Experimental Studies of the Biology of 13- and 17-year Periodical Cicadas

A Laboratory Exercise for University and AP Biology Laboratory Classes.

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This purpose of this exercise is to educate students about the ecology and evolution of periodical cicadas and to enlist their aid in gathering important scientific data on growth rates of cicadas with 13- versus 17-year life cycles. It is designed to be adaptable to a broad range of educational levels: university/college, and grades 7-12.

The first section, introduces the natural history of periodical cicadas. It poses a series of questions that can be answered by accessing www.magicicada.org, a crowd-sourcing, reporting, and informational website dedicated to periodical cicadas created by our collaborator and colleague, John Cooley.

The second section describes a laboratory exercise that involves: 1) Digging 13- or 17-year cicada nymphs, 2) Learning to identify nymphs to one of five juvenile stages (instars), 3) Comparing results to those already published, 4) Summarizing the results and creating new study questions, and 5) Preserving specimens for DNA work and sending them to the Simon lab for archiving and species identification by PCR. Very ambitious classes can try PCR-species-typing using species-specific PCR primer sequences available upon request.

The third section describes alternative exercises that include: 1) Listening for cicada stragglers (off-year emergences) every year during the emergence season in an area that has dense periodical cicada populations, 2) Studying the history and popular culture of cicada emergences, 3) Using egg scars to study population ecology, and/or 4) Learning more about non-periodical cicadas.

Section 1. The Biology of Periodical cicadas. Periodical cicadas are unique, highly unusual and intriguing insects that make “recklessly theatrical” appearances in a given area once every 13- or 17-years. They have been the subject of hundreds of scientific studies involving areas of enquiry as diverse as predator-prey dynamics, life history strategies, species formation, courtship behavior, reproductive character displacement, and symbiosis. Appendix 1 poses a series of questions about periodical cicada biology that can be assigned as a take-home exercise and answered via web research. We recommend that students watch the following videos by Sam Orr at home before the day of the exercise to gain an appreciation for periodical cicadas:

Return of the Cicadas on Vimeo (Return of the Cicada by Sam Orr, 2013, short, 7:24).
Swan Song of the Cicadas. http://www.nytimes.com/2013/07/01/nyregion/during-cicadas-swan-song-many-wonder-if-they-missed-the-show.html?_r=0

Section 2.

2.1. Field studies on the growth and development of periodical cicada nymphs.

The purpose of this nymph-digging project is to 1) Track the development of growing *Magicicada* nymphs, 2) Correlate rates of instar change over time with local climactic factors, and 3) Compare your results to those obtained elsewhere. Read Appendix 2 to learn about growth patterns of periodical cicada nymphs and why they are interesting.

Objectives.

Obtain samples of developing underground periodical cicada nymphs, classify nymphs according to developmental stage, summarize the data, compare data to published studies, draw conclusions and pose new questions.

Materials for digging.

1. Shovels
2. Tarps or trash bags to kneel on and dissect soil samples
3. Tight-seal vials and 95% ethanol for specimens (to preserve DNA)
4. Pencils + white paper in small pieces for labeling, e.g., index cards w/o lines are ideal, avoid pen; the ink will bleed in alcohol, mechanical pencils work well.
5. Tweezers
6. Thin cotton gardening gloves (long gloves if there is poison ivy in the area)
7. Field notebook-to describe activities, location, habitat type, weather, numbers of nymphs found.
8. Rulers or tape measures to measure depth of holes
9. A one-liter beaker or a two-liter soda bottle with the top section cut off and a one-liter measure mark (to measure soil volume

It is also useful to bring at least one GPS locator (e.g., the compass app that comes with every I-phone; or some other navigation device, e.g., Garmin Nuvi).

Figure 1. Two 5-yr old nymphs dug from Tomlinson Run Sate Park, WV, 2009, Photo by C. Simon

Directions for digging.

Find the locations nearest you where the last emergence was most dense. If it occurred within the past 7-8 years, egg scars should be apparent on the branches of trees in the area (Google: “periodical cicada egg scar images”), if you find egg scars, start digging below them or in that area. Dig under egg nests at the edge of the forest rather than inside the forest. Cut rough squares of earth with the blade of the shovel, and carefully pull up the soil. Younger nymphs may be closer to the surface (1st instars can be on surface roots). Most nymphs will be found within 30cm of the surface but they can be found deeper. For this exercise, separate nymphs by depth into three sections; 1-10cm, 10-20cm, and 20-30cm. First, second, and third instar nymphs will be small and whitish (Figs. 1-4); as nymphs age they take on a golden hue (Figs. 5 and 6). Pick up nymphs carefully with tweezers or your hands; avoid crushing them (this is quite easy to do accidentally). In clay soils, you may need to break open clods of dirt with your hands to

find the nymphs sitting inside in their mud cells. Sample one liter of soil at each depth, in each plot by filling the beaker or soda bottle to the one-liter mark. Soil can be sorted on site on a plastic sheet or in the laboratory. Students who aren't digging can search soil for excavated nymphs, prepare labels and place nymphs in the ethanol solution.

A pair of students should sample one plot at all three depths. Soil from one plot can take up to an hour to search, depending on nymph density. Other soil invertebrates can also be surveyed (and identified using on-line resources). Recording date, time, locality, vegetation, weather conditions, should not take more than ten minutes. Nymphs can be identified to instar in the lab using a dissecting microscope, photos immediately below, and the chart in Appendix 3. Specimens should be kept cool (e.g., ice or fridge) when not under the scope. Insect collecting labels look like this (Remember to use pencil!):

County or Parish, State, Country
GPS + Description of location
Date, Collector's name

Note to instructors: when repeating this exercise from year to year, try to keep the digging month/day as similar as possible.

2.2. Gathering Climate Data.
1. Investigate the climate in your area

- CRITICAL THINKING BOX.
  Why would latitude alone be an inexact indicator of growing degree days?
  (Hint: took at maps of hardiness zones and growing degree days on the web.)

  How does the underground habit of cicada nymphs help to buffer them from the aboveground climate?

Growing season and other climactic factors influence the growth rates of nymphs so we need a comparative measure of number of feeding days for each locality where nymphs are sampled. We suggest three below: a) Time between the first and last frost-free day, b) Hardiness zone, and c) The historical average growing degree days (since 1995). These are of course related to each other. To find the first frost-free day for planting and the last frost-free day for harvesting in your area, see http://davesgarden.com/guides/freeze-frost-dates/. To find your Hardiness Zone, see http://www.garden.org/zipzone/. To find your growing degree days, google "Weather Underground". Then enter your zip code or city name" and click on "Today's Almanac", and in the lower right corner, "view more history data", choose "Monthly" and set the date for 1 May 2013. You will see averages based on data from 1995 to present. Record the sum of the growing degrees days, and precipitation.

