Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Volume 39, Article 8, 2018

Behavioral Diversity of Social Insects

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Behavioral Diversity of Social Insects (BDSI) is a modular laboratory exercise designed to introduce students to methods of quantifying animal behavior by acquainting them with a diverse, ubiquitous, and interesting group of insects. Through interactive classroom instruction, field activities, and laboratory assays with termites and ants, students gain appreciation for insect diversity and apply the scientific method to behavioral observations. The BDSI exercise is composed of three main modules: Introduction to Social Insect Behavior, Field Biology, and Laboratory Assays. The introductory module is oriented towards getting students to understand how to measure insect behavior and the importance of its application. This is accomplished through a student demonstration of emergent behavior and an observational video activity. The field module takes students outside to explore the natural habitats of local ants, observe them, and collect specimens that they can identify by applying field biology skills. Finally, the assay module engages students in data collection and analysis by testing hypotheses regarding ant food preference, termite trail following, and ant and termite aggression and phototaxis. These activities are easily modified to fit into a variety of undergraduate laboratory courses such as an introductory course on ecology and evolution, animal behavior, or entomology.

Keywords: entomology, animal behavior, ecology, social insects, modular, directed inquiry

Link to Supplementary Materials: **http://www.ableweb.org/volumes/vol-39/Gordon/supplement.htm**

Introduction

The Behavioral Diversity of Social Insects exercise (BDSI) was developed to instruct foundational topics in animal behavior, entomology, and ecology through guided inquiry and natural observations. BDSI was initially developed in a six hour format for MA high school students participating in the Upward Bound Math-Science Program (UBMS), which provides enrichment in science and math subjects for historically underrepresented, economically disadvantaged, and potential first generation college students (US DoED 2007). BDSI was designed to fulfill part of the UBMS curricula including hands-on laboratory experiences. The laboratory assays (Module 3) were later adapted for a three hour Biology Inquiry and Outreach for Boston University Graduate Students (BIOBUGS) activity, an outreach initiative that serves Boston Public High School students with the intention of increasing interest in biological sciences through laboratory exercises instructed by graduate students on Boston University's campus. The termite trail following assay was further modified and expanded to function as an undergraduate major elective animal behavior laboratory session at Boston University.

BDSI has a modular design so that instructors can select elements that work best for their curricula. The first module, Introduction to Social Insect Behavior, consists of a Swarm Activity and Describing Behavior Video Activity, which are accompanied by an introductory lecture intended to provide a scaffold for the following modules. The goal of Module 1 is for students to understand why social insect behavior is important and practice how to quantify it. These goals are accomplished by having students demonstrate emergent behavior in real time, and practice observational skills through a series of video clips. In Module 2, Field Biology, students apply field techniques to observe and identify local ants through Field Observation and Collection and Voucher Specimen Identification activities, where they locate, observe, and collect local ant specimens outside, and identify which ant genera they observed and collected using a customized dichotomous key. Module 2 addresses the need for incorporating natural history in biology education and research programs (Tewksbury et al. 2014). The exercise concludes with Module 3, Laboratory Assays, in which students test hypotheses, execute experiments, and analyze their results using a variety of assays and different social insect taxa. This Module uses the knowledge, observations, and skills acquired in the

previous modules to engage in a directed inquiry of social insect behavior. Students decide ant food preference by giving a choice of two food items, determine the trail following ability of termite workers at different concentrations of pheromone, quantify aggressive responses of termite soldiers and ants to wingless fruit flies, and assess the light/dark preferences of both termites and ants. Students also gain statistical skills by analyzing their assays through the use of a t-test, analyses of variance, and a Z-test for proportions.

The structure of BDSI permits the modification of these modules to accommodate a variety of instructional needs. BDSI is easily adapted to fit into introductory biology, animal behavior, entomology, and ecology curricula, and scalable to fit a range of skill levels and class sizes, although we provide a list of materials necessary for a 25-person class. In the Notes for the Instructor section we provide detailed instructions to execute these modules and suggestions on how to extend and modify them. By utilizing local social insect species and general lab supplies, instructors eliminate the need for permits, IACUC documentation, and special equipment while encouraging group work, extension of content knowledge, and computational skills through the application of the scientific method.

Student Outline

Module 1.2: Describing Behavior Video Activity

Introduction

To get you acquainted with the many different types social insect behavior and how to record them, we are going to show you a video clip of a small group of *Pheidole morrisi* workers and soldiers tending to some brood (immature ants) and their queen. Pay close attention to the directions you receive and think about the challenges associated with making detailed behavioral observations.

Describing Behavior

Exercise 1:

Watch the video and list all behaviors you observe:

Behaviors

Exercise 2:

Watch the video and tally how many times you observe one of the behaviors described by the instructor:

Name of the behavior	# of times observed

Exercise 3:

Focus on one worker ant and tally how many times you observe her performing the each of the behaviors described by the instructor:

Name of the behavior	# of times observed

Module 2.1: Field Observation and Collection

Field Sheet

Getting to know how organisms act in their natural habitat is vital to understanding behavioral diversity. Look around at the ground, on trees, and under rocks to find ants on the move. Take detailed notes and record where and when you found them and what they are doing. Include information about how many there are, how they are moving, and what happens if you place a piece of cookie in their path.

Name:
Date & Time:
Location:
Microhabitat:

Descriptive observations:

Collect ONE individual specimen in a vial of alcohol for later identification. Assign your specimens to separate numbered vials. These observations are for specimen #_____.

Module 2.2: Voucher Specimen Identification

Voucher Identification

Knowing what kind of ant you are working with is extremely important, different species have different ecologies, life histories, and behaviors. The first step to proper identification is recognizing important features of the ant's head and body.

Look under the microscope: Draw the ant's head from the front and from the side and the body in profile.

Work through the dichotomous key. Which of the nine ant genera in Wisconsin does your ant specimen represent?

Module 3: Laboratory Assays

Hypotheses and Summaries

For each assay, write down a hypothesis that explains your predicted experimental outcome. As you finish each set of experiments, record a summary of what you find. After you conduct the analyses, indicate whether your hypothesis was or was not supported by the experimental evidence.

Ant Foraging Preference
HYPOTHESIS:
SUMMARY:
This SUPPORTED / DID NOT SUPPORT our hypothesis.

Termite Trail Following
HYPOTHESIS:
SUMMARY:
This SUPPORTED / DID NOT SUPPORT our hypothesis.

Aggression Assay
HYPOTHESIS:
SUMMARY:
This SUPPORTED / DID NOT SUPPORT our hypothesis.

Phototaxis Assay
HYPOTHESIS:
SUMMARY:
This SUPPORTED / DID NOT SUPPORT our hypothesis.

Module 3.1: Ant Foraging Preference

Ant Foraging Preference Data Collection Sheet
Instructions:
Count and record how many ants are at each food source every 10 minutes.

Choice of food items:
1.
2.
Colony ID:

Number of foraging ants:

Minute	Food Choice 1:	Food Choice 2:
10		
20		
30		
40		
50		
60		
70		
80		
90		
100		
110		
120		

Module 3.2: Termite Trail Following

Termite Trail Following Data Collection Sheet
Instructions:

- Draw a circle with segments using the stencil with a pencil on filter paper and place the filter paper into the petri dish lid.
- Carefully overlay the circle with one of the HIGH/MEDIUM/LOW concentration extracts using a fresh capillary tube. Let dry for 10 seconds.
- Gently introduce a termite from the "ready" container into the middle of the circle, lidding the dish with the bottom half.
- Observe behavior for 5 minutes after the termite makes contact with the trail.
- Record the distance traveled in # of segments traveled around the circle.
- Gently return termite to the "retired" petri dish after trial.
- Repeat with remaining concentrations and termites until you have tested 2 termites/concentration for your pair.

Record the # of trail segments followed for each replicate at each concentration.

