Protocols for Rearing Fall Webworm (*Hyphantria cunea*) in a Colony and Basic Methods for Laboratory and Field Experiments

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Abstract

Standardized protocols are an essential asset for research requiring the maintenance of live organisms. Ecological studies often involve collaborations between multiple teams that are spread across locations, and these collaborations benefit from sharing successful laboratory procedures. Our research team is studying the ecology of the fall webworm moth (*Hyphantria cunea*, hereafter FW) in North America for >10 years, during which time we have established reliable procedures for starting and maintaining FW colonies under laboratory conditions. FW is a North American species that has been introduced to Europe and Asia where it is a major pest. Here, we present a detailed review of the methods we use to find and collect FW caterpillars in the field, house and rear caterpillars in the laboratory, handle pupae, and initiate diapause for overwintering. We also describe how to end diapause the following summer, care for emerging adult moths and mate them, and tend to eggs. Lastly, we test the effectiveness of some of our protocols related to mating adult moths to determine whether fertile eggs are produced. FW is becoming a model study system for ecological and evolutionary studies related to diet breadth. As more researchers begin studying the ecology and management of FW, laboratory colonies will play an important role for these projects. Our protocols will provide guidance to inform the successful study of this important insect.

Keywords: colony maintenance, immune function, insect herbivore, Lepidoptera, natural enemies

1 INTRODUCTION

Herbivorous insects are one of the most diverse groups of organisms, accounting for nearly half of all terrestrial animal species[1;2]. Most insect herbivores have a narrow diet breadth and feed on only a few host plant species, whereas very few herbivorous species are generalists with a broad diet breadth that feed on many plants[3]. How herbivorous insects have diversified to become so incredibly specious and why so many of them have narrow diets are two key ecological questions. Indeed, specialization to specific host plants has been proposed to drive diversification[4]. A newly emerging model study system for ecological and evolutionary studies related to diet breadth is the fall webworm (*Hyphantria cunea*, hereafter FW[5;6;7]). Additionally, FW has become an agricultural pest in areas of Europe and Asia where it was introduced[8;9], so an understanding of life history and diet breadth is also necessary for biological control. As more researchers begin studying the ecology and management of FW, laboratory colonies will play an important role for these projects, and standardized protocols will be essential for the maintenance of FW colonies. Thus, we present a standardized protocol that we use to rear FW in Colorado.

FW is a broad-ranging North American moth species and has one of the broadest diet breadths of any insect herbivore on Earth, feeding on >400 plant species over its geographic range[9;10]. However, in some places such as Colorado, populations sometimes have a relatively narrow diet breadth and feed on few of the available host plants[5;11;12]. There are two morphotypes of FW that differ in their diet breadth, natural history, behavior, web architecture, geographic distribution, and ge-

netic makeup. These types are named by the color of the head-capsule of the caterpillar: black-headed and red-headed. In Colorado, we only observe the red-headed FW. When the two FW types occur in the same location, such as in the eastern United States, the black-headed FW can have two generations per summer and emerge earlier and later in the season than the red-headed FW, which usually has just one generation per summer; however, timing can vary greatly across geographic regions [10;13]. After FW caterpillars have completed their development, they pupate in the soil or trunk crevices and overwinter as pupae.

To properly comprehend how diet breadth evolves and how invasive generalist species could potentially be controlled, it is important to understand their dynamics with natural enemies. Fall webworm caterpillars can be heavily attacked by parasitoids, which act as important top-down controls of their populations [5;12;14]. Top-down controls can be especially important for the evolution of generalist herbivores, as they tend to be more heavily affected by top-down than bottom-up effects from their host plants [15]. Host plant use has also been shown to affect the immunological function of insect herbivores, which can affect their response to top-down controls [16;17;18;19;20]. As such, protocols to test the immune response of insect herbivores to parasitoids as well as identifying common parasitoid species that attack these herbivores will be necessary.

