Chapter 19

Courtship Reaction Chains and Mate Attraction: A Two-Part Laboratory Activity Using WOWBugs[1], A New Model Insect

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Contents

Introduction

Traditionally, fruit or vinegar flies (*Drosophila* spp.) have been the laboratory workhorse for studies of behavior. They have been widely recommended as model organisms for courtship behavior studies in advanced placement as well as introductory biology courses, based on ease of culture and availability of numerous individuals. However, in practice, beginning students find it difficult to distinguish *Drosophila* sexes, and manipulating the adult insects requires that they be anesthetized. Furthermore, their courtship behavior is sometimes not reliably elicited and their behavioral responses are not dependable. These disadvantages can be frustrating for students and teachers alike, making *Drosophila* less than ideal as a classroom organism for illustrating behavioral concepts.

This activity introduces an alternative insect, *Melittobia digitata*, commonly called the WOWBug. About the same size as *Drosophila*, the WOWBug has the same advantages of rapid life cycle and production of large numbers of adults, and is even easier to culture. Unlike *Drosophila*, the sexes of *Melittobia* are very different and easily recognizable even to the naked eye. WOWBugs rarely fly, preferring instead to crawl slowly. Best of all, their courtship rituals are elaborate and easily observed, and if simple procedures are followed, this behavior is dependably elicited in the laboratory. Thus, *Melittobia* is ideally suited to the demands of the introductory or advanced laboratory in biology.

The activity includes two exercises which complement one another. The first is the longer of the two, but each fits comfortably into a one- to two-hour time frame, with little or no "down time." Because this versatile activity requires no prior training in techniques and poses no safety hazards, it can be used equally well in Introductory Biology, Zoology, Entomology, or Ecology laboratory classes, either to acquaint students with animal courtship or to familiarize students with methodologies used to study behavior.

Student Outline

Melittobia digitata are little parasitic wasps whose tiny stingers are used only to puncture the immature insects which serve as their hosts; they cannot penetrate human skin. Because of their many bizarre but instructive behaviors, these insects have received the common name of WOWBugs, and are rapidly becoming popular as new models for classroom and laboratory studies in biology and ecology.

Found around the world, *Melittobia* raise their young upon the larvae and pupae of over 20 other insect species in several different orders, including bees, wasps, beetles, and flies. Their life is a fast-paced one; only 17-21 days after being laid on the host, eggs mature to adults capable of repeating the cycle (Figure 19.1). Males — which are flightless and blind — spend their short lives entirely inside the host cocoon, but after they have mated, the winged females leave. Up to 700 wasps may emerge from a single host cocoon; however, nearly all will perish before finding a new host.

Courtship and mating are intense affairs that take place inside the host cocoon. Correctly identifying a potential mate's sex and condition is a serious matter. Over 95% of any generation of new adults are females, but they mate only once. Furthermore, the blind and flightless males, greatly outnumbered, reduce their own numbers even further by ferocious battles among themselves. Nonetheless, a highly efficient system must be in place, for almost all WOWBugs mate successfully within a few days. How do the sexes locate and recognize suitable partners in the darkness?

Objectives

After completing this laboratory exercise, you should be able to:

- Define the terms behavioral reaction chain, ethogram, and bioassay.
- Describe the behavioral components that make up the courtship sequence for each sex of *Melittobia* wasp.
- Describe and differentiate two common behavioral sampling methods.
- Conduct a simple bioassay for the presence of mating signals.
- Explain how to design an experiment that would distinguish between visual, auditory, and chemical courtship attraction signals.

Materials (per pair or team):

- Bioassay chamber and surrounding cover (such as a plastic pail)
- Deep-well projection slide containing male and female *Melittobia*
- Dissecting microscope and fiber-optic light

- Fine-gauge felt-tipped marking pen
- Forceps
- Pipe cleaner or fine-bristled brush
- Plastic wrap and cotton balls to make vial stoppers
- Scissors (for cutting small pieces of plastic wrap)
- Timer, stopwatch, or wristwatch that measures seconds
- Transparent tape
- Vials — each exercise uses one vial with 10-15 unmated female *Melittobia* wasps and two empty vials.
- White unlined paper (one sheet)

Obtain from instructor later: filter paper with "male essence"; preprinted data sheets (optional).

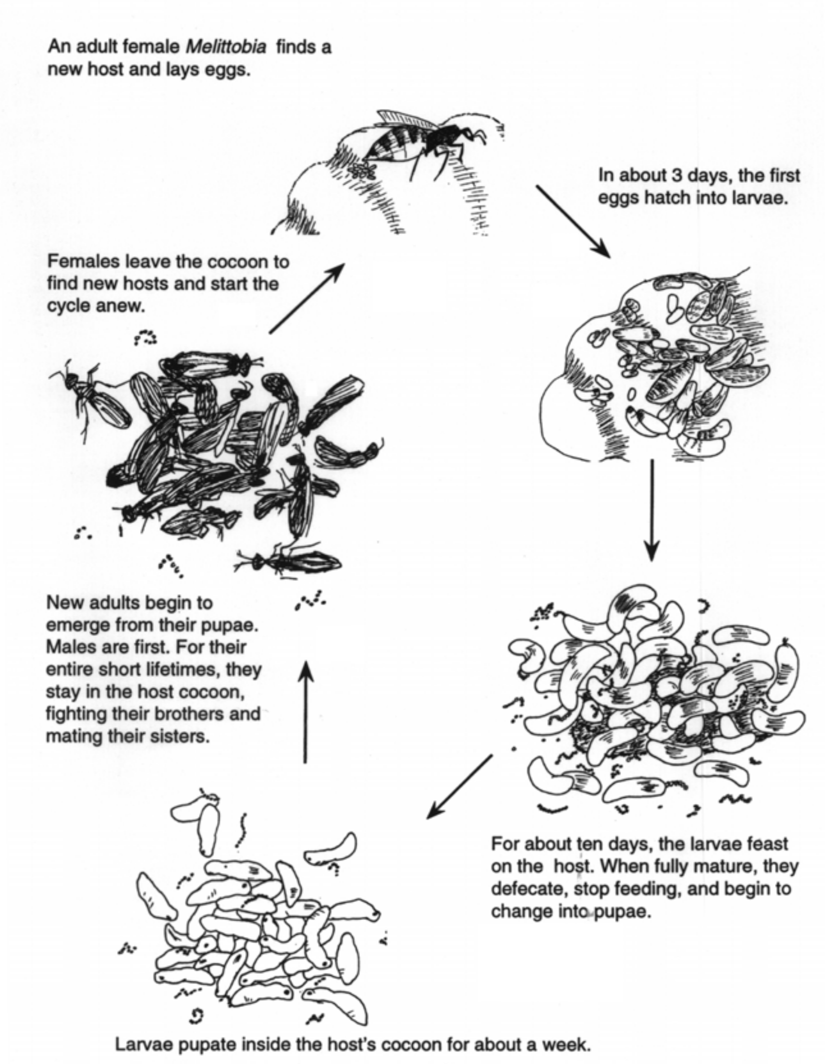

Figure 19.1. The life cycle of the WOWBug, *Melittobia digitata*. Under classroom and laboratory conditions, the cycle usually takes about three weeks. However, developmental timing is influenced by temperature. Outdoors it can be as short as 14 days or as long as several months. Illustration adapted from Matthews et al., 1996.