Replicate	Low	Medium	High
1			
2			

Module 3.3: Aggression Assay

Aggression Assay Data Collection Sheet

Instructions:

- Introduce an individual from the "ready" container to a small petri dish and cover with the lid. Wait 1 minute.
- Add a wingless fly to the dish and cover with the lid.
- For 3 minutes tally how many behaviors are witnessed in each of the following categories:
    - 1 = no aggression/avoidance/running away
    - 2 = olfactory investigation/antennal contact
    - 3 = mandible flaring
    - 4 = biting
- After the 3 minutes of observations are over, return the individual to the respective "retired" container after each trial.
- Repeat procedure for all taxa (*Ant Genus #1, Ant Genus #2*, and *Termites*) with 3 individual replicates of each for your pair.

Taxon	Replicate	1: No aggression	2: Antennal contact	3: Mandible flaring	4: Biting
Ant Genus #1	1				
Ant Genus #1	2				
Ant Genus #1	3				
Ant Genus #2	1				
Ant Genus #2	2				
Ant Genus #2	3				
Termites	1				
Termites	2				
Termites	3				

Module 3.4: Phototaxis Assay

Phototaxis Assay Data Collection Sheet

Instructions:

- Set up a choice chamber with a paper bottom. Draw a pencil line bisecting the dish.
- Introduce an individual from a "ready" container to the center of the dish. Lid the dish, making sure the pencil line and the border of the red film align.
- Start the timer for 3 minutes.
- Record the proportion of time the animal spends under the red section by starting and stopping the stopwatch every time it crosses the line.
- Return individuals to the appropriate "retired" container when finished.
- Change white paper bottom in between trials.
- Reverse the positions of the red and white sides in between trials.
- Repeat procedure for all taxa (*Ant Genus #1*, *Ant Genus #2*, and *Termites*) with 3 individual replicates for each for your pair.

Record the amount of time (in seconds) spent in the RED for each individual ant and termite.

Replicate	*Ant Genus #1*	*Ant Genus #2*	*Termites*
1			
2			
3			

Materials

The following list of supplies and equipment needed for a 25-student class is broken down by module because we acknowledge the fact that most likely individual activities will be adopted rather than the whole exercise. Note that likely only one 8 oz. (0.24 L) bottle of Fluon ® will be needed for all activities combined (it should be diluted four parts Fluon ® to one-part distilled water), but it is listed for each activity because it is essential for animal containment. The materials for both activities in Module 1 are reusable. For Module 2 most of the materials listed can be reused or will extend beyond one iteration of the activity, except the home-made aspirators (first three materials in *Module 2.1: Field Observation and Collection)* are designed for each of the 25 students to keep as their own.

Module 3 requires that you have (at least) two different ant genera and one genus of termites to use in the assays. We collected all of our own animals to use in this exercise. If you plan on collecting your own, do so in the warmer months between April-September (anecdotally, temperatures around 18-27°C (65-80°F) are typically ideal for finding foraging New England ants), there is variability in ant activity across regions, so we recommend that you explore your localities in advance and try to collect at least double what you need for each assay. To collect large fragments (workers without a queen) or colonies you will need minimally an aspirator (BioQuip catalog #1135A), and feather-weight forceps (BioQuip catalog #4748) per collector, Ziploc bags to temporarily hold animals, tape and marker to label bagged collections, and a cooler to control light and temperature while transporting collections. Please note for each colony or fragment ordered or collected, you will need the proper supplies to maintain them as listed under *3.1: Ant Foraging Preference* (plastic boxes, Fluon ®, transfer pipets, test tubes, cotton balls, tin foil, honey). Each ant colony or fragment needs to be housed in a separate plastic box with the walls coated in Fluon ® and provided with nest tubes. Nest tubes can be constructed out of test tubes partially filled with water (at least 2 inches) and plugged with cotton before wrapping the outside with tin foil. Feed them every other day with a cotton ball soaked in a solution of 1:3 honey:water. You should supplement them with a source of protein like scrambled eggs, mealworms, or wingless fruit flies. Maintained like this, even without a queen, your ants should live for a couple of months or longer. Termites can be collected from large pieces of rotted wood, which you should keep mostly intact and housed in a large lidded plastic box. Keep their wood fragments damp by laying wet paper towel over it and spraying it with water occasionally. You should aim to collect a couple hundred to ensure enough soldiers are present. For the assays below we used local *Lasius* and *Aphaenogaster* ants and *Reticulitermes* termites. However, you can easily order *Formica* and *Pogonomyrmex* ants online as well as *Reticulitermes* if you cannot collect your own local genera. It is strongly encouraged to test the assays in advance for each genus you plan on using, as different ants respond to stimuli in different ways.

Module 1.1: Swarm Activity

- 5 cm x 5 cm (2" x 2") paper squares: ~50 squares each of 5 different colors
- Paper slip instructions: 25 (1 per student)
- Open space for the class to move around

Module 1.2: Describing Behavior Video Activity

- Video of ants: supplemental video file
- Describing Behavior student sheet: 25 (1 per student)

Module 2.1: Field Observation and Collection

- Soft PVC tubing for aspirators: 11.43 m (37.5') (45.72 cm (18") per student), McMaster-Carr (5231K355)
- P1000 pipet tips for aspirators: 25 (1 per student)
- Nylon mesh for aspirators, ~250 µm openings: 121.92 cm x 121.92 cm (48" x 48") (~3.81 cm (1.5") square per student), McMaster-Carr (9318T16)
- Scissors: 1
- 1.5mL microcentrifuge tubes or cryovials: 60 (10 per small group)
- 70-95% Ethanol: 60 mL (1mL per tube)
- Sandwich bags: 12 (2 per small group)
- Cardstock (labeling tubes and baiting: 12 note cards
- Pencils: 6 (1 per small group)
- Keebler Pecan Sandies®: 1 box (1 cookie per small group + baiting)
- Clipboards: 6 (1 per small group)
- Gloves (optional): 50 (2 per student)
- Field Sheet: 60 (10 per small group)

Module 2.2: Voucher Specimen Identification

- Plastic petri dishes, 30 mm diameter: 25 (1 per student)
- Toothpicks: 1 box
- Dissection microscopes, <40x with lights: 6 (1 per small group)
- Voucher Identification sheet: 25-50 (1-2 per student depending on collection success)
- Dichotomous keys: 25 (1 per student)

Module 3.1: Ant Foraging Preference

- Fluon ®: 8 oz. (0.24 L), BioQuip (2871B)
- Transfer pipets (to apply Fluon ®)

- Plastic boxes, 10- 1.59 cm x 19.37 cm x 9.52 cm (5/8″ x 7-5/8″ x 3-3/4″) (walls coated in Fluon ®): 3 (1 per colony), Pioneer Plastics (195C)
- Test tubes, 15 mm x 150 mm: 6 (2 per colony or fragment)
- Jumbo cotton balls (for feeding and water tubes): 1 bag
- Tin foil: 1 roll
- Food options: peanut butter, apple, seeds, honey, tuna fish, etc.
- Timer: 1
- Ant Foraging Preference data collection sheet: 3 (1 per colony or fragment)

Module 3.2: Termite Trail Following

- Fluon ®: 8 oz.(0.24 L), BioQuip (2871B)
- Transfer pipets (to apply Fluon ®)
- Plastic petri dishes 90 mm diameter, walls coated in Fluon ®: 14 (1 per concentration per pair of students + 1 "ready" dish + 1 "retired" dish)
- Damp paper towel (to line bottom of "ready" and "retired" dishes)
- Soft paint brushes (for transferring termites): 4 (minimum, 1 per mini-station)
- Razor blade: 1
- 1.5 mL microcentrifuge tubes : 9
- Microcentrifuge tube pestles (or P1000 tips): 3 (1 per concentration)
- Microcentrifuge
- Ice bath or refrigerator
- Freezer (for extract storage)
- Grade 1 filter paper 90 mm diameter: 1 box of 100, VWR (28450-081)
- Melting point capillary tubes 1.5-1.8 x 100 mm long: 1 vial of 100 Fisher Scientific (08-261-2A)
- Circle stencils 30 mm in diameter divided into 8 evenly spaced segments around edge: 3 (50 mL conical tube lids works well)
- Timers: 4 (1 per mini-station)
- Pencils: 4 (1 per mini-station)
- Termite Trail Following data collection sheet: 12 (1 per pair of students per rotation group)