Ecological studies often involve collaborations between multiple teams that are spread across locations, and these collaborations benefit from sharing successful laboratory procedures. Our research team has been studying the ecology of FW in North America for >10 years, during which time we have established reliable procedures for starting and maintaining FW colonies under laboratory conditions and for conducting field research. Here, we present a detailed review of the methods used to find and collect FW caterpillars in the field, house and rear caterpillars in the laboratory, handle pupae, and initiate diapause to overwinter individual pupae. We also describe how to end diapause the following summer, care for emerging adult moths, mate adults, and tend to resultant eggs. Additionally, we include our protocols to assay immune function of caterpillars, identify the most common parasitoid taxa that attack FW, and store immatures for morphological studies. Lastly, we test the effectiveness of some of our protocols related to mating adult moths and determining the optimal container size for successful mating. Our protocols will provide guidance to inform the successful study of this important insect.

2 PROTOCOL METHODS AND EFFICACY

We successfully use these methods to rear FW in the laboratory, find them in the field, and conduct both lab and field experiments. Our research experiences with FW are in Colorado, but our methods likely apply to populations elsewhere. Small details in our protocols may need to vary from one geographic area to another to follow environmental conditions of different locations. In particular, the light regime that we use for rearing caterpillars to induce diapause will need to follow the day length of each place the researchers are maintaining the caterpillars. Some variables might be different in different geographic locations, but the overall protocols we present here are general enough to be applicable to a variety of locations. We start by describing our laboratory protocols and then our protocols for field research.

2.1 Laboratory Protocols

2.1.1 Diapause for FW Pupae

In late October or early November, we put lab-reared pupae in containers with peat moss to overwinter in growth chambers (Percival) at 4°C and 0:24 (light:dark) photoperiod as described in Loewy et al. [11]. We obtain the lab-reared FW pupae from the previous year following the methods explained in this protocol below. We mist the pupae and peat every 6 weeks during the winter to make sure they do not desiccate. In early spring, we start to take the pupae out from the growth chambers so they can emerge from diapause. We usually remove pupae from the chambers every other day and remove about 80 males and 80 females each time, which results in a manageable number of moths emerging at any single time. We remove the pupae from peat and place them in new clean containers (0.5*l*) with a moistened filter paper; some containers can have multiple pupae. We keep track of which date the pupae were removed from the growth chamber and we line them up on a lab bench from earliest removal to latest removal so that we can estimate when the FW moths will begin to emerge for each group. We also organize the containers by geographic location and sex, which helps to select appropriate mating pairs. It is important to keep the pupae moist, so we spray them with water if the containers look dry. We conduct daily checks of every pupal container to see if any moths have emerged. In this process, we check both the bottom and top of the filter paper because moths may blend with the white background. When the adults emerge (Figure 1A and 1B), we record the females and males that emerged on that day (Figure 2A).

2.1.2 Maintaining Mating Chambers

We pair adult moths for breeding depending on their emergence date within each geographic location, and no siblings are paired together to avoid inbreeding. After removing any newly emerged moths from the pupal container, we also remove their chrysalis so that it does

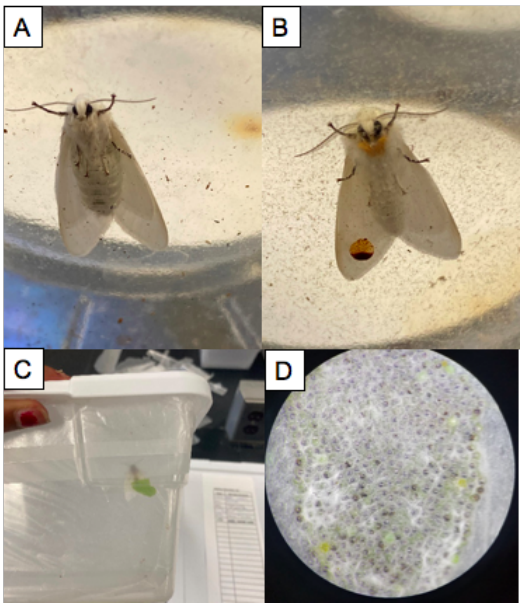

Figure 1. Images of FW depicting how to identify the sex of adult moths. **A)** Female moth with a broader abdomen and more filamentous antennae; **B)** Male moth with a thinner abdomen and more plumose antennae; **C)** Female moth inside a mating container in the process of laying eggs (green); **D)** Eggs right before they are about to hatch when the caterpillar head capsule becomes visible.