2.3. Identifying Instars in Nymph Samples

Both 13- and 17-year *Magicicada* go through five instars during development. Nymphs can be identified using a variety of physical characteristics under a dissecting microscope: Size, shape, hair patterning, antennae characteristics, and femoral comb characteristics tooth characteristics. It is important to remember that the femoral comb alone is not an entirely reliable marker for identifying instar (Thombre 2011), so that it is essential to use Appendix 3 as well as Figs. 2-6 below.

Figure 2. First instar nymph of M. *septendecim* tibia and femur(external side). As noted by Marlatt (1907), no femoral comb is present and the tarsus is extended from the tibia. Soon after entering the ground, the tarsus is folded back against the tibia until the 5th instar nymphs leave the soil. Inset: 1st instar nymph.

photos: E. Dwyer (left), C. Simon (right).

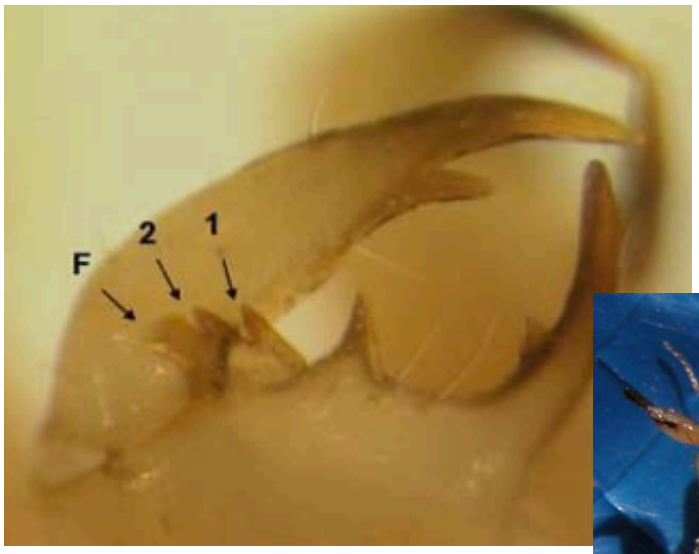
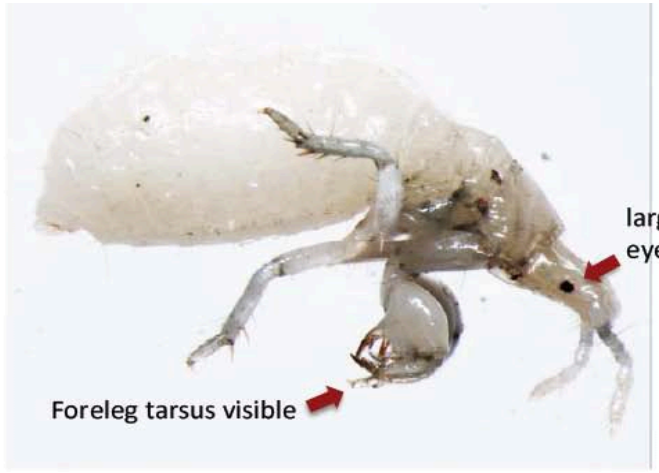


Figure 3. Second instar nymph of M. *septendecim* tibia and femur (exterior side). As noted by Marlatt (1907), four femoral teeth are present; 1, 2, 3, and F (flat), respectively. Photo by R. Thombre. Inset: 2nd instar nymph, photo by C. Simon.

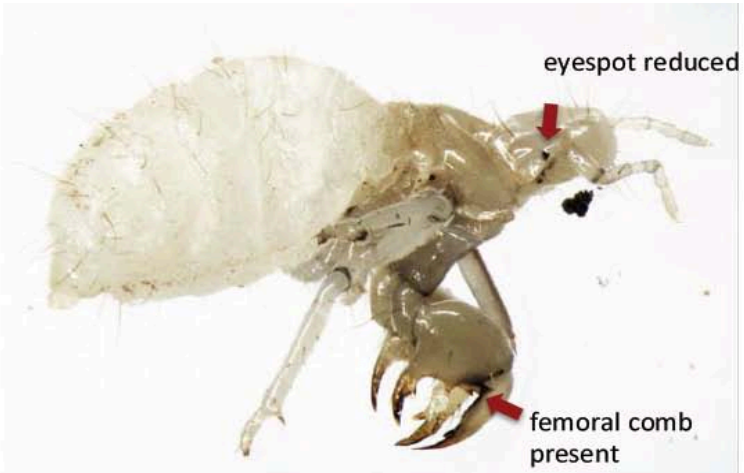
Sweet Briar College, three-month old first and second instar nymphs

collected by Prof. Linda Fink's "Introduction to Organisms" class

Automontage photos by Erin Dwyer



1st Instar Nymph Foreleg femoral comb absent



2nd Instar Nymph tarsus folded back, not visible

Photos of students supplied by Linda Fink

Figure 4. Third instar nymph of M. *septendecim.* Left: tibia and femur (exterior side). Femoral comb displays four teeth, numbered 1, 2, 3, and F (flattened). Photo by R. Thombre. Inset: whole body view of a preserved 3rd instar nymph, photo by Mark R. Smith, www.macroscopicsolutions.com

Figure 5. The fourth instar nymph of *Magicicada septendecim*. a) Femoral comb. Five teeth are present; labeled 1, 2, 3, 4, and F. Photo: R. Thombre. Insect: Preserved 4th instar nymph, photo by Mark R. Smith, www.macroscopicsolutions.com.