Module 3.3: Aggression Assay

- Fluon ®: 8 oz. (0.24 L), BioQuip (2871B)
- Transfer pipets (to apply Fluon ®)
- Plastic petri dishes 30 mm diameter, walls coated in Fluon ®: 12 (1 per taxon per mini-station for assays)
- Plastic petri dishes 90 mm diameter, walls coated in Fluon ®: 10 (1 "ready" + 1 "retired" per taxon + 1 for flies per mini-station)
- Damp paper towel (to line termite assay dishes and "ready" and "retired" dishes)
- Soft paint brushes (for transferring animals): 4 (1 per mini-station)
- Dissection microscopes, <40x with lights (optional): 4 (1 per mini-station)
- Timers: 4 (1 per mini-station)
- Wingless *Drosophila melanogaster* fruit flies: 2 cultures (100+ flies), Josh's Frogs
- Aggression Assay data collection sheet: 12 (1 per pair of students per rotation group)

Module 3.4: Phototaxis Assay

- Fluon ®: 8 oz. (0.24 L), BioQuip (2871B)
- Transfer pipets (to apply Fluon ®)
- Plastic petri dishes 90 mm diameter, walls coated in Fluon ®: 6 (1 "ready"+ 1 "retired" per taxon)
- Damp paper towel (to line termite "ready" and "retired" dishes)
- Soft paint brushes (for transferring animals): 4 (1 per mini-station)
- Plastic petri dishes 90 mm diameter, all external surfaces of lid painted black, external wall of bottom dish painted black (do NOT paint the bottom of the bottom dish): 4 choice arenas (1 per mini-station)
- Black acrylic paint and paintbrush: 1 tube and 1 brush
- Office paper (cut to fit bottom of arena): 108 (1 per trial)
- Red masking film, cut in half circles to cover half of the top surface of choice arenas: 1 sheet 50.8 cm x 60.96 cm (20" x 24") (art and craft supply stores carry this- often called Rubylith®)
- Clear tape: 1 roll
- Stopwatches: 4 (1 per mini-station)
- Timers: 4 (1 per mini-station)
- Phototaxis Assay data collection sheet: 12 (1 per pair of students per rotation group)

Live Social Insects (if you cannot collect your own)

- *Formica*, wood ants: 80-400 individuals, Carolina Biological (144526)
- *Pogonomyrmex*, harvester ants: 80-400 individuals, Carolina Biological (144528)
- Termite workers: 125 workers, Carolina Biological (143736)
- Termite soldiers: 50 soldiers, Carolina Biological (143732)

Notes for the Instructor

This modular exercise was originally designed for a six-hour science outreach program with volunteers to facilitate the small group work. Volunteers guided student

field activities and were positioned at each station to guide the discussions during the activities, enter data, and lead statistical testing. However, to adapt to an undergraduate course, the instructor can lead discussion for each activity as they see fit and the students can perform their own data analysis (see supplemental link to excel files). Students should be in groups of approximately four for the Field Biology module. If you choose to conduct Module 3: Laboratory Assays as rotations in one session, you will need students to organize themselves in three rotation groups. These rotation groups (approximately eight students if in a 25-person class), should be broken into in pairs of students within each station, creating mini-stations, to ensure adequate opportunities for students to conduct the experiments. Below you will find each of the three modules (Introduction to Social Insect Behavior, Field Biology, and Laboratory Assays) described with goals and timing, and the associated activities within each module organized by preparation, instruction, and discussion. Suggestions for how to expand each module into relevant course work are also included.

Module 1: Overview of Introduction to Social Insect Behavior (1 hour total)

The goal of this module is for students understand why social insect behavior is important and how to quantify it. This module should begin with background material that covers the characteristics of eusociality, its taxonomic spread, and behavioral features of the main insect orders that have eusocial species (Isoptera and Hymenoptera). It also should outline the important applications of insect behavior research. There is a swarm activity and a video activity included that aim to illustrate complex insect behavior and the nuances of measuring it.

1.1.1: Swarm Activity Background

Students are introduced to the concept of swarm intelligence through an introductory discussion defining features of social insect behavior. Swarm intelligence is a type of emergent behavior where order arises from simple local rules followed by groups of individual agents. Decisions do not arise from rational choice or from a directive, the agents in the system have no global perspective, and the agents are not aware that they are doing something intelligent. Students will demonstrate this type of behavior in real time as an ice-breaker. Before the exercise, cut out ~50, 5.08 cm (2") squares of five different colors of paper and keep them in an envelope. Print out slips of paper instructions that you will have each student pick from a box/jar/hat/etc. without sharing what the instructions say. Each slip should read: 1. If you are empty-handed and encounter any piece of paper, pick it up. 2. If you are carrying a piece of paper and encounter another of the same color, put yours down.

1.1.2: Swarm Activity Instruction

Take the students to a large indoor open space, this can be a classroom with the desks pushed aside or another suitable space. Spread out the colored paper squares on the floor so that they are roughly evenly spaced, and colors are interspersed. Give the students their instruction slips and tell them not to share with each other. Preface that they are to follow their specific instructions while randomly and continually wandering (this means no stopping, even when performing an action). Once the activity runs for 5-10 minutes (depending on how many students, how fast they are moving, and the number of paper pieces you scatter) the paper will be organized in piles by color. This demonstrates that complex tasks (i.e. sorting paper by color) can arise without hierarchical control when there are many individuals following simple local rules.

1.1.3: Swarm Activity Discussion Questions

What were your local decision rules? Why do you think we had you select your rules without discussion even if they were the same? (Think about the properties of emergent behavior and collective decision making).

Think about the local rules followed and the roles of simple agents. What cognitive and motor skills were required of the simple agents to complete this task through collective intelligence? What is another way to achieve the result of sorted paper without using collective decision making? What cognitive and motor skills would be required in that scenario?

What environmental circumstances might favor collective intelligence? Are there scenarios in which collective decision making is less effective?

What are some other examples of collective intelligence or emergence? In which animal groups do we see this? What characteristics do they share?

1.2.1: Describing Behavior Video Activity Background

Students are presented with a list of "events" and are asked which of the events illustrate behavior. The list of "events" should include reflexes, instincts, and learned responses from a variety of taxa, even including plants. This gets the students thinking about what exactly constitutes behavior and how might we characterize it. After the students are comfortable with the idea that behavior is a response to stimuli, discuss what might be relevant stimuli for social insects and what responses they might elicit. You can continue the conversation to include ways we can measure behavioral responses, paying special attention to natural observations and experimental manipulations.

1.2.2: Describing Behavior Video Activity Instruction and Discussion Questions

This activity corresponds to the Describing Behavior student handout. Students are shown a 30 second video of a small group of *P. morrisi* ants interacting with a queen and some brood three times (provided in the Link to Supplemental Materials). The first time the video is shown, ask the students to describe all the behaviors that they see happening. Check in with the students and see how they describe what they saw. Talk about how similar behaviors were described differently. Someone should mention grooming behavior or describe ants wiping their antennae or putting their mouthparts to another ant's body. Describe grooming in more detail. Ants are extremely hygienic because disease can spread quickly in social groups. Grooming is a common worker ant behavior; ants groom themselves by passing their legs through their mandibles and wiping their antennae with their legs. They also groom each other and the queen, which usually looks like the grooming ant is licking or gently biting other ants' appendages. Finally, ants groom their brood extensively, which similarly looks like licking, biting, or rolling. Now ask the students to count off by four, replay the video, and have the them tally how often they saw (1) an ant groom another worker, (2) an ant groom the queen, (3) an ant groom brood, and (4) an ant groom themselves. Ask around how many grooming acts were observed in each category. Talk about why some counts were more variable than others (Hint: it's easiest to count queen grooming since there is only one queen). Finally, ask the students to each pick one worker ant to watch and tally the number of times that ant grooms another worker, the queen, brood, or itself. Discuss what was difficult about those observations. Did it get easier as you watched the videos again? Ask students what kinds of information are contained in natural observations (e.g., task repertoire, act frequency, time budgets, social networks, colony or individual activity level, etc.) and how they could be used in research.