Exercise A. Ethograms and Behavioral Reaction Chains

Insect courtship is diverse. Some species exhibit almost none. Others practice complex and lengthy rituals that involve a mixture of visual, chemical, and tactile cues. Two general characteristics of almost all insect courtship, however, are that it is stereotyped and it is interactive. **Stereotyped** means the behavioral components follow a prescribed pattern, time after time with little or no variation. **Interactive** means the actions of the sexes are highly interdependent. Each sex responds to the behavior of the other and in turn by its own response triggers the next step on the part of the other. Lack of a proper response at any point in this interactive chain usually terminates the courtship.

Behaviors cannot be placed in an order until they have been described, of course. Detailed studies of animal behavior always begin with "just watching" — observing and listing the actions that define an animal's behavioral repertoire. Thus, a key initial procedure in behavioral studies is compiling an **ethogram**, or catalog of an animal's behaviors (Table 19.1). This is not always an easy task. An ethogram is based upon a determination of individual behavioral units, and may vary according to the way in which these are defined.

Table 19.1. An ethogram of all behavioral acts observed during the courtship activities of a hypothetical insect. This is simply a list, and acts can appear in any order. (Here, they are alphabetical, to emphasize this point.)

Male Behavioral Acts	Female Behavioral Acts
Approaches female	Assumes mating position
Bumps female under head	Bites male
Buzzes wings	Follows male
Inserts penis	Preens
Mounts female	Touches male with her antennae
Preens	Turns toward male
Touches female with his leg	Walks away
Walks away	Waves front and middle legs

Although a simple list of behaviors can be useful, knowing the relationships between them is usually even more useful. A sequence of stereotyped behaviors performed by two individuals, in which the behavior of one stimulates the next behavior by the other individual, is called a **reaction chain**. It usually is schematically represented (Figure 19.2). Helpful tools for visualizing many aspects of animal behavior, reaction chains have received their widest usage in analyses of insect courtship.

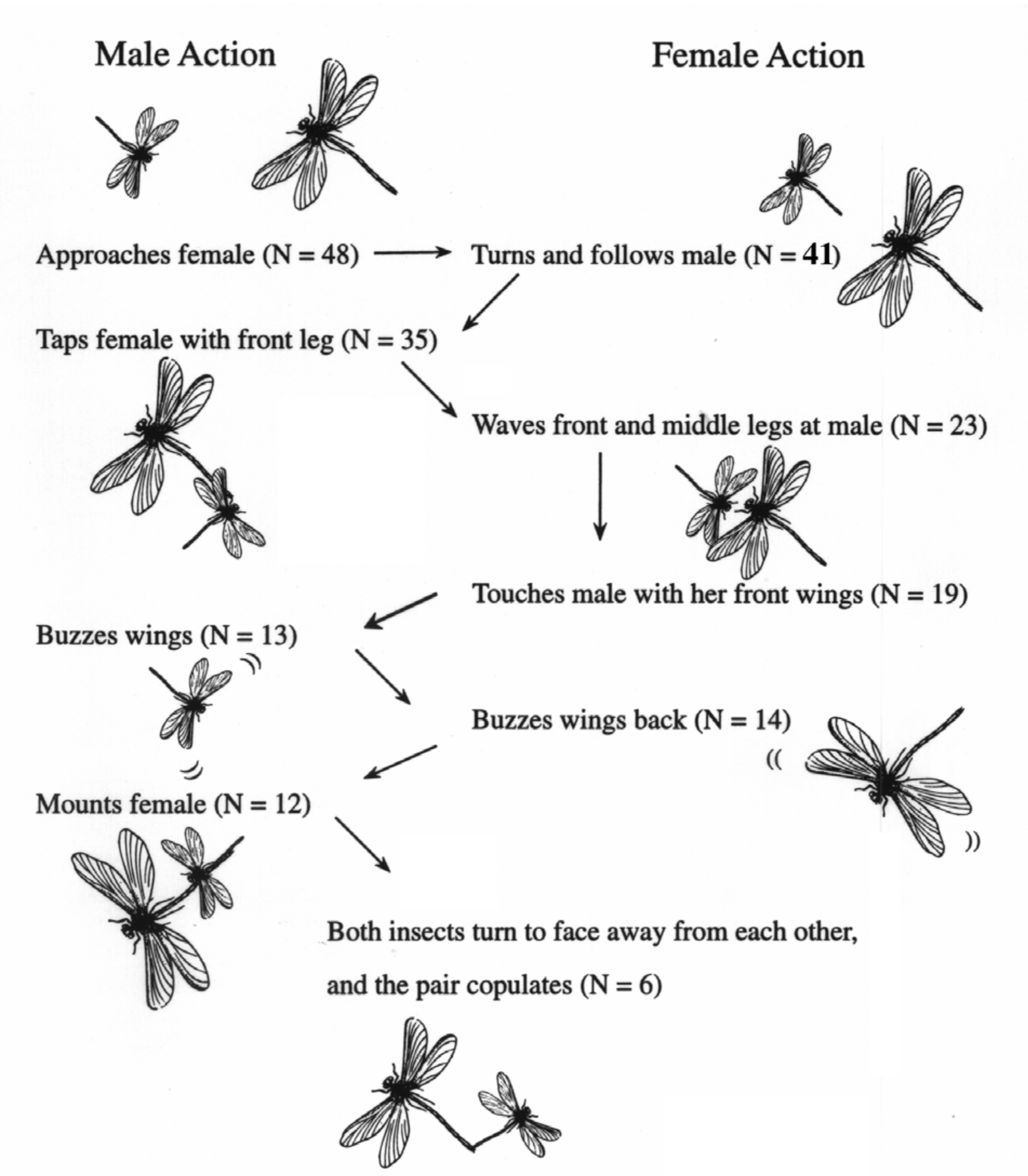

Figure 19.2. A reaction chain for the courtship of a hypothetical insect, illustrating the interactive back-and-forth pattern of the behavioral acts that were listed in an ethogram. The words and arrows alone would be sufficient for a reaction chain, but the sketches in this example add visual impact and interest. Inclusion of frequency values (N) increases quantitative rigor, enabling the reader to know what proportion of the behavioral acts were observed to be successful in each successive stage.

Procedure

1. Working in pairs or teams, obtain your materials and set up the fiber-optic light so that it illuminates your microscope stage.