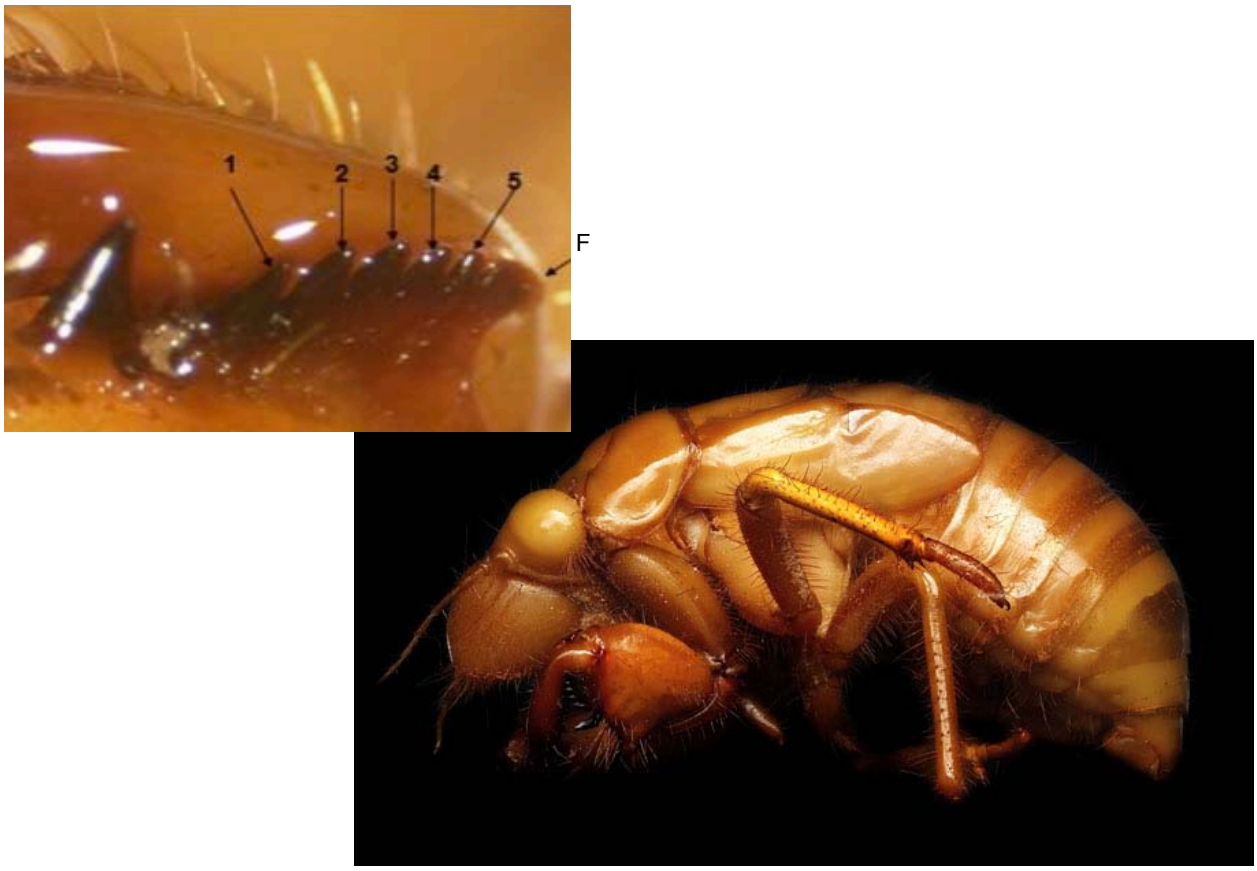


Figure 6. Fifth Instar nymph. Left. Femoral comb with six teeth labeled 1-5, F (flat tooth). Photo by R. Thombre. Right. Preserved 5th instar nymph, note the large wing buds and large colorless eyes. Photo by Mark R. Smith, www.macroscopicsolutions.com.

Data, location, climate, age, and instar can be displayed in a graph, as shown in Fig. 7 (next page) modified from White and Lloyd (1975). In the key above the bar chart, localities are identified by letters. Each location's latitude, USDA Hardiness Zone, and Average Growing Degree Days for the month of May (Average since 1995) are displayed. On the x-axis of the bar graph, the locality code letter is preceded by a number that indicates the age of the nymphs in that sample.

Of course, this is not the only way to display nymph data, but it shows many relevant types of information for 17-year cicadas in one chart; percentages or each instar in each sample, nymph ages, and geographic location. It is important to display all of the data collected together in order to make connections between growth rate, climate, species, and age. When considering the climate for a population of nymphs, it is important to consider both the year of collection as well as the number of years since the nymphs hatched. A change in rainfall or temperature in any of these years may have a significant impact on the population when compared to other populations.

Key to localities on the x-axis of the bar chart below:
A-Pilot Mound, Iowa, 42° 7' N, Hardiness Zone 4b; Growing Degree Days for May (GDD) 10
B-Webber's Falls, Oklahoma, 35° 30' N, Zone 7a, 16 GDD
C-Davenport, Iowa, 41° 47' N, Zone 5a, 13 GDD
D-Raccoon Grove, Illinois, 41° 33' N, Zone 5b, 11 GDD
E-Bessire Orchard, Indiana, 39° 16' N, Zone 6a, 15 GDD
F-Brown Fruit Farm, Ohio, 40° 9' N, Zone 6a, 15 GDD. Localities A-F from White & Lloyd, 1975.
M-Southington Connecticut, 41° 6' N, Zone 6a, 9 GDD, Maier 1996.
N-Ridge, NY, 40° 9' N, Zone 6b, 8 GDD, Dwyer, Cooley, Meister, Banker, Bonaros & Simon, unpubl.