1.3: Expansion Suggestions for Module 1: Introduction to Social Insect Behavior

This module can be expanded to cover quantitative animal behavior methods more thoroughly. Students can observe animals (on video or live) and use those observations to construct ethograms and repertoires, time budgets, and practice focal and scan sampling.

Module 2: Overview of Field Biology (2 hours)

The goal of this module is for students to apply field techniques to observe and identify local ants. It consists of going outside to find ants, observing their behavior in nature, collecting voucher specimens, and identifying the observed ants to the genus level.

2.1.1: Field Observation and Collection Preparation

Scout out sites that have a lot of ant activity around a week in advance of the exercise. College campuses everywhere (urban and rural settings) will have ants active during warm days; you just need to take time to look around to find them. Assemble collection kits and aspirators in advance of the exercise as well. Each collection kit should include a sandwich bag of ten cryovials (1.5 mL microcentrifuge tubes also work fine) of Ethanol (70-95%) that are labeled by submerging a piece of cardstock with a pencil-labeled tag (#1-10) inside each one. Additionally, a cookie should be provided (Keebler Pecan Sandies® are irresistible to ants) in a separate bag. Aspirators are constructed by trimming a P1000 pipet tip (the small end should be ~3mm in diameter) and wrapping the wide end in a 3.91 cm (1.5") square of nylon mesh before inserting it in one end of an 45.72 cm (18") length of food-safe flexible PVC tubing. Each student should have his or her own aspirator to keep. At least an hour before this activity, bait some locations around the site you will visit. Do this by crumbling a tiny bit of cookie on a piece of card stock and place that on the ground in different locations around the site. Before leaving the classroom have students put on gloves (optional) and receive their individual aspirators. Each group of four students should have one collection kit and a clipboard with around ten copies of the Field Sheet student handout.

2.1.2: Field Observation and Collection Instruction

Lead students to microhabitat areas of ant activity- under rocks, decaying wood, cracks in pavement, bait stations, etc. Try to keep groups separate, no more than five people should be at each microhabitat, but students can sample the same microhabitats as long as they do it sequentially. When searching for ants advise students against reaching hands where they cannot see, always roll rocks toward oneself, and exercise caution in general since there is often broken glass and other dangerous trash on the ground. Always try to return the collection site to what it looked like before you disturbed it. Guide students in observing ant behavior and take notes on the Field Sheet student handout before voucher collection. After several minutes of observing ants, groups should collect one specimen from their observation site in alcohol for later identification so that each set of observations has a specimen associated with it. The number of sites visited depends on time allocated to the activity, ant abundance, and microhabitat variation. The goal is to get students to observe and collect a variety of genera.

2.1.3: Field Observation and Collection Discussion Questions

What were some characteristics of the microhabitats where you found ants? Can you think of an ecological explanation for this? Were there microhabitats that you expected to find ants and didn't?

What did you observe the ants doing? Was there much variation in what they were doing? How did they respond to the cookie bait?

At the baits were there many ants? How did they interact?

2.2.1: Voucher Specimen Identification Preparation

A dichotomous key for Wisconsin ant genera is included (Appendix A), but you should make your own based on local ant fauna. There are many state- and region-specific ant keys published that you can adapt, but we recommend only including the top ten most common ant genera of your area for simplicity. If you are without a published key, Antweb.org is a fantastic resource for ant distribution by region and informative specimen images. If you combine your state list of genera with the information in Fisher and Cover (2007) you can make a custom key for any group of ants in North America.

2.2.2: Voucher Specimen Identification Instruction

Groups should be able to collect at least four different specimens (and up to ten) to bring back to the lab for identification. Each group of four can work at a microscope station with dichotomous key and members can take turns identifying what they collected outside and complete the Voucher Identification student handout. Toothpicks can be used to manipulate the dead specimens in petri dishes to examine their features. It is important to review ant anatomy as there are specific anatomical features (e.g., post-petiole, clypeus) that will be important in distinguishing your specimens. To that end, a guide of Ant External Anatomy is provided before the dichotomous key (Appendix A).

2.2.3: Voucher Specimen Identification discussion questions

Which genera are represented at each site? Were certain genera associated with specific microhabitats? Engage in a discussion about where students found them.

Is there anything about the natural history of the ants found that explains their distribution? Using personal observations and the resources provided, can we make any connections between the ecology of the ants (e.g. where they are found) and their behavior (what we observed them doing)?

2.3: Expansion suggestions for Module 2: Field Biology

This module can be expanded to serve learning objectives of ecology, entomology, animal behavior, or similar undergraduate courses that utilize field biology. This field component can be extended to encompass several sessions. More sites can be compared, and a variety of collection methods can be introduced such as pitfall traps, black lights, and Winkler bags. Furthermore, students can be instructed on the proper curation of specimens, can assemble personal collections of pinned specimens, and can use such collections to identify genera (or species) using local and regional keys (Fisher and Cover 2007), or they can construct their own dichotomous key for their set of specimens. Ecological sampling methods can be used to explore topics in biodiversity, such as species richness, diversity, evenness, etc. And field experiments can utilize ant recruitment to test hypotheses regarding foraging behavior using wild populations.

Module 3: Overview of Laboratory Assays (3 hours)

The goals for this module are for students to test hypotheses, execute experiments, and analyze their results using a variety of assays and different social insect taxa. This is accomplished through three 40-minute rotations (Termite Trail Following, Aggression Assay, and Phototaxis Assay) and one continuous (Ant Foraging Preference) behavioral assay station. Students should be grouped into three rotation groups (one at each of the rotation stations), with each rotation group consisting of pairs of students, which will work in parallel at mini-stations within each rotation station. Each student is provided with a Hypotheses and Summaries student handout to keep track of their hypotheses and each pair of students should complete a data sheet per station. After the three rotations have completed, each group remains at their last station. The three student representatives at the Ant Foraging Preference station for the last time point should remain at that station as well. There the students can input the data from all three rotation groups (excel worksheets supplemental) and perform the suggested statistical tests as described for each activity. As a class share the results of each test and complete the Hypotheses and Summaries student handouts.

3.0: Animal Handling

Each rotation station should have a “ready” and “retired” 90 mm diameter petri dish labeled for each of the taxa used at the respective stations. If you use a large-bodied ant like *Formica* small boxes (7.30 cm x 7.30 cm x 2.70 cm (2-7/8” x 2-7/8” x 1-1/16″) Pioneer Plastics 006c) can be used instead. All dishes that contain insects should always have the walls coated in Fluon ®. Additionally, “ready” and “retired” dishes should have a small piece of a cotton ball soaked with honey water. This supplement is important as animals will likely desiccate and die without it during the course of a day. Termites should also have a lightly dampened piece of paper towel lining the bottom of the dish. This is because they have trouble walking on plastic and will desiccate quickly. Students should only take animals from the “ready” dish and return them to the “retired” dish after each replicate. Ants can be transported from dish to dish via dry paint brushes and termites can be

transported using damp paint brushes (soft bristles minimize injury).

