histogram of intensity (the number of symbionts per individual; in this case the number of spots per leaf). Was tar spot randomly distributed on leaves? If so, the mean and SD of intensity should be similar – a defining characteristic of Poisson distributions. A more rigorous test can be done by calculating expected values for each interval in the histogram. Sample calculations are provided in Table 2. Next, calculate prevalence and mean + SD intensity separately for each habitat, for each location, and for each leaf color, and compare these values using analysis of variance (ANOVA).

The following are results from actual implementation of this lab activity. Data were collected from 1550 leaves; the mean number of spots was 3.75 and the SD was 3.84, which is relatively consistent with a Poisson distribution (Figure 2). Whereas ~60% of leaves in the forest were green, ~72% were green in the residential habitat (Table 3). About 94% of leaves sampled from trees were green versus only 36% on the ground (Table 3). The more important analyses are on tar spots. There were more tar spots on leaves on trees than on leaves on the ground (Table 4). Also, the number of spots did not differ between green versus yellow leaves. Finally, there were far more spots on leaves of forest trees than on leaves of residential trees (Table 4). These analyses do not control for repeated sampling of a tree; this would require using a mixed statistical model with tree identity as a random factor.

○ Final Remarks

MacArthur (1972) wrote that "to do science is to search for repeated patterns." If this perspective on science is not already awake in your students, the exercise described here has the potential to awaken it. The content of the lab introduces students to the entire range of the scientific process, beginning with simply

Table 2. Calculating expected frequencies under the null expectation of a random distribution of tar spot fungi based on a hypothetical sample of 1000 leaves.

Number of Spots	Observed Frequency	Expected Frequency	Contribution to Chi-square
0	109	23.6	309.0
1	188	88.5	111.9
2	166	165.7	0.0
3	143	206.9	19.7
4	112	193.7	34.5
5	74	145.1	34.8
>5	208	176.5	5.6

Notes: To calculate the expected frequency, you first need to calculate the mean, which for these data was 3.746. Expected frequencies for a Poisson distribution are $e^{-\bar{x}} * \bar{X} / x$ factorial * N. For the first cell, this is $0.0236 * 3.746^0 / 0! * 1000$. Note that 0! is 1. The contribution to the χ^2 statistic is (Observed-Expected)^2/Expected; the final χ^2_6 stat is the sum of these values. Without doing the calculations, the first line indicates significant deviation from a Poisson distribution; P will be much less than 0.001.

Table 3. Analysis of the association between leaf color and habitat (χ^2_1 = 19.8, P < 0.001), and between leaf color and whether the leaf was on a tree or on the ground (χ^2_1 = 601.9, P < 0.001).

Color	Forest	Residential
Green	247	815
Yellow	166	322
	On the Tree	**On the Ground**
Green	820	242
Yellow	52	436

Table 4. Comparison of the number of tar spots on leaves with different attributes (ANOVA).

Category 1	Mean Spots	Category 2	Mean Spots	$F_{1, 1548}$	P
On tree	4.2	On ground	3.2	25.3	<0.001
Green	3.7	Yellow	3.8	0.4	0.54
Forest	5.3	Residential	3.2	100.6	<0.001

collecting data, perhaps with no hypothesis in mind, followed by testing for patterns, and, when confronted with patterns, trying to explain them. In addition, students will get a glimpse into the complexity of symbiotic relationships. The exercise can be used for a variety of levels of students; it is up to the instructor to decide how far to take the presentation of data and the analyses.

○ Acknowledgments

I thank the Acadia student newspaper, the *Athenaeum*, for publishing an article about tar spot fungi many years ago that inspired me to create this lab. I also thank the many students who have now collected data in multiple years; the patterns that emerged encouraged me to publish this article. Finally, I thank a very insightful reviewer for a wealth of valuable suggestions and guidance that substantially improved the paper.

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