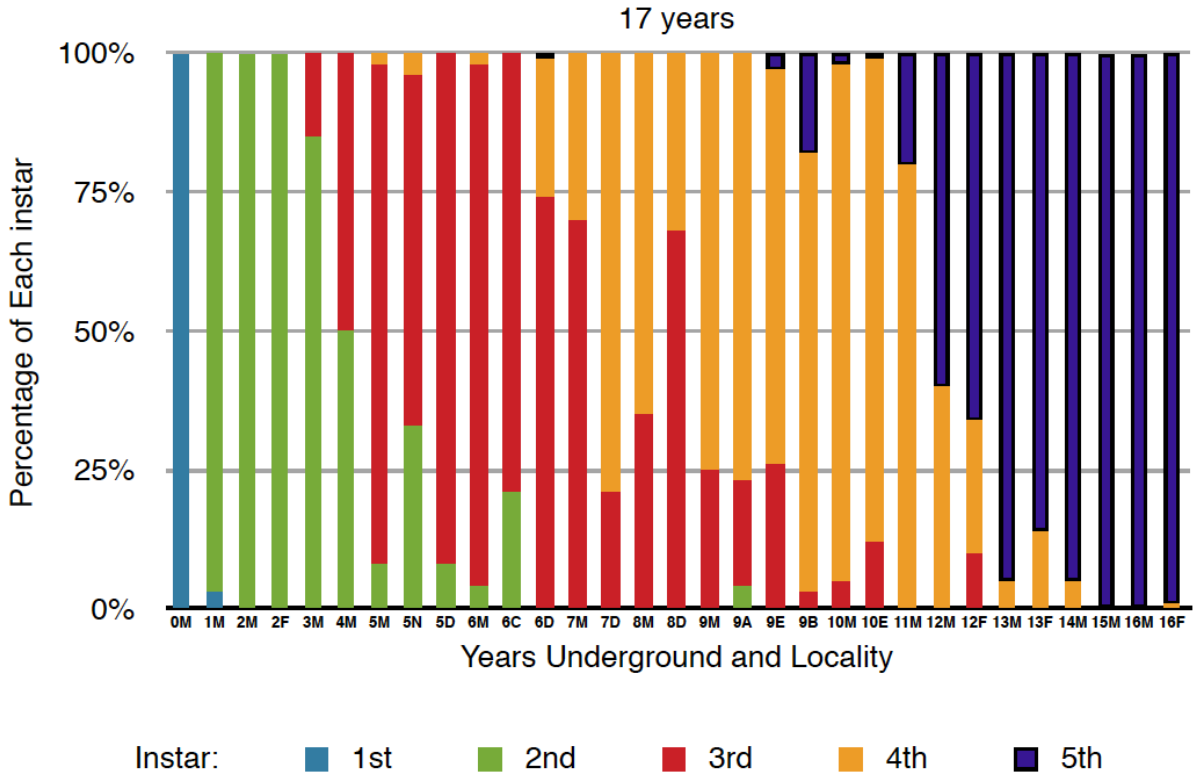


Figure 7. The proportions of nymphs of each instar (y-axis) at different ages and different locations (X-axis with key above chart). This is similar to Fig. A2-1 in Appendix 2 (Connecticut nymph growth only), but appears more irregular because of the addition of multiple localities. Sample sizes vary among locations and years: M (all ages): 100 nymphs in each year sampled, 2F: Unknown, 5N: 24 nymphs, 5D: 50 nymphs, 6C: 42 nymphs, 6D: 94 nymphs, 7D: 102 nymphs 8D: 124 nymphs, 9A: 590 nymphs, 9E: 116 nymphs, 9B: 386 nymphs, 10E: 1040 nymphs, 12F: 344 nymphs, 13F: 249 nymphs, 16F: 150 nymphs. Data from White and Lloyd (1975) and Maier (1996). See Fig. A.2.1 for a bar chart showing nymphs collected once per year for 17 years in a row at a single location in Connecticut.

2.5. Statistical Data Analysis

Use a contingency table like the one below (Fig. 8) and those in Appendices 1, and 4 to test for significant differences in the proportions of each instar in different samples. The table is completed by using the totals of each sample and each instar to calculate an ideal value for each instar for each sample.

	Sample 1	Sample 2	Total
5th instar	Number of 5th instar nymphs in this sample	Number of 5th instar nymphs in this sample	Add up the number of 5th instar nymphs
Expected	The percentage of fifth instar nymphs multiplied by the total of this sample	The percentage of fifth instar nymphs multiplied by the total of this sample	The percentage of total nymphs that are in all samples that are 5th instar
4th instar			
Expected			
Total	Total nymphs in the sample	Total nymphs in this sample	Total of all nymphs in all samples

Figure 8. A template for a table to calculate the statistical differences between samples. Further analysis can be done by finding the difference between the expected and experimental values, and then the percent difference of that. The percent difference can be found by dividing the difference by an average of the two values, experimental and expected. These values can be used to assess and compare the differences between different samples. The null hypothesis is that there is no significant difference between the samples.

The statistical significance of the difference between the observed and the expected result can be determined using the G test. The G-statistic is calculated by taking the natural log of the number of nymphs divided by the expected number of nymphs, and then multiplying this value by the observed value, taking the sum of these values, then multiplying by two: G=2 x sum [observed x ln(observed/expected)]. The significance of the G-statistic can be found by comparison to statistical tables for the G-test (e.g. Rohlf and Sokal 1995). After the G-statistic is calculated, its significance can be found through a statistical table, using the degrees of freedom of the test. The degrees of freedom of the test are calculated by taking the number of variable possibilities, and subtracting one. In this case, this is calculated by finding the number of instars represented in each sample, and subtracting one. For the above example, the degrees of freedom would be one, because there are only fourth and fifth instars shown.

2.6. Conclusions and Questions

Now that your data is organized, can you draw any conclusions?
Did your results mirror those of White and Lloyd (1975) or Maier (1996)?
Think of reasons why this may or may not be. Maier (1996) collected in Connecticut, where only *M. septendecim* was present. Is your climate warmer?

Looking at records of emergences in your area, do you have a mixed population of the three species instead of just one? Note: Records can be found through newspaper archives, museum specimens, or interviews of experts who witnessed the last emergence in your area. It is important to have records from exact locations. What is the predominant instar in your sample, does this fit your prediction? Could your results be influenced by sampling error? Discuss in groups or as a class the ways in which climate, growing season, crowding, and other factors could have influenced the growth rates of your nymphs. (e.g., see Lloyd, Kritsky, and Simon, 1983). Appendix 5 reports preliminary results from this lab exercise from Sweet Briar College.

Section 3. More questions to investigate

3.1. Do ovipositing (i.e., egg-laying) periodical cicada females prefer some trees over others? Egg scars are noticeable up to 9 years (or more) after emergence and easily visible up to five years after an emergence. Students can learn to identify scars (Google periodical cicada egg scars; see also Fig. A-3.3) and mark them. Eggs are laid in pencil sized branches generally in the previous year's new growth. Each year, students can follow egg scars as the trees grow. Many studies have involved censusing and determining ecological preferences in ovipositing cicadas. Particularly ambitious classes can read the following papers and attempt to gather similar data: Simon et al. (1981), White (1980), Cook and Holt (2006). Full citations are listed below on page 11.

3.2. Are periodical cicadas more abundant at forest edges? Sample nymph densities along a transect from the inside the forest to just beyond the canopy edge using the protocol from Section 2. Compare your results to those of Maier (1980, 1982).

3.3. How do ant predators affect the density and patchiness of *Magicicada* nymphs? Another interesting experiment, that has not been done previously, involves monitoring predator ant populations along a transect that crosses the nymph survey area (density enhanced plots and natural plots). This predation is especially relevant to the smaller instars (1^{st}, 2^{nd}, 3^{rd}). The larger 4^{th} and especially 5^{th} instar nymphs are favorite food for moles (see Lloyd and Dybas 1966a). Quantify ants within plots and between plots by monitoring ¼ tsp tuna baits on plastic yoghurt lids every day for a week (sample at different times of the day to be sure not to miss them). Record ant density at each station for each time of day (e.g., 9 am, 11 am, 3pm, 4pm—avoiding the hottest parts of the day). Correlate the ant population densities with the nymph densities at each location to help understand the impact that ant predation might have on nymph populations underground.

3.4. How do attitudes towards periodical cicadas differ among people? Investigate the history of past periodical emergences in your area. Search for information related to periodical cicadas, e.g., local newspaper archives and museum collections. **I**nterview citizens who have experienced past emergences**.** Discover ways in which periodical cicadas been incorporated into the local culture (hint: Check out Cincinnati, OH or Nashville, TN, Ri-Bhoi, India, and Navosa or Serua, Fiji for interesting examples.)