3.1.1: Ant Foraging Preference Preparation

Set up three boxes of ants (1 per rotation group), each box containing ~100 workers from your collected colonies or order live ants to arrive at least three days before the exercise. Do not feed them for the three days leading up to the exercise but be sure to provide them with water-soaked cotton. Each box should be sufficiently large (at least 10- 1.59 cm x 19.37 cm x 9.52 cm (5/8″ x 7-5/8″ x 3-3/4″)), the walls should be coated in Fluon ®, and a tin foil-wrapped nest tube in the center should be provided. Nest tubes can be constructed out of test tubes partially filled with water (at least 2 inches) and plugged with cotton. This provides a humid and dark microhabitat for the ants. These ants do not have to be the same genera you use for the other assays, nor do they have to be from different sites or colonies. Be aware that not all ant species will recruit to food sources readily, so testing this in advance is recommended. Finding ants outside using baits would help discriminate which to collect- those that cover a food item *en masse* are good choices (e.g., *Lasius*).

3.1.2: Ant Foraging Preference Instruction

As a class, decide which two food items you wish to test (given limited options such as honey, apple, peanut butter, seeds) and present each of the three boxes with each of the food items equidistant from the opening to their nest tube. An Ant Foraging Preference data collection sheet should accompany each of the three boxes of ants or however many you use for the experiment. Each of the rotation groups should be assigned one box to monitor. Groups should send up a representative every 10 minutes (use a timer), so that all students have a turn recording the number of ants on each food source. A clip of foraging ants is supplied in the Link to Supplemental Materials as an example.

3.1.3: Ant Foraging Preference Statistics

A t-test comparing the cumulative means for the final time point between the food sources can be used to determine preference for a food source. Use one- or two-tailed p-values depending on how the hypotheses were phrased. A graph of the cumulative number of ants on each food source can be plotted against time to illustrate differences.

3.1.4: Ant Foraging Preference Discussion Questions

Why did we choose these two food items? What kind of nutrients do the different types of food offer? Which do you think they will prefer? Why?

Do you ever get a craving for a specific type of food? Does your craved food item ever change? Would you expect a colony to change their food preference over time? What might mediate changes in food preference?

Why do we measure the cumulative number of ants?

What can you conclude about the ants' food preference? Why might this be the case? Was your prediction correct?

3.2.1: Termite Trail Following Preparation

In advance (at least one day before) prepare pheromone extracts for a final concentration of 6x (high), 3x (medium), and 0.3x (low) glands per mL. Isolate 10 mature termite workers with light brown substances in their abdomens (do not use reproductives, soldiers, or immatures). Prepare three microcentrifuge tubes with 1mL of 100% ethanol each. Working in a petri dish, hold a termite securely with forceps and use the razor blade to cut the termite in half along its 'waist' (the constriction just above its legs). Discard the head, and carefully place the abdomen in the ethanol. Repeat this five times, pooling six abdomens into 1mL of ethanol for the 6x (high) concentration. Repeat the process with three termites for the 3x (medium) concentration, and one termite for the 0.3x (low) concentration (this will be diluted later). Using a clean microcentrifuge tube pestle or P1000 pipet tip, pulverize the tissue within the ethanol for each concentration. The goal is to homogenize the mixture as much as possible. Let the homogenized mixtures incubate on ice or in a fridge for 10 minutes. Centrifuge the mixtures at high speed (~20,000 rpm) for 10 minutes. Pipet off 400 µL of the supernatant (ethanol layer), being careful not to disturb the pellet of organic matter at the bottom, into a clean 1.5mL microcentrifuge tube for each concentration. Repeat, so that you have two sets of 400 µL aliquots per concentration. To dilute to 0.3x concentration, add 800 µL of 100% ethanol to each of the aliquots from the mixture of using one termite. Store extracts in a freezer (-20°C) until ready to use.

3.2.2: Termite Trail Following Instruction

On the day of the exercise, you should have at least 75 termite workers in the "ready" container. Briefly, students should draw a circle with segments using the provided stencil with a pencil on filter paper and place this filter paper into an assay petri dish lid (invert the petri dish). Using a fresh capillary tube, they should carefully overlay the circle with the appropriate concentration extract and let dry for 10 seconds. Then, students should introduce a termite from the "ready" container using a damp paint brush into the middle of the circle and place the petri dish bottom on top of the lid fitted with the filter paper. Once the termite makes contact with the trail, students should start a timer and record how many segments the termite travels in five minutes. The termite does not have to travel in the same direction. Video clips are provided in the Link to Supplemental Materials to give

examples of following behavior at each concentration. Students should gently return each termite to the "retired" petri dish after each trial. Each pair should complete a Termite Trail Following data collection sheet by repeating this protocol two times for each concentration. A possible expansion of this assay is to use both workers and soldiers as the focal individuals and compare following ability.

3.2.3: Termite Trail Following Statistics

A one-way ANOVA can be used to determine if the concentration of pheromone affects the number of segments the termites travelled. Post-hoc pairwise comparisons can be implemented if there is a significant overall effect of concentration to differentiate which concentrations were different from each other. A bar graph of the mean number of segments followed for each concentration can be plotted with standard deviation to illustrate any differences in following behavior.

3.2.4: Termite Trail Following Discussion Questions

What are the termites using to follow the trail? What is the chain of behavioral events that leads to following?

Why do we use the number of segments to measure following ability? What other measure could we use?

Do you expect there to be differences between individuals within a colony in their ability to follow trails? Why or why not?

Which trail concentration do you think will be most effective? Why? What did you observe?

Which trail concentration was followed best? Was your prediction supported? Why do you think this is the case?

3.3.1: Aggression Assay Preparation

Prepare assay dishes in advance by coating the walls of twelve (30mm diameter) petri dishes with Fluon ®. Label with the name of the genus (four each). Line the bottom of four dishes with dry paper for the termites. If you are using a larger-bodied ant, like *Formica*, you may have to adjust the size of the petri dish you use. You can set up the aggression station by placing four low magnification (10-20x) dissection scopes with lights or three bench-mounted lighted magnifying glasses around a lab bench. Although magnification makes it easier to observe, it is not absolutely necessary. Each of these mini-stations will be assigned to a pair of students. You should have at least 40 individuals per ant genus and at least 40 termite soldiers in the respective "ready" containers. Tap out enough flies (at least 30) in Fluon ® prepared 90mm petri dishes (1 per mini-station). Each mini-station should have three assay dishes (1 per taxon) and one petri dish of wingless fruit flies.

3.3.2: Aggression Assay Instruction

Students should work in pairs within their rotation group, so that all three taxa are being sampled in parallel and all students are engaged. The pairs should alternate which taxa they sample for each trial within the rotation station so that everyone observes each taxon. Each pair of students should introduce an individual from the "ready" container to a small petri dish and wait 1 minute for the animal to acclimate. They should transfer one wingless fly to the dish and put the lid on. For 3 minutes have the students tally how many behaviors are witnessed for each of the behavioral categories. Be familiar with how to identify these behaviors. After each observation period is over have the students return the individual to the respective "retired" container using a paint brush. Each rotation group should repeat this protocol six times per taxon to complete an Aggression Assay data collection sheet.

3.3.3: Aggression Assay Statistics

A one-way ANOVA can be used to determine if there are differences in average aggression score between taxa. Aggression scores are calculated by weighting each act by its relative aggression level and dividing by the total number of acts to control for general activity level. The formula to calculate this score per individual is: [(# of non-aggressive acts *1) + (# of olfactory investigation acts *2) + (# of mandible flaring acts * 3) + (# of biting acts *4)]/ total # of acts across all categories. Post-hoc pairwise comparisons can be implemented if there is a significant overall effect of genus to differentiate which genera are different from each other. A graph of the mean aggression score for each taxon can be plotted with standard deviation to illustrate any differences between genera in aggressive responses to fruit flies (Figure 3).

3.3.4: Aggression Assay Discussion Questions

What kinds of signals might the social insects be using to determine if they should attack or not?

Why would being aggressive be useful for an animal? What are different contexts in which aggression is displayed? In what scenarios might being too aggressive be harmful?

Why might we use termite soldiers instead of workers? What differences might we find if we test workers instead?

Which do you think will be more aggressive, ants or termite soldiers? Why?

Do you think the different ant genera will be different? Why or why not?