2. Place the deep-well slide containing male and female wasps on the stage. Adjust the focus and observe the two sexes. Describe each sex's body color, wing length and form, and antenna structure. How do the sexes differ? The situation in which two sexes of a species differ in such physical characteristics as size, color or shape is called **sexual dimorphism**.

3. Working over the white paper, use your fingernail to open the lid of the deep-well slide carefully. With your pipe cleaner reach in and snare the female using a slight twisting motion. Gently place her in the empty vial and label it "Mated Females." Replace the lid on the slide to keep the male inside.

4. Hold the vial containing the unmated females over the white paper. Flick your fingernail sharply against the side of the vial. This snapping motion will dislodge the females, causing them to fall to the vial bottom. Carefully remove the plastic-wrapped cotton stopper. Reach into the vial with the pipe cleaner and gently pick up one female. Replace the stopper in the vial promptly. Should extra females leave the vial, invert your extra empty vial over them until you can pick them up and return them to the original vial.

5. Reopen the lid of the deep-well slide. Let the female crawl off the pipe cleaner into the well with the male. Replace the lid, and place the deep-well slide back under the microscope.

6. Working in pairs, begin observing and recording the wasps' actions as described below. A brief period of exploration and acclimatization to the chamber may be necessary before sexual activity is initiated, so observe carefully and be patient. However, if no courtship is initiated within eight minutes, record this fact on your data sheet, remove the female and replace her with a new unmated female.

7. Using either a preprinted data sheet or a page in your laboratory notebook, write a continuous narrative. Jot down each different behavior you see, and include its duration (in seconds) whenever possible. Scientists call this **ad libitum sampling.** (See the Glossary.)

As you go, group the behaviors that you observe into these four stages:

- *Latency:* The length of time between placing the male and female together and the first contact.
- *Preliminary Courtship:* All behaviors that occur between first contact and mounting. How long did this stage take? What sorts of actions did you see? Which sex initiated this contact? What body parts made actual contact?
- *Advanced Courtship:* All behaviors from male mounting to the beginning of copulation. Record the orientation of the male and female, movements of body parts, and the sequence in which these are done. Identify each movement as precisely as you can. For example, if one insect brushes the other, note which body part does the brushing (e.g., middle leg) and which body part of the partner is brushed.

- *Copulation:* Stage from penis insertion to male dismounting. How does the length of this stage compare with previous stages?

7. Use the pipe cleaner to remove the female gently. Place her in the "mated females" vial. (Never return a mated female into the vial with the unmated ones!) Plug the vial tightly with a cotton stopper wrapped in a small piece of plastic wrap.

8. Consider what you have written. Does the ongoing stream of behaviors divide naturally and easily into distinct components? How are the behaviors of the two sexes different? Working together in pairs or as a class, attempt to define and describe behavior units that could be used for further study. (For examples, refer to Table 19.1.) For easier note-taking, decide what abbreviation you will use for each component that you observed.

9. Repeat your observations of courtship and mating at least three times, until you feel that you have sufficient information to construct an ethogram and a reaction chain diagram for your wasps. For each trial, time each of the five courtship phases. During these trials, also count (and time, when possible) repetitions of each behavioral component that you have identified.

10. Conduct a similar trial, reusing one of the now-mated females from your labeled vial. Record these observations. What effect does mating appear to have upon subsequent female courtship behavior? How would you characterize an ethogram and reaction chain for a mated *Melittobia* female?

11. When you have finished collecting data for this exercise, record your values on the master data sheet for the class. You may wish to read briefly over the Discussion Questions below, but wait to answer them until later in your laboratory report. You'll want to move quickly on to Exercise B.

Discussion Questions

A thorough knowledge of the courtship behaviors of animals is important because reproduction is a critical aspect of behavior with implications for conservation management and control of animal populations. In your laboratory report, show that you have mastered the objectives for this exercise by using both your own data and that from the class master data sets to discuss the following questions. Include your ethogram and your courtship reaction chain with your report.

1. How do the two sexes of *Melittobia digitata* differ in appearance? What correlation can you draw between male antenna structure in *Melittobia* and courtship behavior?

2. For both your own data and that of the entire class, list the mean times (in seconds), and the ranges, for each of the four stages of *Melittobia* courtship behavior observed. Which stage had the longest average duration? The shortest? From an evolutionary standpoint, what advantages might there be for what you observed? Which stage was the most variable in length? The least? How might this be explained?

3. Which behavioral stage included the greatest number of different components? The longest chain of reciprocal interactions between the male and female? Why are these particular stages crucial to successful reproduction?

4. We've said courtship behavior is stereotyped. In what way? Did the same behavioral components occur in every successful courtship reaction chain, or not? Which was more similar from one trial to the next: the types of acts performed or the number of times they were repeated and how long they took?

Exercise B. Mate Attraction

How do male and female insects locate one another in the first place? Across the insect world, many sorts of courtship attractants have been documented. There are visual cues, such as the flashes of fireflies. There are auditory cues, such as the chirps of crickets. And there are chemical cues, or **pheromones**, such as the perfumes by which moths attract one another.

Does *Melittobia* courtship begin purely with random encounter, or with such a cue? If there is a cue, what form does it take? To answer such questions, biologists often use a **bioassay**, which is an analysis that uses the behavior of living organisms to measure response to a variable. We will use an experimental apparatus called a bioassay chamber (Figure 19.3) to examine the possibility that male WOWBugs may be actively attracting females.

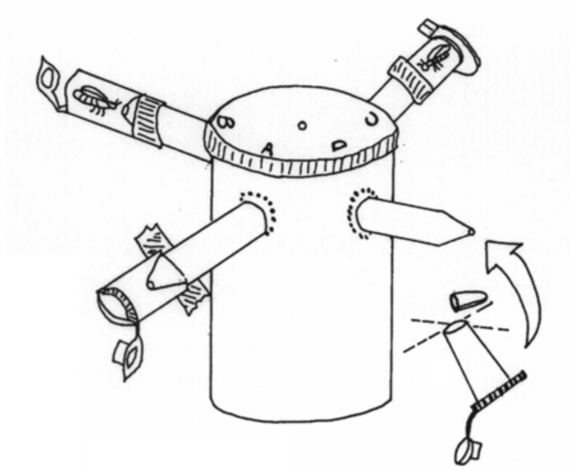

Figure 19.3. This bioassay chamber uses four microcentrifuge tubes as replaceable housing for the experimental subjects and substances. Nonwoven polyester caps separate the interior of each tube from the interior of the bioassay chamber. These barriers allow odors and sounds to pass through, but *Melittobia* will remain separated — unless, as occasionally happens, a determined individual chews a hole through it!