3.5. What proportion of periodical cicadas emerge in "off-years"? Listen for stragglers (cicadas appearing in off years) in April, May or June (depending on your hardiness zone) in areas that experience heavy emergences of periodical cicadas. To detect and describe stragglers you will need to learn to identify periodical cicada species by their songs which you can find here: http://www.magicicada.org/about/behav.php

Experiments that can be set up by instructors in the year of a periodical cicada emergence:

3.6. Create square mater plots at the forest edge under trees with egg nests; label with a permanent label and stake at each corner. Enrich these plots with hatching cicadas from an adjacent area. To do this watch for hatching 6-8 weeks after the eggs are laid this would place hatching in June-August, depending on hardiness zone. Cutting the egg nests too early can kill the eggs, but cutting a week before hatching should be fine. The safest bet is to wait until some eggs hatch and then cut many branches. Count the number of egg nests. Place a quantity of cut twigs in a pile inside each plot. The eggs will hatch, nymphs will jump out of the branches and crawl into the ground. Create replicate plots with different densities of nymphs. Have your lab classes sample nymphs once or twice per year using the same protocol described in section 2. Compare nymphal growth rates under different density conditions. Alternatively, all plots can be supplemented with the same densities of nymphs. Increasing the density of egg nests in a plot will increase the probability of finding nymphs in that plot in later years.

Figure 9. Eggs dissected from branches. From left to right; healthy, dead, and hatched.

Experiments that can be set up by instructors in the year prior to the emergence: sample 5th instar nymphs to determine exactly when the eyes change from colorless to red. Marlatt (1907) reports that the eyes change to red in the fall prior to the emergence spring but it may not happen simultaneously in all individuals. More data is needed to understand this phenomenon.

Studies of Non-Periodical Cicadas Worldwide

Information about other species of cicadas can be found online by following the links to other cicada sites consolidated at Magicicada Central Resources…
http://hydrodictyon.eeb.uconn.edu/projects/cicada/resources/resources.php

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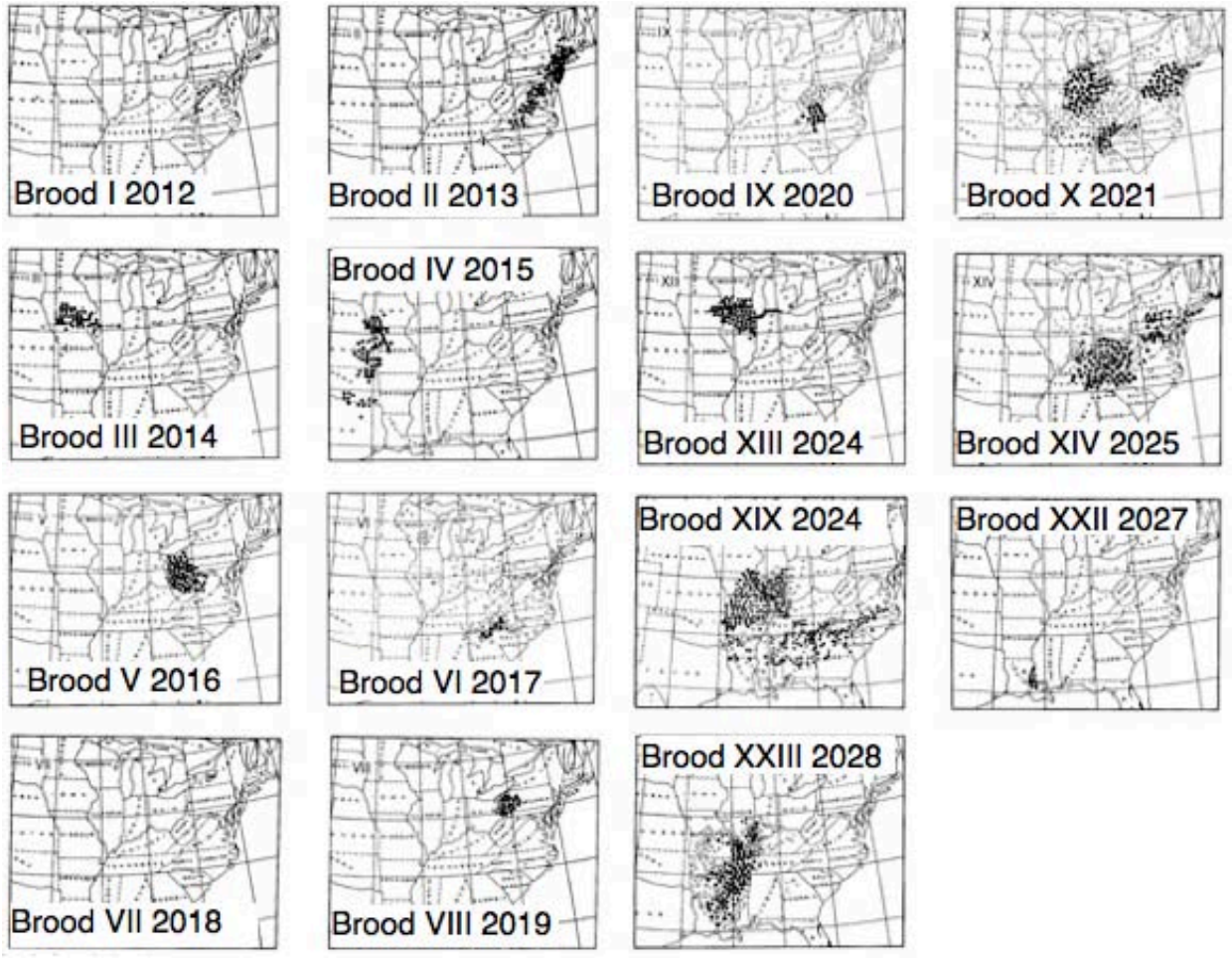
Appendix 1. Homework study questions. After watching the videos mentioned in Section 1, students should prepare answers to the following questions by reading information at www.magicicada.org. For younger students, the class could be divided into groups each of which takes a set of questions and becomes the expert in that section. In class, the instructor can ask the questions and allow the relevant student experts to explain the answers to their peers.

I. General Periodical Cicada Information

1. In what region of the world are *Magicicada* found?
2. How many broods of *Magicicada* have a 17-year lifecycle?
   a. 13-years?
   b. What brood occurs in your area and when will it emerge next?

Figure 3A-1. A historical map of brood distributions. Larger map www.magicicada.org
See also: Maps of individual broods



3. At what time of year do *Magicicada* emerge?
4. Name a species of non-periodical North American cicadas; How often does this species emerge?
5. What is the best way to differentiate male and female *Magicicada* though their morphology?
6. Are cicadas dangerous? Why or why not?
7. How can Cicadas damage tree branches?
8. How many juvenile stages (instars) exist in the multi-year development of *Magicicada* nymphs?
9. At what time of day do fifth instar nymphs typically emerge and begin eclosion into the adult stage? What does eclosion mean?
10. What aspect of soil can be used to predict an emergence?
11. How many eggs can one female lay?
12. How long does it take for *Magicicada* eggs to hatch?
13. What is the greatest population density that has been observed in *Magicicada*?
14. By what phenomenon do periodical cicadas maintain their population in the face of predation?
15. Name a fungal parasite that preys on *Magicicada*.
16. What circumstances can cause wing deformations in *Magicicada*?