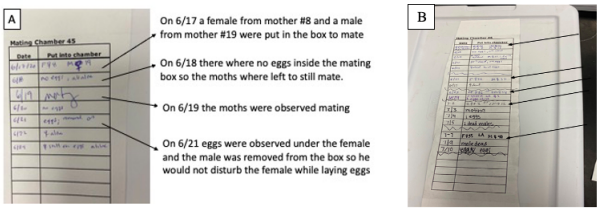

Figure 2. Mating container labels for FW. **A)** How to read the mating container lids and what information should be recorded; **B)** A label for a container that has been used multiple times for different pairs of moths; the arrows indicate each time a new pair was put into the container.

not interfere with any later emerging moths and we again moisten the filter paper if it is dry. Adult moths, especially males, can fly quickly, so care is taken when removing moths from containers so as not to lose them; we keep an insect net in the lab in case moths need to be retrieved from the ceiling.

To mate FW, we use plastic shoebox containers and line the entire container with wax paper, including the lid; it is best to wrap as much of the container as possible with a single piece of wax paper so that moths cannot get stuck under an edge of the wax paper. FW are very sensitive to light and will not mate overnight if the lights are left on, so it is important to turn off all of the lights when not in the vicinity. Once a female and male are chosen for mating, we place them inside the mating container; if we have an abundance of males, sometimes more than one male can be included to increase chances of mating in case females are overly choosy. On each mating container's lid, we record the female and male maternal line identification numbers and the county from which they were collected (Figure 2). The females oviposit on the wax paper (Figure 1C), and we cut around the egg mass and patch the hole with a piece of wax paper and tape. There should not be any holes in the wax paper allowing access to the container's surface; eggs laid directly on the plastic container cannot be retrieved without damage.

When checking the mating containers, it is important to understand what to expect inside before opening it as moths can quickly fly out of the container (Figure 2). First, we look from the outside of the container to see if any egg masses can be seen on the inside of the container, often with a female moth sitting on them (Figure 1C). Then, if necessary, we carefully open the lid to avoid any moths escaping and locate the moths. We then record the following information on the datasheet located on the lid of the mating container: date mating was first observed, date female begins oviposition, if the male has escaped or died and is replaced with another male, and if all moths died in the mating container. If the male is dead in the container but the female is alive and did not lay eggs, we add another male from the same county to give him a chance to mate with the female. When the female has laid eggs, we carefully remove the male to not disturb the female, and record that the male was removed on the lid datasheet. If both moths have died, the container can be cleaned out by removing the dead individuals and wiping out any frass, and is then restocked with new moths (Figure 2B).

As the size of the container could affect moth behavior, we assessed the effect of container size on mating success. We tested 3 container sizes: small deli container ($0.5l$, $n = 34$), medium deli container ($2l$, $n = 21$), and a large plastic shoebox ($6l$, $n = 162$). We included a total of 217 containers and used a Chi-squared test in JMP Pro version 14 to compare the percentage of fertilized eggs produced by successfully mated females housed in the three different container sizes. We found no significant effect of container size on mating success, estimated as the proportion of fertile eggs produced ($\chi^2 = 1.56$, $df = 2$, $p = 0.46$; Figure 3). This may be due to an unbalanced design in which we had 162 large containers yet only 34 small and 21 medium containers. Although differences among treatments were not statistically significant, we found that large and medium containers produced 50% more fertile eggs than small containers, which is biologically relevant for rearing efforts.

2.1.3 Monitoring Eggs

We monitor the mating chambers daily to look for the presence of eggs (Figure 1C). Females are often found with eggs they have laid, and we allow females to re-

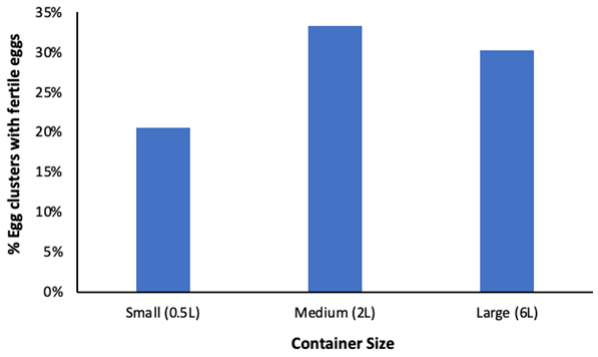

Figure 3. Percentage of egg clusters with fertile eggs depending on the size of the container in which the adult moths were mated.