Did you see differences in aggression scores across genera? How do you explain?

3.4.1: Phototaxis Assay Preparation

Set up the phototaxis station by arranging four arenas around a lab bench, creating mini-stations. For each arena we used a 90 mm plastic petri dish with the external aspects of the lid painted with black acrylic paint and the external side wall of the bottom painted black as well. The external bottom should not be painted. Invert the dish to create the arena. The internal lid (now at the bottom of the arena) should be lined with white office paper, with a pencil line drawn down the center, which is changed out each trial. To the petri dish unpainted bottom (now the top of the arena), attach a half circle of red masking film (these insects cannot detect red and thus this appears dark) with clear tape (Appendix B, Figure 1). Each of these arenas will be assigned to a pair of students. You should have at least 40 individuals per ant genus and at least 40 termite workers in the respective "ready" containers.

3.4.2: Phototaxis Assay Instruction

Students should work in pairs at each mini-station within their rotation group, so all students are engaged. Students should introduce an individual from a "ready" container to the midline of the bottom arena and lid it immediately, matching the pencil midline with the border of the red film. Have one student per pair start the timer for 3 minutes. Record the proportion of time the animal spends under the red section by starting and stopping the stopwatch every time it crosses the line. Students should return individuals to the appropriate "retired" container when finished. In between trials, change the white paper bottom and reverse the side covered by the red film. Each pair should repeat the protocol three times for each taxon to complete a Phototaxis Assay data collection sheet.

3.4.3: Phototaxis Assay Statistics

A Z- test for proportions, which is robust against continuous data, can determine if the percent time spent in different light environments deviates from 50:50 in each taxa with the null hypothesis that the time spent in each light environment should be equal. A graph of the percent time spent in each light environment can be plotted per taxon to illustrate the strength of preference for light or "dark".

3.4.4: Phototaxis Assay Discussion Questions

Why do we switch the sides of the film in between trials?

Why might it be important for animals to detect light from dark and different colors?

Why might there be changes in light preference within an animal over its lifetime?

Do you think there will be a difference between individuals? Genera? Orders? Why or why not?

Were there any differences in choice made between individuals/genera/orders? How can you explain?

3.5.1: Expansion suggestions for Module 3: Laboratory Assays

This module can be expanded into multiple sessions in an animal behavior undergraduate course for students to elaborate on hypothesis testing, experimental design, and statistical analysis. Each assay could be a stand-alone laboratory activity with an increased sample size. This module can function independently of the other two modules or be integrated by having students collect their test subjects by modifying the collection procedure from the Field Observations and Collections activity.

3.5.2: Overall Module 3 Discussion Questions

Which assays produced results that supported your hypotheses? Which did not support your hypotheses? Can you think of other experiments to follow up what we tried?

Did you observe any behavior that surprised you? What questions do you have remaining? How might we find the answers through an experimental approach?

Think about levels of analysis. Did you see different patterns of behavior on an individual level (within genera), a genus level (between genera), or on an order level (between ants and termites)? Which level did we analyze? What other statistical methods or data could we use to test the other levels?

Think about our experimental design and execution. How might have our methods, taxa selection, sample size, and possible human error affected our results? What could we do differently next time?

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Acknowledgments

Thank you to Upward Bound Math and Science staff and students for providing the opportunity for the development of this curriculum. We are grateful for Dr. Sara Arganda, Andrew Hoadley, Alejandra Zelaya, Evan Razdan, Clayton Ziemke, Joslyn Mills, and Boston University graduate students for instructing adaptations of this curriculum.

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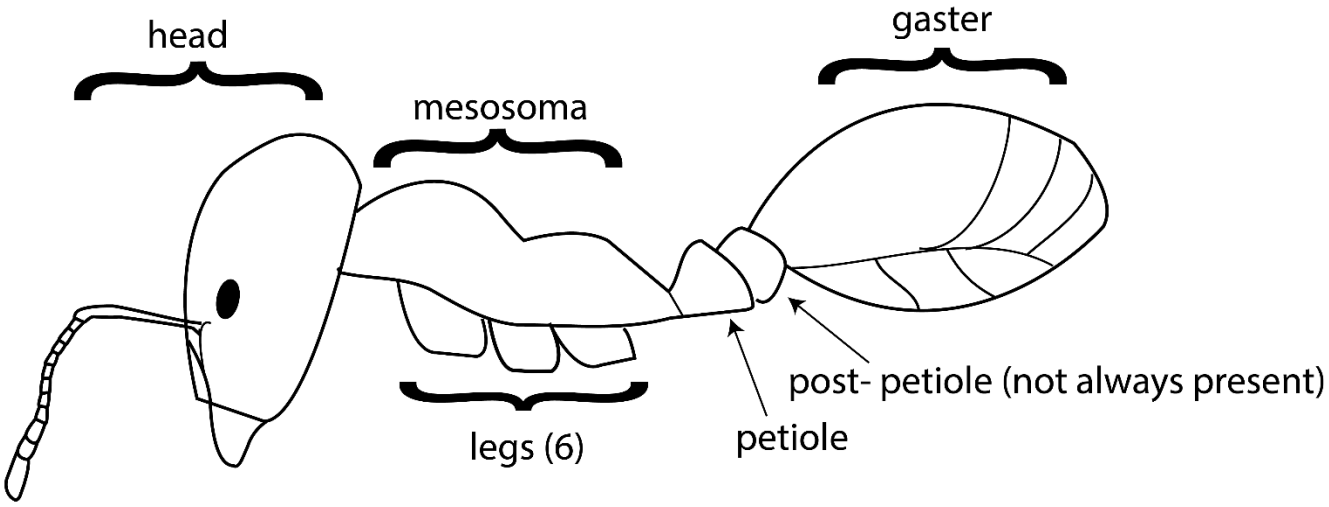
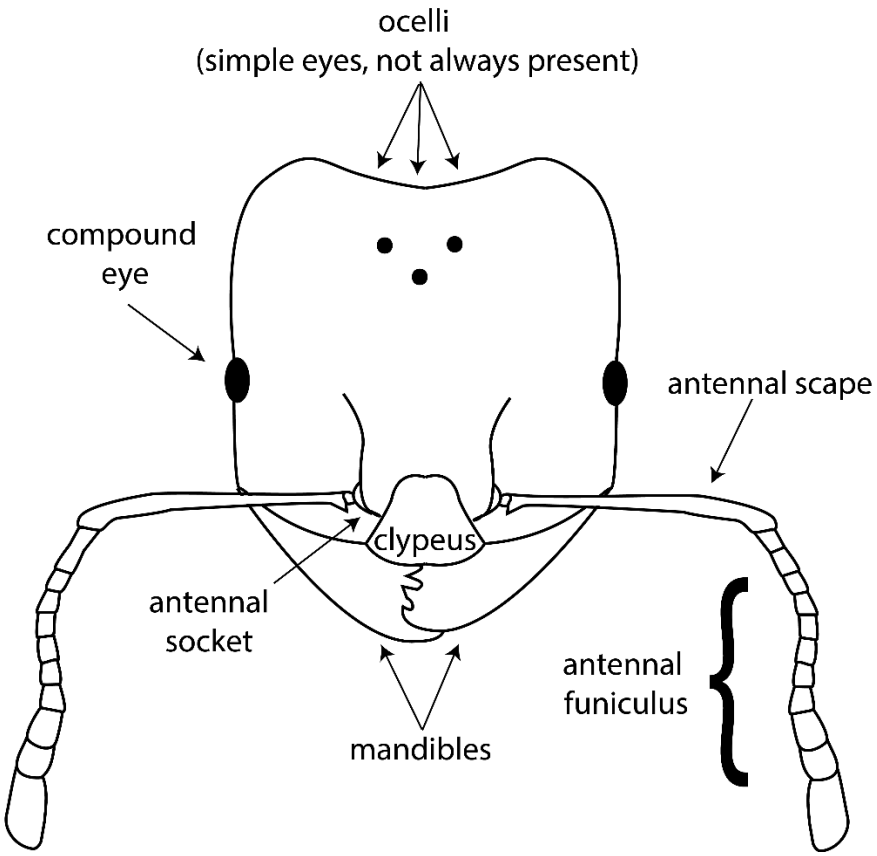
Darcy Gordon designed BDSI as a PhD candidate at Boston University. She completed her degree in 2017 and is currently a Digital Learning Lab Postdoctoral Associate in the Biology Department at Massachusetts Institute of Technology. There she uses evidence-based principles to support the design and management of residential and online courses.