II. Species

1. What is the name for the dense aggregations of adult *Magicicada* that form during mating?
2. Name the three species of 17-year *Magicicada*
3. Name the four species of 13-year *Magicicada*
4. In which broods or sections of broods are only one species present?
5. Name three characteristics by which the morphological species groups are differentiated?
6. How are call songs different in areas of species group overlap?

III. Broods and distributions

1. When will brood VI next emerge?
2. What brood(s) are in your state?
    a. Does it appear from the brood maps that there are overlapping broods in or around your state?
    b. When next will this (or these) brood emerge?
3. What are stragglers?
4. How do stragglers complicate or invalidate some historical records of *Magicicada* emergences?

IV. Behavior

1. What is the name of the ribbed membrane that male *Magicicada* use to produce sound?
2. What is the name of the hearing organ of *Magicicada*?

3. What are the three types of calls emitted by male *Magicicada*?
4. What is the female response to the male call?
5. How do these calls promote speciation and restrict hybridization of the co-existing species? Listen to the different species-specific male and female courtship songs

V. FAQs

1. What are the months of the next *Magicicada* Emergence?
    a. What brood is emerging?
    b. What is the range of this brood (you may have to refer back to the Broods section)
2. In what months do annual cicadas emerge?
3. How long does an emergence typically last?
4. How far will *Magicicada* typically travel to lay their eggs?
5. How do the colorations of M. *septendecim* and M. tredecim differ?
6. How do *Magicicada* feed?
    a. What part of the tree do cicadas feed on?
    b. Can cicadas chew?
    c. What is the name for the cicada sucking mouthparts?
7. What is another name for newly emerged cicadas?
8. When during the day do *Magicicada* sing?
9. How many eyes do *Magicicada* have? How are the two types of eyes different from each other?

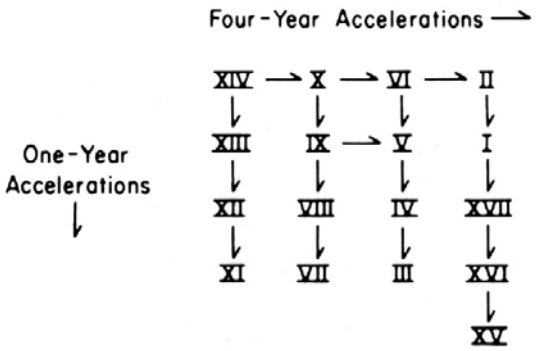
VI. Links

1. Name five species of New Zealand Cicadas
2. To which insect order do cicadas belong?
3. What is the usual length of the life cycle of a cicada?
4. What is the soil temperature that signals 5th instar *Magicicada* nymphs to emerge?
5. Describe the purpose of these sites listed on the *Magicicada*.org Links Page:
    a. Periodical cicada (*Magicicada*) mapping project homepage
    b. Periodical cicada (*Magicicada*) record database
    c. World cicada record database
    d. New Zealand cicada record database
    e. The department of Ecology and Evolutionary Biology at UCONN
    f. Cicada Central
    g. Tim McNary's Cicada Bibliography
    h. Cicada Mania
    i. Gerry Bunker's Massachusetts Cicadas Site
    j. John Zyla's Mid-Atlantic Cicadas site

Appendix 2: Living Life in Four-year jumps.

Background: Lifecycle Switching and four-year stragglers

Periodical Cicada broods are arranged in a jig-saw-like pattern; some overlap, some don't. They are numbered sequentially by year of emergence (17's: I-XVII and 13's XVIII-XXX). Twelve broods of 17-year cicada and three broods of 13-year cicada exist today. The largest broods of 17-year cicadas are separated from each other by 4 years in adult emergence time. These broods overlap geographically and likely formed from each other by 4-year changes in the life cycle (Lloyd and Dybas 1966b, Simon and Lloyd 1982, Cooley et al. 2009; see figure to right). Broods separated in time by one year are smaller, often adjacent but never overlap These broods are often arranged in geographic chains that fit together like pieces of a jig saw puzzle (Alexander and Moore 1962, Lloyd and Dybas 1966b, Simon 1988, Cooley et al. 2009, 2011, 2013).



In general, Northern climates have 17-year cicadas and southern climates have 13-year cicadas (exceptions- 17-year cicadas in Georgia, Texas and Oklahoma; 13-year cicadas in Illinois, Missouri and Iowa) suggesting that it is difficult to complete development in 13-years given northern growing seasons. An alternative explanation is that there is more of a premium on larger body size and higher fecundity in the northern US with its colder climates. Karban (1997) found that 17-year females had more eggs than 13-year females living at similar latitudes. Size variation among adult individuals within populations is substantial (Simon 1979b).

Periodicity is not perfect. Four-year early emergences of 17-year cicadas are common and well documented (Dybas 1969; Lloyd and White 1976; Simon, Karban, and Lloyd 1981; Simon and Lloyd 1982; Lloyd, Kritsky, and Simon 1983; Heliovaara et al. 1994, Marshall 2001, Cooley et al. 2009, Marshall, Hill, and Cooley 2011). Four-year early 13-year cicadas have been observed less often and in smaller numbers (Marshall, Hill and Cooley 2011). There has been at least one well-documented sizable emergence of 17-year cicadas four years late and numerous records of a few individuals of both 13- and 17-year cicadas four years late, one year early or one year late. Other deviations are rare (Marshall et al. 2011). Periodical cicadas have a predator-foolhardy behavior and safety-in-numbers strategy for survival ("predator satiation"; Karban 1982, Williams et al. 1993); low-density populations are in danger of extinction so life-cycle- switching individuals are often short lived.

The cause of life cycle shifts is not clear; hypotheses range from nymphal crowding limiting nutrient availability in the immediate environment (Lloyd et al. 1983), to hybridization between 13- and 17-year cicadas (Yoshimura 1996), to genetic variation

for life cycle plasticity that is maintained by interdemic selection (Simon et al. 2000;Marshall and Cooley 2000). Nutrient (xylem fluid) quality and quantity must be related to soil fertility, endosymbiont health, and growing season length.

Nymphal and adult nutrition in cicadas is dependent on two obligate bacterial endosymbionts species from two genera: *Sulcia* that manufactures 8 of the 10 amino acids essential to cicadas and *Hodgkinia* that manufactures the remaining two (McCutcheon et al. 2009a,b). All cicadas suck xylem fluid exclusively that supplies water and nutrients to themselves and their bacterial endosymbionts.

Nymphs grow at highly variable rates underground. Nymphal growth is not synchronized despite the fact that adult emergence is highly synchronized; thus instar timing is independent of eclosion timing except that all members of a population must be in the last instar after 13- or 17-years if they are going to emerge together. Nymphs of 17-year cicadas grow slower than the nymphs of 13-year cicadas particularly in the second and third instars; growth rates vary with latitude and within populations (White and Lloyd 1975; Maier 1996).