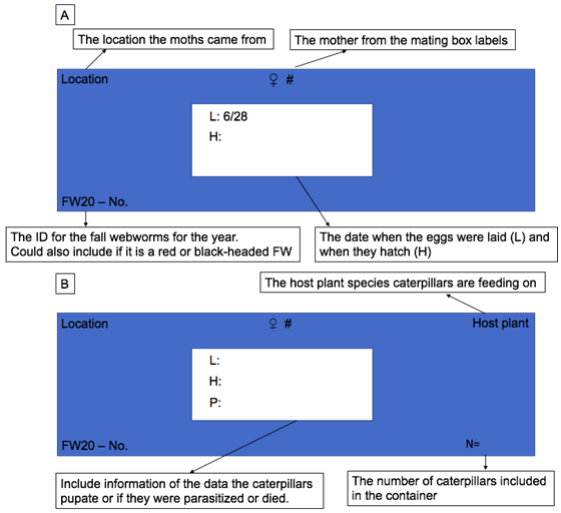

Figure 4. Example of how to make a label for a caterpillar container on lab tape that goes on the lid of the container. **A)** The label when only the eggs are in the container and includes a date when the eggs were laid (L) by the female moth; **B)** The label after the eggs have hatched and that now includes the date the eggs hatched (H) and in the future the date that the caterpillar pupates (P). Label colors differ among host plants such that all caterpillars on a single host have the same color label (e.g., choke cherry is always pink).

main on their eggs for up to 3 days after eggs are first observed. Once the female dies or she has been on her eggs for over 3 days, we remove the egg cluster from the mating chamber by cutting the wax paper lining the container with an X-acto knife and transfer it to a 0.5*l* container using forceps to avoid damaging the egg cluster. We then assign each egg cluster a new container with a label including a unique identification number and information about its provenance (Figure 4A). Before putting the lid on the container, we spray a mist of water into the air, then scoop the container upwards into the mist to get a small amount of moisture into the container; we repeat this egg misting every day. We store the eggs in chronological order to be able to anticipate when head capsules would be visible (Figure 1D), which indicates that the eggs will hatch soon. The eggs of red-headed FW take about 14 days to hatch[11]. We check eggs underneath a dissecting microscope to determine whether the dark coloration was head capsules emerging or if the eggs are starting to decay. When the eggs are decaying, they will often become concave or shrivel, and develop a yellowish color. We dispose of any egg clusters that are dead.

Once an egg cluster has visible head capsules (Figure 1D), we can split it using Castro-Viejo scissors if this is required for the experiment. We frequently split egg clusters into 4 equal parts in order to rear a single maternal line on four different host plant species to test for factorial differences in genetic lineages. We place the egg masses in 0.5*l* containers with a host plant leaf and a moistened filter paper disc to maintain the correct humidity (Figure 5A and 5B). Laying the leaf flat on the bottom of the container allows us to carefully place the piece of egg cluster flat on the leaf, which helps to ensure the cluster will not fall off the leaf accidentally. It is important for the hatching eggs to be immediately near host plant foliage because wild FW emerge from the eggs directly onto a leaf, so we try to replicate this in the lab.

We use different colors of laboratory tape to be able to easily identify different experimental conditions (e.g., host plant species) and organize containers so that we can feed all of the caterpillars from a single host plant species at once. Each label has the following information: the date the egg clusters were split, the date they hatched, host plant species, the identification number of the maternal line, and county or the word 'lab' if lab reared from our colony (Figure 4A).

2.1.4 Maintaining FW Caterpillars

As the FW caterpillars emerge, we give them leaves with small portions of the branch attached as a food source. When adding leaves to the containers, we check the leaves thoroughly for other animals, such as predators (e.g., spiders, hemipterans, etc.). We collect plants from the field as branches and store them in vases filled with tap water. We cover the tops of vases with plastic bags and place them in the fridge to reduce wilting of the leaves. The water in the vases is checked daily and water should be replaced before the leaves start to desiccate.