To use the bioassay chamber, cut the tapered ends from four microcentrifuge tubes. Insert the cut end of a newly cut tube over one of the protruding spokes on the bioassay chamber. Wrap a piece of transparent tape once around the joint. Load each chamber with an appropriate variable, then close its lid. When ready to begin the experiment, place unmated female *Melittobia* in the central cylinder and close the lid.

Procedure

1. During this experiment, a certain amount of handling of the bioassay chamber is inevitable. There is no evidence that this affects *Melittobia* behavior in any way. However, you may

wish to wash your hands before beginning, particularly if you have recently applied hand cream, eaten something, or otherwise potentially added odors to your skin.

2. To tell the tubes apart, use the felt tip pen to mark the cannister lid above each tube with a letter from A to D (see Figure 19.3).

3. Insert a tube into location A by sliding it firmly onto the open end of one of the spokes on the bioassay chamber. Tape the connection between the two sections. This empty tube will serve as a control.

4. Repeat the above procedure with another tube at B. After attaching the tube and taping it on the bioassay chamber, use a pipe cleaner or brush to introduce a live male *Melittobia*. Then close the cap.

5. Follow the same procedure again, but place a live mated female *Melittobia* into a tube at C.

6. Tape tube D onto the last spoke of the bioassay chamber. From your instructor, obtain a piece of filter paper containing "male essence" derived by squashing a dead male. Use your forceps to place it inside tube D. Then close the tube.

7. Double-check the interior of the bioassay chamber. All the spokes should be flush with the inside of the canister so that *Melittobia* can easily enter them, and be securely sealed around their perimeter so that the wasps will be unable to escape.

8. Devise appropriate data sheets for this experiment, or obtain pre-printed ones. First, you will be collecting data (number of wasps in each tube) at one-minute intervals for 15 minutes. Then you will be collecting behavioral data (wasp entries and exits for the four tubes) continuously for five minutes.

9. Count and record the number of *Melittobia* in a vial of unmated wasps. Then remove the cotton plug from the vial and tip it into the central canister of the bioassay chamber. Snap your fingernail against the side of each vial a few times, until all the wasps are bounced out of the vial into the canister.

10. Replace the cap, being careful not to smash any wasps between the cap and canister. Make sure the letters on the cap are realigned correctly with the tubes (with A over the empty tube and D over the filter paper).

11. Cover the bioassay chamber with the bucket and start your timer. Once a minute for the next 15 minutes, lift the cover briefly and record the number of females visible in each of the spokes of the bioassay chamber. Scientists call this the "on the dot" or **instantaneous sampling** method.

12. Are you seeing the same or different individuals at each count? Remove the bucket and watch the wasps in the spokes of the bioassay chamber for five minutes. Record what you see. This is **ad libitum sampling**. Consider what additional information ad libitum sampling might reveal as compared to instantaneous sampling.

13. Dismantle the bioassay apparatus. First, remove tube B from its spoke, and return the male to his deep-well slide. Return the mated female in tube C to her vial. Discard the "male essence" paper.

14. Open the canister and count the wasps as you transfer them back into vials. Work over your white paper. Start by bouncing out the wasps with a flick of your fingernail. As before, you can place the open end of an empty vial over individuals on the paper. As each wasp moves up the vial, pick up the vial and place it over another one. When all have been collected, plug the vials securely with cotton stoppers.
15. Return the bioassay chamber and the emptied tubes to the place designated by the instructor.

16. Record your data on the master data sheet at the front of the room. Copy the master data sheets from Exercise A and Exercise B into your notebook for use in writing your laboratory report.

Discussion Questions

l. Why did we cover the bioassay chamber? What variable did this address?
2. By quickly reviewing the natural history information given in the Introduction and reading through the procedures, you should be able to eliminate one communication mode from *Melittobia* mate attraction. Which one, and why?
3. Compare and contrast the contents of the four experimental tubes. What types of communication signals were being tested with each? What new information did tube D provide that was not evident just from tube B? What evidence for pheromone usage did these experiments provide? Suggest a further experiment that would be relevant to this topic. What would you expect it to show?
4. When *Melittobia* enter a spoke of the bioassay apparatus, do they stay at that location? Were you seeing the same individuals at each count during your instantaneous sampling?
5. In this experiment we used a single male in captivity to assay the behavior of 20-30 unmated females. Predict what would happen if we were to reverse the experiment, using a single captive female in the spoke, with 20 male *Melittobia* in the central canister.

Glossary

ad libitum sampling: Continuous recording of behavior in a running commentary.
bioassay: An analysis that uses behavior of organisms to measure response to a variable.
ethogram: A catalog of an animal's behaviors based upon determination of individual behavioral units.
instantaneous sampling: Recording behavior by its presence or absence at precise pre-determined points in time (e.g., once each minute); also called "on-the-dot" sampling.
interactive: Describes behavior in which one organism responds to the behavior of the other and in turn by its own response triggers the next step on the part of the other.
pheromone: A chemical secreted to the outside of the body of one animal which affects the behavior of another animal of the same species (for example, a mate attractant pheromone).
reaction chain: A sequence of stereotyped behaviors performed by two individuals in which the behavior of one triggers the next behavior of the other.
sexual dimorphism: Situation in which two sexes of a species differ in one or more physical characteristics, such as size, color, shape of body parts, etc.

stereotyped behaviors: Behaviors which are uniform, repetitive, and ritualistic.

Notes for the Instructor

Laboratory Safety Considerations

There are no special safety considerations for this exercise. The animals used in this activity are harmless to humans, occur naturally around the world, and present no environmental hazards.

Advance Preparations and Tips

Advance Preparations: Timing is Everything

Time your order (or your ongoing cultures) to have adult organisms available when you need them for the activity. Commercially available *M. digitata* are shipped by mail as pupae. Like all invertebrates, their development is influenced by temperature, and can be slowed down or speeded up by appropriate environmental manipulations. At about 78° F (26° C) pupae will become adults in about a week. Adults usually will live about two weeks. During this time, they can be easily cultured (Appendix B). The entire life cycle is about 24 days at usual laboratory temperatures. If you are maintaining a continuous culture, or wish to rear male-only cultures, work backward from the anticipated laboratory activity date to determine when to set new cultures. In general, plan to have pupae available 10-14 days before the activity is scheduled.

One of the many visible differences between male and female WOWBugs is that adult males lack compound eyes (Figure 19.4). When the cultures arrive, or as soon as you are able to distinguish the red-eyed female pupae from the eyeless males, sort the pupae into separate containers to provide males and unmated females. *This must be done before the adults emerge.* This is a simple task under low power of a dissecting microscope. Gently break open the host cocoon, shake the culture out onto a piece of white paper, and use a camel's-hair brush or a white pipe cleaner to push the pupae into two piles.