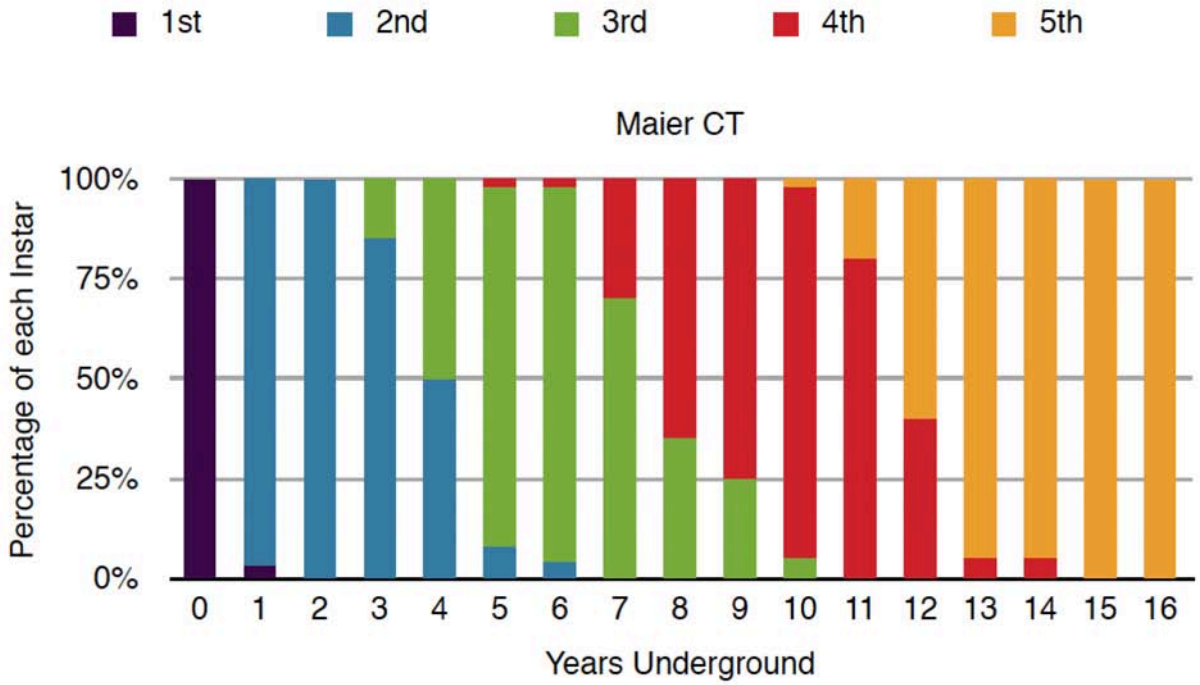


Figure A2-1. the proportions of nymphs collected by Chris T. Maier in Southington CT from 1979-1995 (Maier, 1996). Each year in October or November, Maier collected 100 nymphs from the same population. The even distributions of instars in this chart shows how the nymphs developed as a group over time. The instars are not synchronized, but it is unclear whether individual nymphs have consistent rates of development throughout their growth period. Note that second instar nymphs were found over a 6-year period, 3rd's over an 8-yr period, 4th's over a 10-yr period, and 5th's over a 7-yr period.

Why do broods separated by one year never overlap geographically. A number of hypotheses have been put forward to explain this lack of overlap: 1) Intense nymphal competition for feeding space prevents nymphs from becoming established on roots occupied by a previous-year brood (Lloyd & White 1976, Bulmer 1977). 2) Intense competition for oviposit-tion sites above ground prevents females from laying in branches heavily scarred in a previous year- (White 1980, 1981). 3) Predator build-up (Hensley 1986, Strehl and White 1986) from the previous year reduces lagging population densities resulting in extinction of populations too sparse for survival given their high-density-adapted life history strategy (Karban 1982, Williams et al. 1993).

Why can broods separated in time by four years overlap successfully. Simon et al. (1981) studied two well established 17-year broods (I & XIV) that overlapped in the same forest on Long Island (Simon & Lloyd 1982). Brood I was smaller in extent and contained entirely within the range of brood XIV. In forest areas where the two broods overlapped, the forest supported twice as many individuals as in the adjacent forest area where Brood XIV occurred alone. Census data was obtained by measuring egg-nest density of each brood following the 1978 emergence of Brood I (Brood XIV appeared in 1974). Lloyd and White (1976) proposed that nymphs were different enough in age and size to avoid competition by partitioning feeding sites by root size.

How are the current broods related to each other? Lloyd and Dybas suggested that the large overlapping broods were formed from an ancestral brood that was synchronized with present day Brood XIV. They suggested that small broods budded off the larger broods by coming out one year early. The diagram (right) summarizes their hypothesis. The success of 4-year life cycle shifters depends on having either 1) sufficient numbers of individuals co-emerge to satiate predators or 2) early emergers joining an overlapping protective “nurse brood” with the same life cycle as the switchers. The nurse brood would offer protection from predators (Lloyd and Dybas 1966, Marshall and Cooley 2000). Evidence for the success of 4-year life cycle shifts in populations that do not join nurse broods can be found in modern day Long Island (Simon & Lloyd 1982) in Broods I, V, and IX (although these now appear to be extinct or nearly extinct they were healthy self reproducing populations throughout the 1900’s and probably well before that); and the healthy self-perpetuating northeast TN disjunct population of brood I (Cooley in prep.). Evidence that 1-year accelerations sans nurse broods do survive is the existence of modern day parapatric brood groups [X, IX, VIII, & VII]; [VI & V]; [IV & III]; [II & I]; and [XXIII & XXII] (see diagram right from Lloyd & Dybas 1966b).

Various Hypotheses have been put forward for the evolution and control of life cycle length. The day of adult emergence in the 13^{th} or 17^{th} year is influenced by ground temperature (Heath 1987) but the year of emergence is hypothesized to be controlled by an internal clock that counts the years (Williams and Simon 1995) most likely by monitoring and counting host plant annual cycles (Alexander and Moore 1962, Karban, Black, and Weinbaum 2000). Hypotheses for the evolution of the long periodical prime-numbered life cycle from a non-periodical ancestor are summarized

and ordered chronologically (Williams & Simon1995). The feasibility of some of these hypotheses has been examined with deterministic mathematical models (Lehmann-Ziebarth et al. 2005, Yoshimura et al. 2008).

When do 13- and 17-year cicadas make their decision to shift life-cycle length? Empirical data (White & Lloyd 1975; Maier 1996) suggests that each nymph's decision on whether to emerge after 13 or 17 years appears to occur prior to the fifth instar. As discussed above, four-year early and late emergences of subpopulations of both 13- and 17-year cicadas are common.

Figure A-3.2. Left. Erin Dwyer digging nymphs, in Ridge, Long Island, NY 7 Oct 2012. Brood XIV emerged at this location in 2008.