From the time of emergence until day 10, the caterpillars remain together in a 0.5*l* (16 *oz*) deli container with filter paper placed in the bottom. In each of these containers, we place a few leaves to supply an ample food source for the small FW and minimize caterpillar handling at this sensitive stage (Figure 4B). We check the containers thoroughly twice a week with spot checks in between to ensure the FW caterpillars always have enough fresh foliage to eat. During the thorough feedings, we inspect the containers for mold on the leaves or

excessive moisture on the sides. If there is any mold, we carefully remove caterpillars from the molding leaves using soft forceps and place them back into the container with fresh foliage. If there is an excess of condensation on the sides of the container, we wipe it off using paper towels to reduce the chances of leaves molding. FW often skeletonize leaves, so we remove leaves without any green parts remaining and replace the filter paper if it has mold or is falling apart. We clean the containers of frass during the thorough cleanings, at least once per week. While it is important to ensure the FW caterpillars have enough food, it is also critical that the containers are not overstuffed so they have room to move.

At 10 days old, FW caterpillars are large enough to be moved easily. Using soft tweezers or our fingers (pulling on webbing, not the caterpillars), we transfer the caterpillars to other containers and divide them into smaller groups; by the time the FW caterpillars are 3 weeks old there should never be more than 5 caterpillars in a container. Depending on the experiment, we either split caterpillar groups up into smaller groups and keep them in $0.5l$ containers or move them to larger $1l$ (32 oz) containers. Each container needs to have a moist filter paper on the bottom to keep the humidity level appropriate and keep the plants from desiccating. We place new leaves with portions of the branches attached (to help avoid desiccation) in each of the larger containers. As caterpillars are split into new containers, we duplicate labels using the same tape color to ensure all original information is maintained through the subdivision process (Figure 4B); as caterpillars grow, new information will be added to the label and as containers are split, the number of caterpillars in each container should be noted in the lower left corner (Figure 4B). All of the information on the labels is also recorded in a logbook that contains a row of information for each FW caterpillar that is reared in the lab; our logbooks often have thousands of entries. While the logbook is a paper record of our data used in the lab, we also scan it frequently and make digital copies for permanent records. FW caterpillars are similar to the moths in that they are sensitive to lights; if lights are left on in the lab overnight, they receive a photoperiod cue that tells them to not enter diapause and it is therefore extremely important to turn off the lights in the laboratory every evening.

When the caterpillars pupate, they form a cocoon with their body hairs around where they will form their pupa. Ten days after pupation, we remove the pupa from their cocoon and determine the sex of each pupa based on Loewy et al.[11]. We then place them in peat in a $0.5l$ container (Figure 5C) and spray the container with water. If individual information is not necessary for future experiments, we can include more than one pupa in the same container, making sure that they are from the same maternal line, fed on the same host plant, and of the same sex. If more than one pupa is included in a container, we write the number of pupae on the container, and their individuals' log numbers. These pupae will be placed into diapause as explained above.

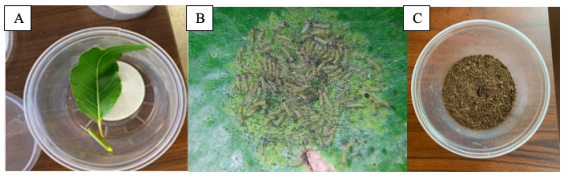

Figure 5. Different stages of the FW life cycle housed in deli containers. **A)** The deli container with leaf and moistened filter paper right before eggs are put into it; **B)** FW caterpillars in container; **C)** The pupal stage in peat before being misted with water and put into a growth chamber set for winter conditions.

2.1.5 Immunological Protocols

Immunological protocols are important tools to understand caterpillar response to parasitoid attack. We use two main methods for assessing the immune function of FW caterpillars: nylon filaments and hemocyte counts. We insert filaments into the interstitial body cavity, between the cuticle (skin) and intestinal tract, and remove them 24 hours later to measure the proportion of the filament that has been melanized by the caterpillar's immune system. High levels of melanization are associated with a strong immune response[20;21]. Counting the hemocytes gives us an additional measure of immune response; an abundance of hemocytes indicates an increased immune function.