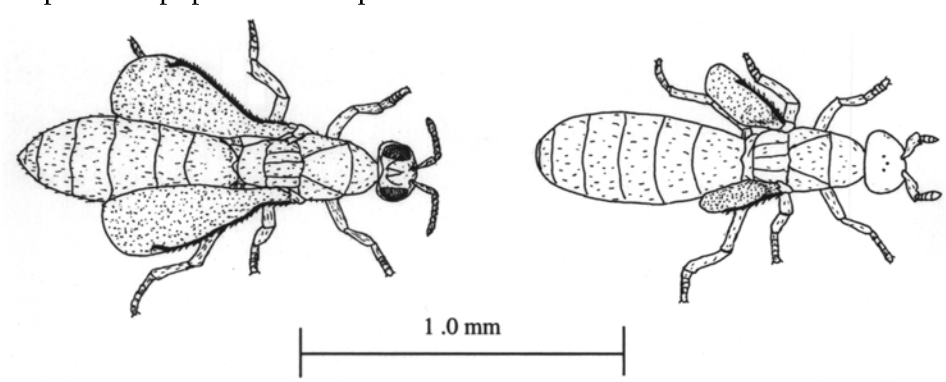

Figure 19.4. The black female WOWBug (left) has compound eyes and typical wasp-like antennae; it also has normal wings, although it seldom flies. The caramel-brown male WOWBug (right) has tiny wings, and lacks compound eyes; each antler-like antenna bears the conspicuous "digit" which gives the species its name.

Female pupae (which will outnumber male pupae in the culture by about 20:1) should be placed in groups of approximately 15 per vial. They can be picked up on the brush hairs or with a white pipe cleaner, then flicked into the vial. Cover a cotton ball with a small piece of plastic wrap and tightly wedge it into the opening of the vial. The plastic wrap will keep the insects from becoming entangled in the cotton.

Because they fight with one another, *males must be individually housed.* Place each male pupa (or newly emerged adult) directly into one of the deep-well slides that will be used for this exercise. Add a female pupa to the slide with the male and replace the cover. (The reason for this is that the presence of a female or late pupa seems to "prime" the male for sexual activity; a completely isolated male may become aggressive toward females when placed with them for the laboratory activity.) Have the students remove these "primer" females to their "mated" vial after first observing and comparing the differences between the sexes in step two of the procedure. Neither sex requires food or water at any time for these exercises.

Making Bioassay Chambers

To save classroom time, assemble the bioassay chambers prior to the laboratory exercise. (To make the replaceable chambers, students will use a razor blade to cut the tapered ends from four more centrifuge tubes; if you prefer, you may also do this step in advance for them.) Appendix A provides a materials list for the chambers; Figure 19.5 gives construction steps. The basic chamber can be reused indefinitely, but be sure to remove the spokes for storage or the film canisters eventually will split where they are inserted.

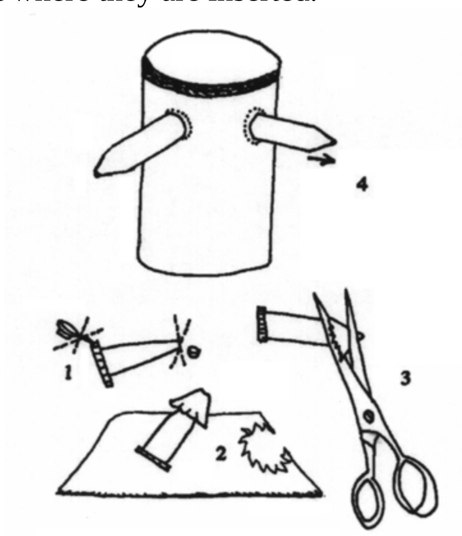

Figure 19.5. Constructing the bioassay apparatus:

1. Use a single-edged razor blade to cut the tips and caps from four microcentrifuge tubes.
2. Place a drop of nail polish on a piece of wax paper, and dip the cut tapered end of one of the centrifuge tubes into the polish. Blow gently on the end to break the small bubble of polish that will form. Allow the polish to set for about 10 seconds, and then lower the polish-covered end onto a small piece of interfacing material.
3. When the nail polish is dry, trim off the loose edges of the permeable interfacing barrier.
4. Remove the cap from a black film canister. From the inside, punch four holes equally spaced around the canister. (As an aid in spacing the holes evenly, you can mark the top lip of the canister and use the marks as a guide for positioning the hole punch.) Push the tubes through the holes from the inside to make four spokes.

The "essence of male" papers used in Exercise B should be prepared immediately before class. Place 10 live males in a freezer or on ice for a few moments to anesthetize them, then using one tip of the forceps, squash them individually in different places on a piece of filter paper. Cut the paper around each smear into a small disk that will fit inside a microcentrifuge tube.

Conducting The Laboratory Exercises

As a prelaboratory assignment have students view the first part of the WOWBugs Biology videotape (see appendix for source) in order that they come prepared knowing the differences between *Melittobia* sexes and something of their basic life history. To facilitate compliance, some instructors give a very short (10-minute) quiz on the background at the start of the laboratory .

Approximately 90 minutes is needed to complete Exercise A successfully:

- The first 10-15 minutes of Exercise A will be spent introducing the students to the viewing chamber, demonstrating techniques for transferring the wasps to and from it, and setting up the microscopes.
- Allow at least 20 minutes for initial observations necessary to develop a list of behavioral acts (procedure, number 6), another 10 minutes for the class to reach a consensus on these (procedure, number 8) and the terminology to be adopted. It sometimes helps to replay the brief actual courtship and copulation sequence in the videotape to clarify issues or disagreements that will arise (e.g., whether it is the middle or hind legs that are rhythmically spread during mounted courtship). Data collection (procedure number 9) will require about 20-25 minutes total as students make observations and record their data for three introduced females. For retesting an already mated female (procedure, number 10), another five minutes is needed.
- Finally, allow about 10 minutes for clean-up and recording data on the class master data sheet.

If laboratory periods are shorter, a logical break is to spend one session introducing the background and techniques, with the actual observation and data collection done in the subsequent session.

Exercise B can be easily completed in a one hour session.

At times, mating activity may seem slow to begin. Individuals are quite variable. Like many organisms placed in unfamiliar surroundings, WOWBugs may need a period of time to acclimatize before they begin mating activities. Encourage students to be patient, but to replace any female who has not engaged in mating behavior within eight minutes.

An alternative method that may increase the chances of each team observing several successful mating attempts is to have each student start his or her own deep-well projection slide with a *Melittobia* pair and watch them individually until one pair begins active courtship. At that point, both partners can begin to watch and record data on the active pair. Note that this alternative requires twice as many insects and projection slides.

Appendix 4 provides some sample results and expected outcomes. Note that about 20% of females fail to be mated during the allotted observation time; this is normal.

On rare occasions, a female is attacked (and sometimes partly dismembered) by a male. Males behaving in this fashion will generally court the next female in the normal fashion. The possibility of such an attack should be mentioned to the students during the background introduction. Reasons for this behavior are unclear. It may be an artifact of the isolation and manipulation. Alternatively, it may be related to hunger. WOWBugs typically do all of their feeding as larvae, but the victors of male battles have also occasionally been seen to turn cannibalistic.