Figure A-3.3. Below. Tomlinson Run State Park, 2007. Brood VIII emerged at this location in 2002. Left- 5-yr old egg nest scars, Right- Chris Simon searching for nymphs.

Appendix 4. Statistical comparisons among nymph populations sampled at different locations. Data and locality codes as in Figure 7.

The first analyzes 13- and 17-year cicada nymphs that were 9 years old when dug from the ground. It asks the question, do they have the same distribution of nymph instars? The null hypothesis is that they all grow at the same rate so that they do have a similar distribution of nymphs. The null hypothesis is not supported.

Table A1-1. A statistical comparison among 9-year old nymphs from different locations and life cycles (both 17- and 13-year cicadas included). Locality codes are given in the key to Fig. 7. The Average Difference of this chart is 51.61. The G-Statistic is 1196.2, and there are three degrees of freedom, so the percent certainty is >99.99% that the samples are statistically different (or we can say that the result is significant at the 0.01 level). It can be seen from this contingency table that population 9GT (the 13-year population) is an outlier. This 13-year cicada nymph sample has many more 5th instar nymphs. In the next chart, the 13-year cicadas have been removed.

	9A	9E	9M	9B	9GT	Total
5th	0	2	0	68	334	404
Expected	152	29.9	25.8	99.6	96.8	25.8%
Difference	152	27.9	25.8	31.9	237.2	Av: 94.96
% Difference	2.0	1.75	2.0	0.38	1.1	Av: 145%
G-Statistic	0	-5.41	0	-25.95	413.65	382.30
4th	457	83	75	306	39	960
Expected	361.7	71.1	61.3	236	229	61.3%
Difference	95.3	11.9	13.7	70	190	Av: 76.18
% Difference	0.23	0.15	0.201	0.258	1.42	Av: 45.1%
G-Statistic	106.88	12.84	15.13	79.48	-69.04	145.29
3rd	106	31	25	12	2	176
Expected	66.1	13	11.2	43.2	42	11.2%
Difference	39.9	18	13.8	31.2	40	Av: 28.58
% Difference	0.464	0.409	0.762	1.13	1.82	Av: 91.7%
G-Statistic	50.06	26.94	20.07	-15.37	-6.09	75.61
2nd	27	0	0	0	0	27
Expected	10.2	2	1.7	6.6	6.5	1.72%

	9A	9E	9M	9B	9GT	Total
Difference	16.8	2	1.7	6.6	6.5	Av: 6.72
% Difference	0.903	2.0	2.0	2.0	2.0	Av: 178%
G-Statistic	-5.09	0	0	0	0	-5.09
Total	590	116	100	386	375	1567
Av. % Difference	89.9%	107.7%	124.1%	94.2%	158.5%	Av: 114.95%

Table A1-2. A statistical comparison of the four samples of nine year old 17-year cicada nymphs. Locality codes are given in Fig. 7. This contingency table excludes the 13-year nymph sample and, while this does make some of the values, especially the 4th instar values, fit better to the null hypothesis of no difference in growth rates, there is still a strong difference in growth rates among localities (as measured by the number of individual cicada in each instar). The average difference of the table below is 14.04. The G-Statistic is 254.2, and there are three degrees of freedom, making the certainty >99.99% that the samples are statistically different (or we can say that the result is significant at the 0.01 level). We hypothesize that the difference may be caused by differences in temperature and rainfall at different locations.

	9A	9E	9M	9B	Total
5th	0	2	0	68	70
Expected	34.6	6.8	5.87	22.6	5.87%
Difference	34.6	4.8	5.87	45.4	Av: 22.67
% Difference	2.0	1.09	2.0	1.0	Av: 152.3%
G-Statistic	0	-2.45	0	74.9	72.46
4th	457	83	75	306	921
Expected	456	89.7	77.3	298	77.3%
Difference	1	6.7	2.3	8	Av: 4.5
% Difference	0.0022	0.0776	0.0302	0.0265	Av: 3.41%
G-Statistic	1.00	-6.44	-2.26	8.106	0.406
3rd	106	31	25	12	174
Expected	86.1	16.9	14.6	56.4	14.6%
Difference	19.9	14.1	10.4	44.4	Av: 22.2

	9A	9E	9M	9B	Total
% Difference	0.207	0.589	0.525	1.298	Av: 65.5%
G-Statistic	22.04	18.81	13.45	-18.57	35.73
2nd	27	0	0	0	27
Expected	13.4	2.6	2.3	8.7	2.27%
Difference	13.6	2.6	2.3	8.7	Av: 6.8
% Difference	0.673	2.0	2.0	2.0	Av: 166.8%
G-Statistic	18.52	0	0	0	18.52
Total	590	116	100	386	1192
Av. % Difference	72.0%	93.9%	113.9%	108.1%`	Av: 96.97%

Table A1-3. A comparison of 13-year and a 17-year cicada nymph populations four-year prior to emergence; 17-year population F is 13 years old and 13-year population GT is 9 years old. When comparing these two populations with the g-test, we chose to remove the single 3rd instar nymph to make the samples more conducive to the test. The g-test results show that there is not a statistically significant difference between these two samples. Both populations appear to be in a similar stage of development four years before emerging. This makes sense because in both groups all individuals are expected to be at the same stage of growth by the end of this four year period.

	13F	9GT	Total
5th	214	334	548
Expected	218.6	329.2	87.8%
G Statistic	-4.55	4.83	0.28
4th	35	38	73
Expected	29	43.5	11.6%
G Statistic	6.58	-5.14	1.44
Total	249	375	624

Appendix 5. Collection of three-month-old nymphs at Sweet Briar College in Sweet Briar, Virginia, 15 October 2013

Dr. Linda Fink and her "Introduction to Organisms" class collected periodical cicada nymphs in mid October, three months after they hatched on campus. In July Dr. Fink cut branches with mature egg nests of all three species and laid them in plots along a transect already populated by cicadas of the same Brood II emergence. She thus created areas with concentrations of egg nests of about 700 nests per square meter. In October the class revisited these concentrated sites and dug out nymphs, separating them into three categories based on the depth at which they were found.

Of the nymphs found in the top layer of soil (0-10 cm deep), 17 (47.2%) were 2nd instars and 19 (52.8%) were 1st instars.

Of the nymphs found in the second layer (10-20 cm), 13 (37.1%) were 2nd instars and 22 (62.8%) were 1st instars.

Of the nymphs found in the third layer(20-30cm), 2 (67.7%) were 2nd instars and 1 (33.3%) was a 1st instar nymph.

Population totals were 22 (34.4%) 2nd instar and 42 (65.6%) 1st instar nymphs.

These results show plainly that, contrary to previously published statements (Marlatt 1907), nymphs can reach the 2nd instar of development long before their first year underground is complete. The 1st instar samples display the extended tarsus evident in newly hatched 1st instar nymphs, suggesting that this feature is maintained throughout much if not all of the 1st instar.