Before a filament can be inserted into a FW caterpillar, the caterpillar must be large enough to survive the filament insertion process; caterpillars typically reach this body size approximately 25 days after hatching. Host plant identity can affect the development time and thus the length of time necessary before the caterpillar reaches a large enough size. Using sandpaper, we sand monofilament fishing line to create grooves along the filaments, tie knots every $3mm$, and cut the line into pieces that are 3mm in length with the knot off-center. This way, each line has a knot surrounded by two ends (Figure 6A), one short, that we call the "head," and one long that we call the "tail;" if both ends are equal in length (Figure 6A, top) the filament should not be used because it cannot be inserted deep enough into the caterpillar's body for the assay. A range of filament diameters is necessary if caterpillars of different sizes are being tested. We use smaller diameters (e.g., $0.1mm$ or $0.15mm$) for smaller caterpillars and larger diameters (e.g., $0.2mm$) for larger caterpillars. To ensure there are enough filaments for an experiment when caterpillars reach the appropriate size, we begin tying filaments in early spring, before any caterpillars are reared; there

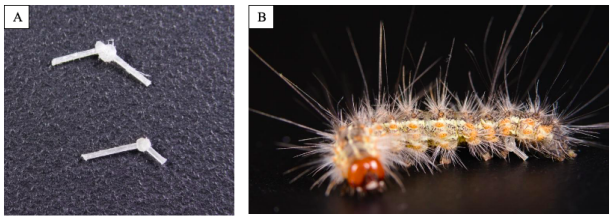

Figure 6. Examples of 0.2*mm* filaments. **A)** Filament that is not cut well and cannot be used because both ends are equal in length and there is no long end to insert into a caterpillar (top), and filament that is cut well with a shorter "head" end and a longer "tail" end that can be inserted into the caterpillar (bottom); **B)** A filament inserted into a FW caterpillar.

can never be too many filaments, so we always make as many as possible.

To insert a filament, we first remove a caterpillar from its container using soft forceps and restrain it under the microscope using wax paper. The wax paper can be laid perpendicular on the caterpillar to minimize the caterpillar's ability to move during insertion. The caterpillar is restrained on its side so that the filament is properly inserted. Once the caterpillar is restrained, we pierce it with an insect pin (size 1 or 0) next to its penultimate prolegs; we always use the same spot to ensure that we remember where the hole is in which to insert the filament (Figure 6B). Using forceps, we pick up the filament by the head end and insert the tail end of the filament into the caterpillar through the hole made by the insect pin. The knot and head of the filament should be external and visible outside of the caterpillar (Figure 6B). We use care because if the filament is inserted too harshly the caterpillar will not survive. We insert filaments at an angle that is parallel with the length of the caterpillar; perpendicular insertions risk rupturing the gut, which will contaminate the hemocyte sample. We then place the caterpillars with inserted filaments into separate, labeled Eppendorf tubes to be certain they are not mixed up with the rest of the colony and also to make sure no other caterpillar jostles the filament out of the focal caterpillar. We do not provide any plant food as the small piece of cut plant material desiccates in the Eppendorf tubes; caterpillars are able to survive without food for 24 hours. We secure the caterpillar inside the tube using cotton instead of the tube's lid as the cotton allows air flow.

After 24 hours, we first check for movement to make sure the caterpillars survived. Using forceps, we take the caterpillar from the tube and place it under the microscope; wax paper can again be used to restrain movement. We use forceps to grab the "head" of the filament above the knot and gently pull the filament from the caterpillar; this movement should be done smoothly to avoid harming the caterpillar. Once the filament is extracted, we dry it, if necessary, by laying a KimWipe flat on a table and placing the filament on top for 1-2 seconds. We then place the filament back into the Eppendorf tube to be frozen until we have time to measure percent melanization, which can be many months later; the tube should be labeled on the top of the cap and side of the tube with the caterpillar identification number. To measure how much of the filament was melanized while inside the caterpillar, we first photograph each filament on a sterilized watch glass under 40X magnification on a compound microscope. We use the image processing program Fiji[22] to quantify both the part of the filament that is melanized and the total area of the filament so that we can get the proportion of filament that is melanized. The sample section that we use (1.5*mm* x 0.17*mm*) is centered in the filament and helps to avoid shadows created during the photographic process.