Angela Seliga has been the Physiology Laboratory Manager at Boston University since 2009, where she divides her time among teaching large laboratory courses in physiology for upper level undergraduate students, training graduate and undergraduate students in pedagogical techniques, and advising students in educational outreach programs.

Appendix A
External Anatomy of Ants

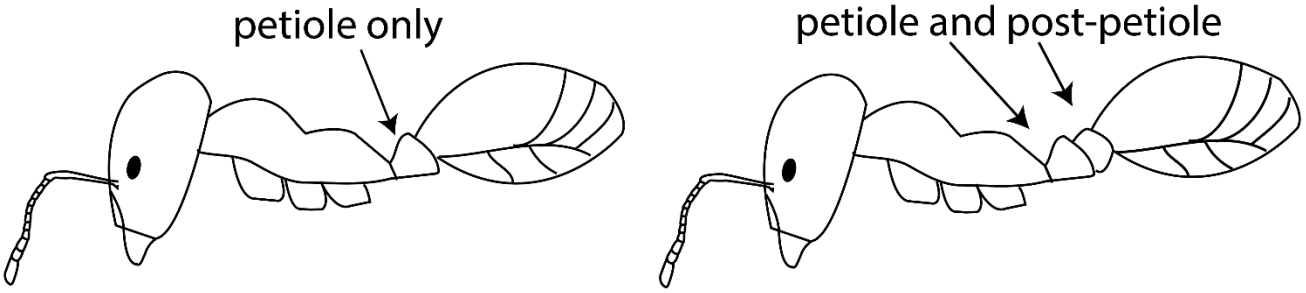
Figures adapted from Fisher and Cover (2007)



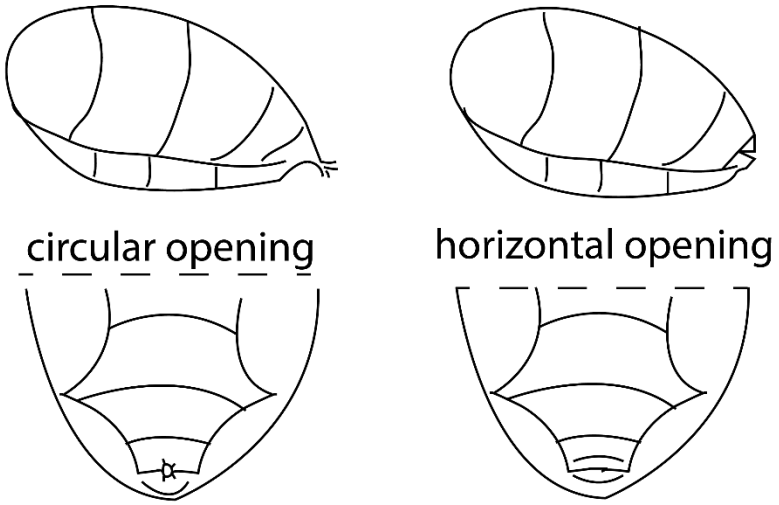


Appendix B
Dichotomous Key to Wisconsin Collected Ants

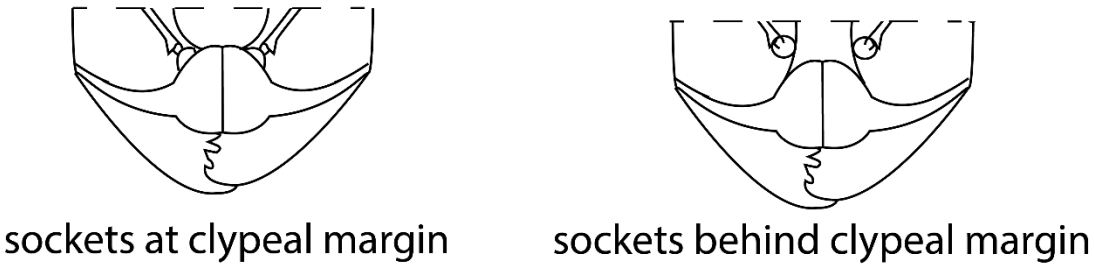
1. Post-petiole
    a. Petiole only, post-petiole absent (2)
    b. Post-petiole present (8)