Figure 19.6 illustrates typical results for a student-generated ethogram and courtship reaction chain. Expect some variation. To facilitate some uniformity in terminology, after each team has collected data on at least one mating, you may wish to call students together to discuss what they are seeing. Which sex was the first to make contact? How would that contact be described? What body parts of each sex were involved in each behavior? Once the students have reached some agreement on the types of behaviors which they are observing and how they will be described, they can continue their observations until they have sufficient information to construct their ethograms.

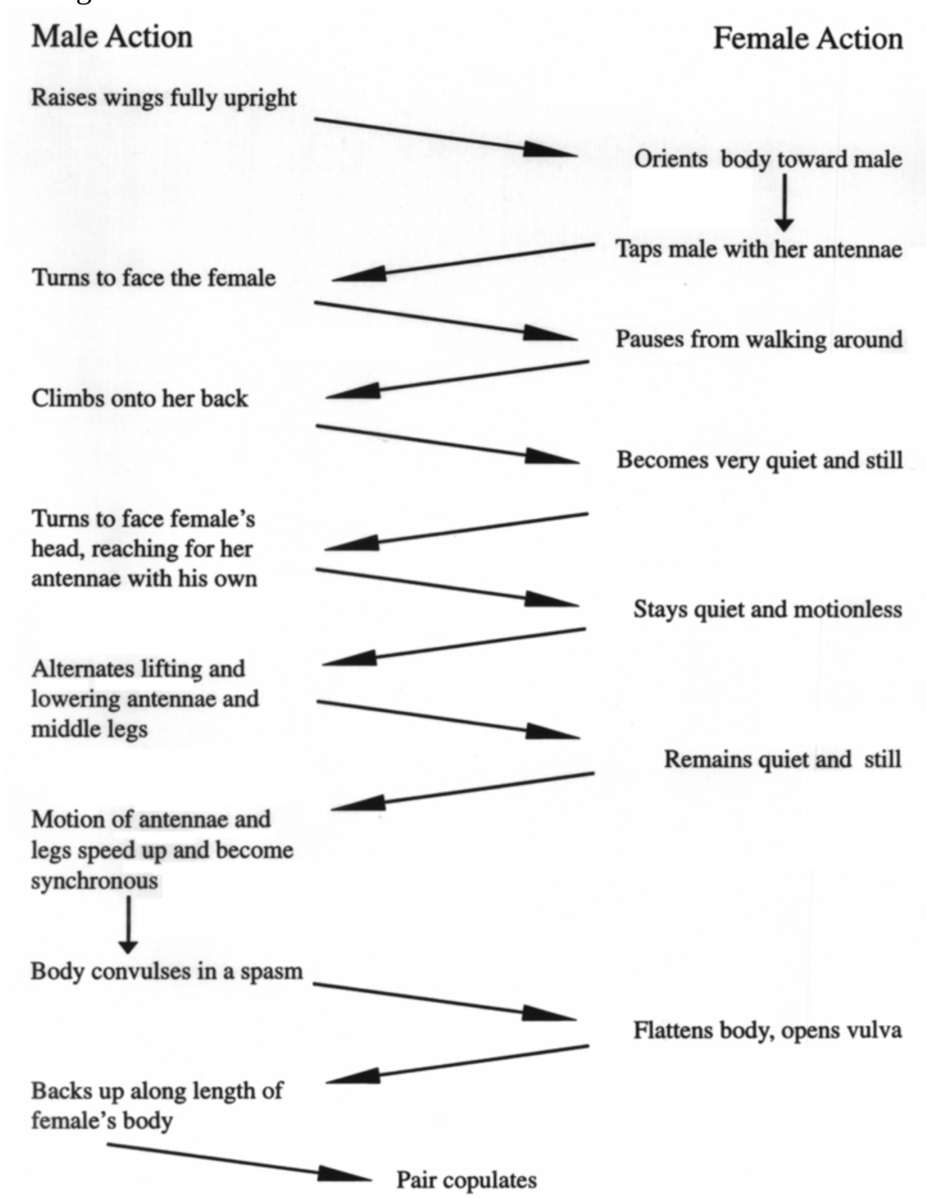

Figure 19.6. A typical courtship reaction chain for *Melittobia digitata*. Adapted from Evans and Matthews (1976).

Both parts of this activity lend themselves to some interesting extensions. For example, in Exercise A, students may wish to test the effects of male experience, either upon female attraction or upon the duration of courtship. For additional background, videotapes which support and supplement the information in this laboratory exercise include *WOWBug Biology* (17 minutes), *WOWBug Rearing and Maintenance* (14 minutes), and *Organ Pipe Mud Dauber Biology* (21 minutes). All are available from Riverview Press (PO Box 5955, Athens, GA 30604; 800-847-6540), which also publishes *WOWBugs: New Life for Life Science* and a quarterly newsletter, *The WOWBugs Bulletin.*

We encourage the use of this exercise as the basis for a formal laboratory report. If students are familiar with procedures for statistical data analysis, remind them to use non-parametric statistics to perform any analyses, as the data will likely violate the normality assumption of parametric tests.

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Appendix A: Materials And Suppliers

Materials Required For 20 Students Working In Pairs on Both Exercises:

- Bioassay chambers[1] (10)
- Cotton balls to use as vial stoppers (one small package)
- Plastic wrap (one small roll)
- Deep-well projection slides[2] (10 - 20)
- Dissecting microscopes (10) with "cool" fiber-optic lights
- Filter paper for "male essence" (one piece, size not critical)
- Fine-tipped black markers (10)
- Forceps (10 pair)
- WOWBugs *(Melittobia digitata*) from commercial[3] or natural[4] sources (one[5] to six[3] cultures)
- Opaque plastic buckets, gallon size or similar size plastic flower pots (10)
- Plastic or glass shell vials, one dram (at least 80)
- Timer, stopwatch, or wristwatch that measures seconds (10)
- Transparent tape (one roll)
- White paper, unlined (10 sheets)
- White pipe cleaners or chenille sticks (one package)

Materials Needed To Make 10 Reusable Bioassay Chambers[6]:

- Black 35-mm film canisters with lids (10)
- Fast-drying nail polish to use as glue (one bottle, any color)
- Hand-held single hole paper punch (one)
- Lightweight nonwoven nonfusible interfacing (approximately 1/8 yd. — available from fabric shops)
- Microcentrifuge tubes, 0.5 ml size (80)
- Scissors (one pair)
- Single-edged razor blade or utility knife (one)
- Small sheet of wax paper (one small sheet to use as a work surface)

Materials Needed For Each WOWBug Culture You Plan To Establish:

- 5 - 10 female *M. digitata*, obtained either commercially[3] or from a naturally occurring population[4]
- One or two hosts such as blow fly puparia[7] or a mud dauber[8] or paper wasp larva or pupa
- A small jar, vial, or plastic box with a tight-fitting lid to house the culture
- Alcohol or other disinfectant

[1]Reusable; initial construction requires the materials listed separately.