In addition to filaments as a method for assessing immune function of FW, we also count the number of hemocytes in a sample of hemolymph to better understand the immune response; an increased number of hemocytes indicates a greater immune response. Before inserting the filament into a caterpillar, hemolymph can be extracted and used for hemocyte counts. First, a tub of ice is necessary to keep materials cold, a beaker of anticoagulant solution placed in the ice, a 10-microliter micropipette, and an Eppendorf tube; these items are chilled for about 30 minutes. Using the micropipette, we place 4 microliters of anticoagulant solution into an Eppendorf tube and place the Eppendorf tube in the ice to keep it cold. After piercing the caterpillar with the insect pin as explained above, a bubble of hemolymph will emerge from the hole. We use a micropipette to extract 2 microliters of hemolymph from the caterpillar and add it to the same Eppendorf tube with the anticoagulant solution, for a total of 6 microliters of solution. To mix the hemolymph and solution, we set the micropipette to 6 microliters and draw and expel the hemolymph and anticoagulant solution into the tube multiple times. Once the solution is mixed, we use the micropipette to transfer the liquid to a hemocytometer in order to count the hemocytes under magnification; we use a compound microscope at 40X. We count both granulocytes and plasmatocytes; granulocytes are a more rounded shape while plasmatocytes resemble an oval shape with pointed ends. Both granulocytes and plasmatocytes are important for cellular immunity and are involved in immune function. After counting, we record the number of hemocytes on a data sheet.

2.1.6 Caterpillar Blanching for Morphological Studies

A common way of preserving immature specimens for permanent collections is to freeze them overnight and subsequently store them in 80 or 95% ethanol. While this is a humane and effective way of preserving caterpillars, it results in shrinkage, which is problematic

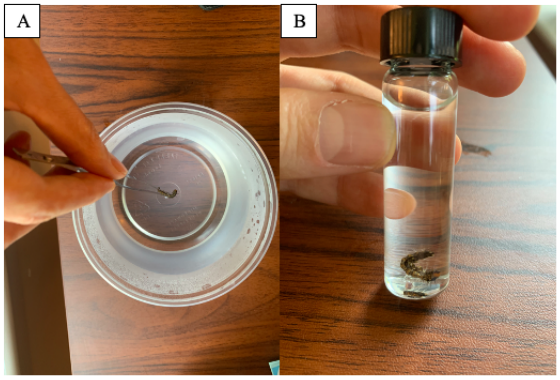

Figure 7. Caterpillar blanching. **A)** Submerging the FW caterpillar in hot water; **B)** Storing the blanched caterpillar in a glass vial with 95% ethanol.

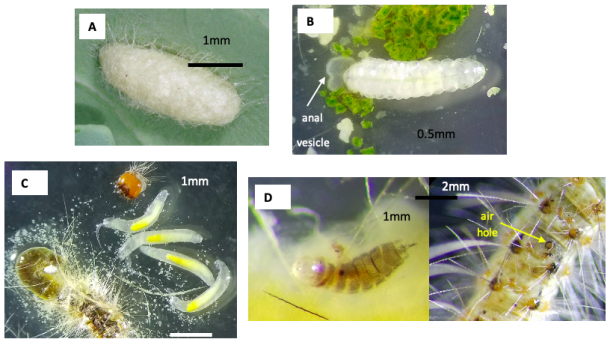

Figure 8. Distinguishing characteristics of four families of parasitic insects that attack FW. **A)** The white cocoon; **B)** Immature larva of braconid wasps; **C)** Yellow stomach contents of immature ichneumonid wasps; **D)** Speckled cuticle and black mouthparts of immature tachinid flies (left); tachinid fly larvae also create air holes that are visible on the outer skin of fall webworm caterpillars (right).

when conducting morphological studies and when designing permanent displays. To preserve a caterpillar's original shape, it must die while being dipped in near boiling water. To do this, we boil water, remove it from the heat source and submerge caterpillars in the water for at least 60 seconds (Figure 7A). Water from standard hot water dispensers is typically over 90°C and can be used for blanching. Because both water temperature and duration of immersion can affect the rate of post-mortem decomposition and larval volume[23], we recommend consistency in both of these variables when conducting comparative studies. Moreover, it is important to dip the caterpillars in enough water so they die instantly; 200ml will suffice for FW but 400ml may be needed for species of larger size. After hot water exposure, caterpillars can be stored in 80-95% ethanol (Figure 7B). To minimize stress, it is important to fully submerge caterpillars in the water; fuzzy caterpillars tend to float, so we use forceps to push and submerge them individually. We avoid pinching or squeezing the caterpillar because pressure during blanching alters their shape. Debris (e.g., FW hairs, feces) can accumulate in the ethanol, so we replace the ethanol about a week after blanching and subsequently if needed to keep the caterpillar submerged.