2. Tip of gaster
    a. Circular opening which might have fringe of hairs (3)
    b. Thin horizontal opening (7)

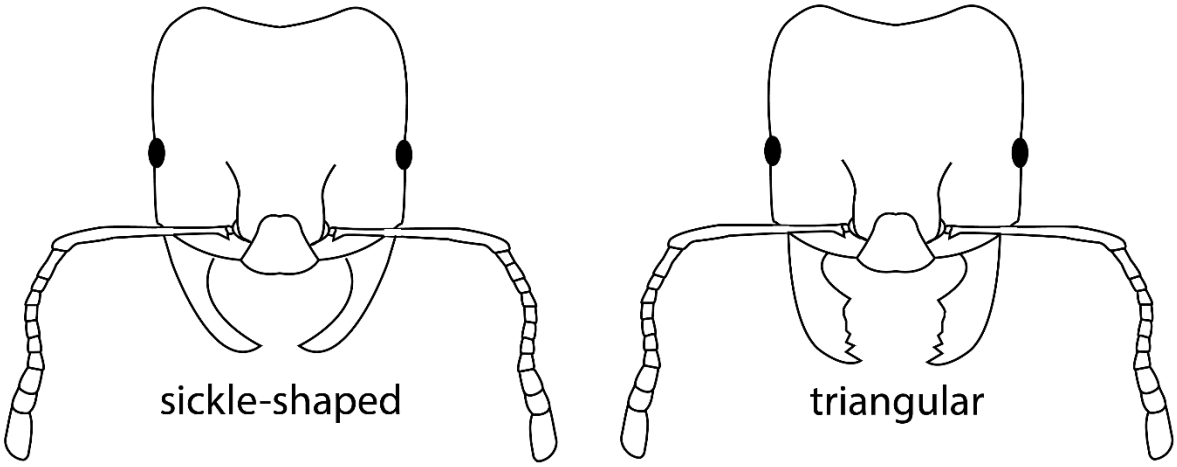


3. Antennal sockets
    a. Located behind the clypeal margin (*Camponotus*)
    b. Located at the clypeal margin (4)

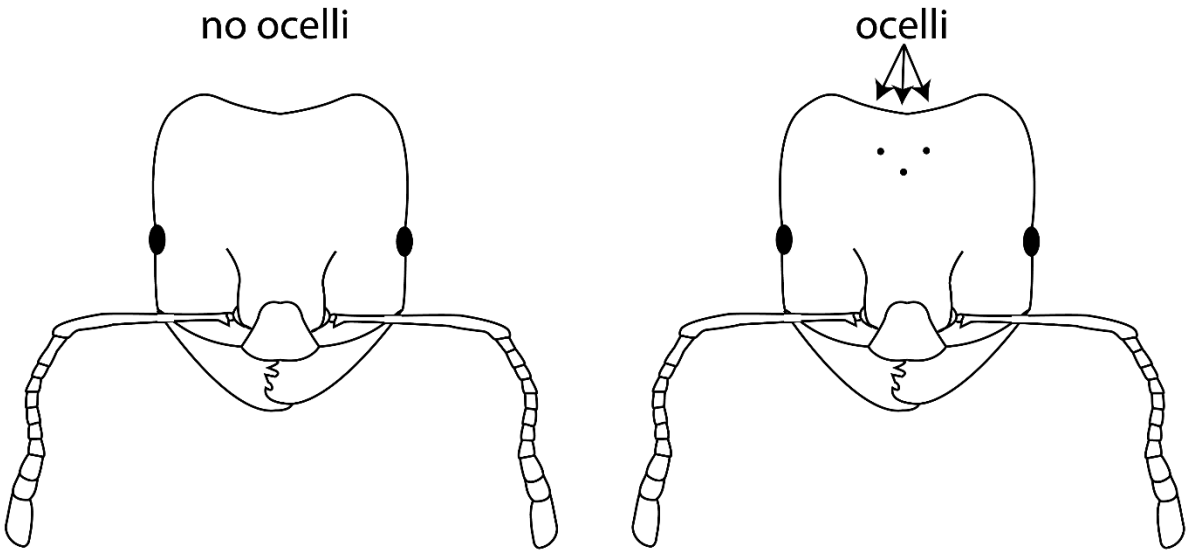


4. Mandible shape

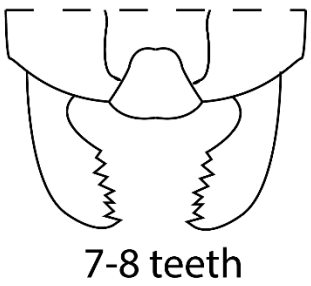
a. Long and sickle-shaped, minutely serrated (*Polyergus*)
b. Not long, roughly triangular, with many serrations or teeth (5)

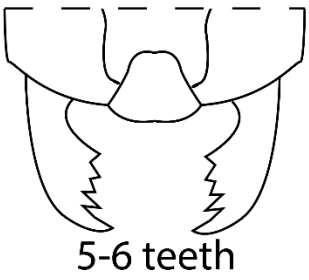


5. Ocelli
    a. Absent (6)
    b. Present (*Formica*)

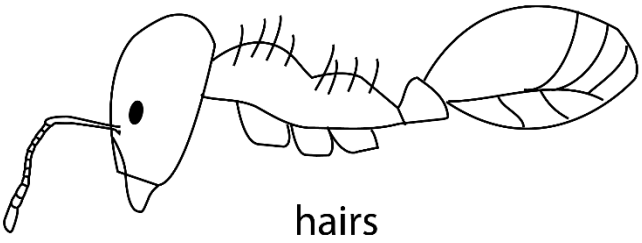


6. Mandible teeth
    a. 7-8 teeth (*Lasius*)
    b. 5-6 teeth (*Prenolepsis*)





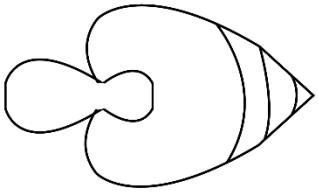
7. Hairs on mesosoma
    a. Lateral view without erect hairs (*Tapinoma*)
    b. Lateral view with erect hairs (*Liometopum*)

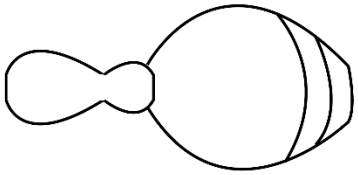


8. Post-petiole attachment
    a. To dorsal side of gaster (*Crematogaster*)
    b. To anterior face of gaster (*Myrmica*)

dorsal attachment

anterior attachment





Appendix C
Wisconsin Ant Genera Descriptions

Camponotus: (Formicinae) > 50 North American species: Worldwide distribution and one of the most common and species rich genera. Known commonly as "carpenter ants" they can be a nuisance to wooden structures. They typically have 2 size classes of workers and are rather large.

Crematogaster: (Myrmicinae) ~ 30
North American species: Truly an unmistakable ant. Their heart shaped gaster can flex over their body functioning to apply a defensive secretion. Their colonies are quite large and are subsisted on a diet of animal prey and carbohydrate rich secretions from aphids, which they tend like sheep.

Formica: (Formicinae) > 100 North American species: These can be confused at first with *Camponotus* or *Lasius*, but *Formica* ants have a certain way of moving that sets them apart. These are diverse ants that are quite abundant in forests and are known for their relationships with social parasitism.

Lasius: (Formicinae) > 50 North American species: Found in almost every North American habitat, these ants are generalist scavengers and predators, but also are known to tend aphids. This genus contains the "citronella ants" which give off a strong citronella odor when disturbed.

Liometopum: (Dolichoderinae) 3 North American species: These ants form enormous colonies, often in the dead centers of mature trees. They actively forage in conspicuous trails, and are armed with defensive compounds. These aggressive workers will release disagreeable chemicals, which some liken to the smell of blue cheese!

Myrmica: (Myrmicinae) 50-75 North American species: Found often in temperate forests, these ants are primarily carnivorous, but are also known to drink sap from plants. They play important roles in boreal ecosystems.

Polyergus: (Formicinae) 2-7 North American species: These are obligate dulotic ants that prey upon *Formica* host colonies. They steal larvae and pupae from their hosts on afternoon raids, which are then in turn used to perform colony tasks for the social parasites.

Prenolepsis: (Formicinae) 1 North American species (*P. imparis*): Also known as "winter ants" these workers are known for their ability to forage at cold temperatures. During the hottest months, these ants stay in their nests and subsist off of fatty liquid reserves contained in the swollen abdomens of specialized workers called "repletes."

Tapinoma: (Dolichoderinae) > 5 North American species: This genus contains a native species that has the largest geographical range and ecological tolerance of any ant in North America, living in every habitat including man-made structures.

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Appendix D
Phototaxis Arena

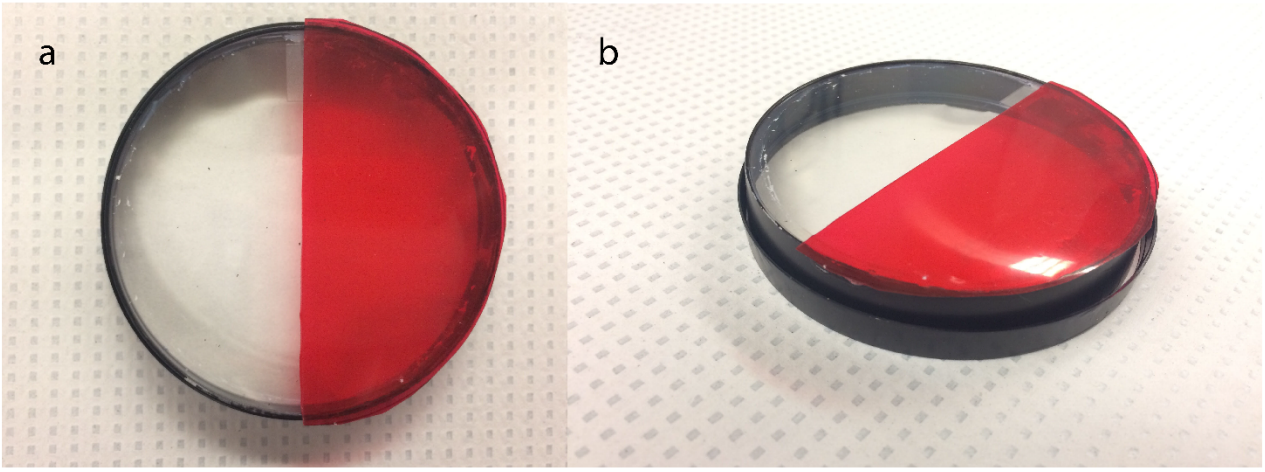

Figure 1. Phototaxis arena (a) from above, and (b) from an oblique angle.

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Citing This Article

Gordon DG, Seliga AM. 2018. Behavioral Diversity of Social Insects. Article 8 In: McMahon K, editor. Tested studies for laboratory teaching. Volume 39. Proceedings of the 39th Conference of the Association for Biology Laboratory Education (ABLE). **http://www.ableweb.org/volumes/vol-39/?art=8**