[2]Durable, reusable. # B3-60-3730, $45.00 per set of 20, Carolina Biological Supply Company, 2700 York Road, Burlington, NC 27215. Phone (800) 334-5551. Fax (800) 222-7112.

[3]Carolina Biological Supply Company is the sole distributor (#B-3-L1162, $9.95). These cultures arrive by mail as late-stage pupae, and generally contain 75-150 individuals, including

two to six males. If you do not plan to rear your own cultures, you may need to order as many as six cultures to have enough males for this activity.

[4]For tips on establishing cultures from nature, see Matthews et al., 1996 or visit:
http://entomology.ent.uga.edu/wowbugs/

[5]Order one culture plus blow fly puparia (#B3-17-3480; $9.70/100; Carolina Biological Supply) to arrive four to five weeks before the activity if you plan to use it as a starter to rear your own cultures (See Appendix B for techniques).

[6]See Figure 19.5 for construction procedure.

[7]Available from bait or aquarium shops, or order from Carolina Biological Supply (see above) or other biological supply houses. Extras can be kept in a closed jar in the refrigerator almost indefinitely.

[8]For techniques on obtaining and using these hosts, refer to Appendix B and the videotapes *WOWBug Rearing and Maintenance* and *Organ Pipe Mud Dauber Biology* ($29.95 each) from Riverview Press, PO Box 5955, Athens, Georgia 30604; 800-847-6540.

Appendix B: Instructions For Rearing *Melittobia digitata*

By investing a few moments of time once a month, you can have a continuous culture to provide WOWBugs in abundant quantity whenever you need them. By raising your own, you can also manipulate the normally female-biased sex ratio.

Assemble the materials listed in Appendix A, part three. For these laboratory exercises, you will need at least one predominately female culture, and one or two all-male cultures. It is prudent to set up a few extra cultures, however, because occasionally one will "crash" or will fail to develop due to mold spores. To minimize the chances of mold, wash your hands before handling the cultures and avoid touching the inside of the culture container after it has been disinfected.

Starting cultures a month before your laboratory activity is scheduled will almost always ensure that you have an ample supply of virgin female *Melittobia* adults on hand. However, because insect growth and development is temperature-dependent, it is sensible to start some cultures four to five weeks before the exercise and a few more about five to seven days later.

Clean the culture containers thoroughly by washing the interiors with soap and hot water, rinsing well, and disinfecting with alcohol. Allow them to dry. Then place one or more hosts in each container. The number of offspring will be directly related to the amount of food available. Two blow fly puparia will usually yield about 150 parasites; a single mud dauber larva, being larger, can yield up to 600 or more.

You do not need to open the host. *Melittobia* can chew through its outer covering. However, seeing the end of the white host inside lets you know that your host is fresh and healthy, and later will allow you to check on the progress of your culture visually, so that you will know when the pupae are ready to sort. To open a blow fly puparium or a mud dauber cocoon, remove the very tip of one end with your fingernail by applying gentle pressure. If you see any fluid after puncturing the end, you have damaged the host. Discard it and open a new one. If the visible end is dark or smells bad, it is sick or dead; discard it and use another host.

If you use mud daubers as hosts, you may sometimes find that they have already been parasitized by any of a number of other insect species. (For identifications, see Matthews, 1997, and the videotape, *Organ Pipe Mud Dauber Biology*). These parasites can include species of *Melittobia* that look superficially like *M. digitata*. Dispose of these hosts and their unidentified parasites to prevent your cultures from becoming contaminated.

Establishing A Prolific Culture That Is 95% Female

Within a few days of adult emergence from the original stock culture, females will have mated and you can remove and use batches of 5-10 of them to begin new cultures. Lift the parasites carefully with a pipe cleaner and tap them into the container with the host(s) without touching the interior with your fingers. This reculturing process will ensure that you have a continually available supply of these insects.

Cap the culture tightly, and store it in a moderately warm part of the laboratory or classroom, away from direct sun or drafts. In about two weeks, you should have numerous new *Melittobia* pupae. If you are simply maintaining the population, no effort is required beyond beginning some new cultures when this generation reaches adulthood.

If you need unmated females for the laboratory exercise, start cultures three to four weeks before the laboratory exercise is scheduled. At the two-week point, if you haven't yet done so, carefully break open the host cocoons to expose the *Melittobia* pupae. As soon as the red eyes that signify female pupae become visible, separate out the number you need. You will be able to obtain a few males from this culture as well; remember, males lack compound eyes, even as pupae.

Establishing A Smaller, Male-Only Culture

Like all Hymenoptera, female *Melittobia* arise from fertilized eggs, but unfertilized eggs produce males. As above, isolate female pupae and let them emerge without allowing any access to males. Place groups of 10-15 females with hosts, and each of these unmated wasps will produce two to five male eggs. Isolate the male pupae in individual deep-well slides or vials before they emerge as adults, to prevent them from fighting with each other and mating with their mothers.

Appendix C: Data Sheets

Melittobia Courtship: Behavioral Components

Trial # ________ **Team # ________**

Courtship Stage *(preliminary, etc.)*	Start/End Time *(seconds)*	Duration *(end time minus start time)*	Behavior *(Describe specifically)*	Count *(# times behavior done)*	By *(sex)*

Melittobia Mate Attraction
Bioassay Results

Team # ________

I. Instantaneous Sampling:

Minute	*Tube A*	*Tube B*	*Tube C*	*Tube D*
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
Totals:				

II. Ad libitum sampling: On the other side of this sheet, write a continuous narrative for 5 minutes of the WOWBugs' behavior in the bioassay chamber.

Appendix D. Sample Results and Responses to Discussion Questions

Sample Results

Exercise A

Behavioral acts performed by both sexes and recognized in initial observations include: touching with antennae, remain motionless, copulating, and preening. Behaviors performed by males included mounting, antenna stroking, hind leg spreading, wing quivering, antenna spreading, backing, and hind leg stretch (just prior to backing). Behaviors exclusive to the female included abdomen tilt, and head tilt to horizontal position, both seen only just prior to copulation.

All students should be able to see the four basic stages of insect courtship, and appreciate the diversity of their duration (Table 19.2) and the stereotypy of their execution.

Table 19.2. Typical averages for a group of 20 unmated females placed individually in viewing chambers with a male. The large ranges recorded reflect the fact that individual wasps vary greatly in their behavior; note that more wasps began courtship than finished it.

Courtship Stage	Duration (seconds)	No. of Times Observed
Latency	82 (range 5-310)	18
Preliminary courtship	174 (range 1-584)	18
Advanced courtship	87 (range 7-289)	15
Copulation	6 (range 3-10)	10

In the typical laboratory session summarized in Table 19.2, a quarter (25%) of the females that were tested failed to mate within the eight-minute time limit allowed. Two females (10%) were cannibalized by their partner. Four mated females retested with the same male all failed to mate a second time, although males were very amorous. It is likely that some chemical or mechanical stimulus provided during the first courtship and mating induces a switch in behavior; in nature, mated females exit the host cocoon very soon after copulation and disperse to search for new hosts.

Exercise B

Individual teams will typically tally low numbers for each choice tube of the bioassay chamber. This is because most females will not make any choice before the experiment concludes. However, when the combined class data are tabulated and the average number of females choosing each tube is calculated, a clear preference for the live male (and/or the crushed male essence) is usually evident (Table 19.3).

Table 19.3. Results averaged from 12 bioassay experiments, each using 10 wasps.

	A. Control (Empty)	B. Live male	C. Mated female	D. "Male essence"	Remained in canister
Females sighted over 15 on-the-dot samples	0.08	1.9	0.18	0.35	7.49

The fact that the differences between the summed totals for each choice tube are relatively small in numerical terms reflects the real world more realistically than might be supposed. Across many fields of science, data often show comparatively small numerical differences between groups. However, when the data are subjected to appropriate statistical analyses, the differences often prove to be significant statistically. Depending on the level of your students, you may wish to suggest analyzing the sample results statistically using a Chi-squared test, which is quite straightforward.

Responses to discussion questions

Exercise A

1. The black female WOWBug has compound eyes, typical wasp-like antennae, and normal wings. The caramel-brown male WOWBug has tiny wings, and lacks compound eyes; each antler-like antenna bears a conspicuous "digit" (see Figure 19.4).

    The extreme development of the male antennae is an example of sexual dimorphism. Recalling that *Melittobia* mate in the dark, students should be able to postulate that the different shapes of male and female antennae help the sexes to recognize one another. Undoubtedly, the male gives tactile stimuli when he repeatedly touches the female's antennae with his own. He also touches her antennae with the thumb-like antenna "digit" that gives this species its name. Such a tactile stimulus would be different from that experienced by the female when being touched by the antennae of another female or a male of another species.

    Students often remember that male deer fight with their antlers and, knowing that male *Melittobia* are aggressive toward one another, propose that the bizarre antennae are for male combat. You may wish to encourage some or all of them to investigate this possibility first-hand, by way of a "Darwinian Selection" activity (Matthews et al. 1996). They will be rewarded with some exciting displays of male aggression, but will confirm that males don't joust with their antennae.

    Interestingly, pheromone glands have been found in the male antennae (Dahms, 1984), and may be involved in the mutual antenna-touching that is readily observable during courtship. The chemical differs from the attractant chemical studied in the bioassay chamber. The latter appears to come from glands near the base of the abdomen (Gonzalez, Matthews, and Matthews, 1985). Behaviorally, this is suggested by the fact that usually a female initially contacts a male by approaching from the side and touching his abdomen with her antennae.

2. Answers will vary, but advanced courtship will generally last between 50 and 120 seconds. Latency and preliminary courtship phases will vary the most. Copulation is the briefest, usually 3-10 seconds and often so short it is difficult to time. (See sample results in Table 19.2.)

   A major selective advantage of long and variable latency and preliminary courtship stages is that they allow individuals to assess their potential partner in some detail before rushing into a sexual encounter. For any animal species, sexual encounters include such potential risks as putting oneself into a vulnerable state, possible risk of injury, or at the least, wasting time and energy. It pays for individuals to assess their potential partner's sexual status, species, vigor, genetic competency, etc. as best they are able before becoming heavily involved.

3. Advanced courtship should show the most reciprocal interaction. By the time the wasps enter the advanced courtship stage, numerous reciprocal acts give either participant a final opportunity to "just say no" and decamp. This is illustrated in the trials with the mated female as compared to unmated ones.

4. The same components will generally occur in every successful courtship and in the same sequence (Figure 19.5). The sequence will thus be more similar than the number of repeats of individual behaviors.

Exercise B

1. Covering the bioassay chamber removes the variable of light as a possible confounding factor in the experiment. *Melittobia* females tend to move toward light and lighting conditions in most classrooms are not uniform. In fact, current evidence is that female *M. digitata* become positively phototactic only after mating, a behavior which helps them leave to find new hosts. However, well- controlled studies of phototactic behavior in unmated females have yet to be done.

2. Since attraction, courtship, and mating all occur within the darkness of the host cocoon, vision would not be expected to be important. Furthermore, males lack compound eyes.

3. Tube A, the control — tells whether females are choosing the spokes randomly or not. Tube B, a live male — if most females come here, they may be attracted by smell (a pheromone or a respiratory byproduct), or by an inaudible (to us) sound he produces, or by some movement they can detect even through the barrier. It is unlikely that females can see the male, or that vision plays a role in attraction, given that they court in the dark. Tube C, a mated female — If movement or presence of a live respiring insect is all that is required for attraction, one would expect equal numbers of unmated females to choose tubes B and C. Tube D contains "essence of male" — If most of the females come here, it can only be odor which attracts them, since "dead males don't sing."

   Although data will vary, students should be able to conclude that male *M. digitata* possess a sex attractant pheromone (see sample results in Table 19.3). Usually the greatest response is to tube B, followed by tube D. It often turns out that the "essence" from a dead male is more attractive than a living male. This can be quite puzzling to students. Scientists hypothesize that rupturing the source gland frees the pheromone in abundant quantities. No longer being released in controlled amounts, it acts as a "supercue."

One of many possible further experiments would be to attempt to isolate the source of the pheromone production by cutting apart males and testing different body parts (see Gonzalez, Matthews and Matthews, 1985).

4. Without marking individual wasps, one cannot be sure whether one has been seen more than once. However, it should be possible to note the amount of movement by ad libitum sampling. Sometimes the first spoke chosen by a female seems to become a focus for other females to follow. Each female may be independently responding to a "super" male signal. Alternatively, the first to arrive may produce a signal which attracts the subsequent females, and with each additional female the intensity of this aggregate signal increases. Different experimental procedures would be needed to distinguish between these hypotheses.

5. Because male *M. digitata* are highly aggressive toward one another, they would probably spend the time fighting instead of responding to female cues. Furthermore, such an experiment would be illogical, for students' reaction chains show that the female initiates the preliminary courtship. Finally, males move slowly compared to females, so most would probably not arrive at the spokes of the bioassay chamber during the time allotted.

   Although bioassays with multiple males in the canister are not to be recommended, tests of female attractiveness to males can be conducted as an additional class project. Each team should place one male in a bioassay chamber armed with such variables as a living female and "female essence" produced as before. The test must be run for an hour or more. Class results can then be pooled.