Blanched caterpillars can also be preserved in small glass containers filled with gel hand sanitizer. We use this technique when designing displays for outreach and to facilitate observation under the microscope. However, we do not recommend this method for long-term storage of research specimens because it leads to degradation of the larval tissue over time.

2.1.7 Identifying FW Parasitoids in the Laboratory

FW are attacked by numerous parasitic flies and wasps (i.e., parasitoids) that target FW eggs, caterpillars, and pupae[24;25;26]. Adult parasitoids lay their eggs inside or on the FW eggs or caterpillars, which are then consumed by the immature parasitoid. We suspect that at least one parasitoid attacks FW eggs and emerges from the FW caterpillar to form a white cocoon on or next to the caterpillar host (Figure 8A). Field-collected FW can be either unparasitized or parasitized, but parasitism status is often unknown for field-collected FW. We observe most of our parasitoids after they have emerged from the caterpillar as adults, but we can also observe them inside a FW caterpillar as larvae if we dissect the caterpillars under a microscope.

We begin dissections by placing the caterpillar on its ventrum on a clear base, such as a glass petri dish. Next, we decapitate the caterpillar immediately behind the head. Once the head is removed, we add 1-2 drops of water where the head used to attach to the body. We hold the caterpillar's posterior end with forceps and place the dissecting scissors across the dorsum above the anus. We then move the scissors towards the anterior end while gently pressing down to squeeze out the internal contents. Internal contents will begin to flow out, beginning with the foregut, into the water droplets. We remove the remainder of the internal contents by restraining the posterior end with scissors while pulling the foregut with forceps. We then cut open the empty skin so that the internal cuticle is fully visible, and inspect the water and cuticle for immature endoparasitoids.

We identified three taxonomic categories of endoparasitoids that attack FW: braconids (Hymenoptera), ichneumonids (Hymenoptera), and tachinids (Diptera). Each taxonomic category has distinct features that help with identification. Braconid offspring are distinguished by their translucent bodies that are segmented (Figure 8B). Young braconids have mandibles and a pointed caudal "spike." As they grow, braconids develop a round structure called the anal vesicle (Figure 8B). Ichneumonid offspring have a slender body with

a yellow-colored gut and a long tail (Figure 8C). Tachinid offspring have black spines on their cuticle and the mouthpart is a black pointed structure that often moves back-and-forth (Figure 8D). Tachinids are often attached to the inside of the FW cuticle and create an airhole that is visible on the outside of the FW skin (Figure 8D). Once parasitoids are identified, we discard the endoparasitoid and the data are stored in record books. We preserve adult parasitoids as dry specimens in display boxes.

2.2 Field Protocols

2.2.1 Field Transects

As FW becomes a model system to understand diet breadth evolution of insect herbivores, a major part of studying FW involves quantifying the diet breadth of each population in the field. To determine which plant species are available and used by FW, we use a transect method to assess relative abundance of plant species. First, we identify a focal host plant that contains a FW web in areas where we know they occur (e.g., iNaturalist or historical records) or by haphazard sighting. We measure the host plant diameter at breast height (DBH) using a soft meter tape or diameter tape. We measure the diameter by wrapping the tape around the tree trunk at a height of $1.35m$. If the tree has multiple trunks, we measure the diameter below where the trunks separate. When the host plant is a shrub, we note it as "shrub" on our data sheets and do not record a DBH. In addition to recording the species identity of the host plant, we also record the sampling date, the number of webs found on the host plant, the location (both GPS coordinates and town/county), as well as other relevant notes, such as any information that can help locate this transect and any information relevant to FW studies (e.g., presence of spiders and other predators in the FW web).

FW are almost always found along the edge of a habitat, whether it be a forest fragment, riparian corridor, property line, utility corridor, roadway, or neighborhood sidewalk. Thus, we set our transects along the habitat edge where they occur and record the abundance of host plants that would be available to FW along this transect. On each side of the focal host plant, we use a $10m$ transect tape and set it on the ground parallel to the habitat edge; we align the zero mark of the transect tape with the focal host plant and then record transect information for two transects, one to the left of the focal host plant and one to the right of the focal host plant (for a total $20m$ transect). For each transect, starting from the focal host plant moving toward the $10m$ mark, we annotate every plant species that we find, within a $2m$ area perpendicular to the transect tape; thus, the transect area on each side of the host plant encompasses $20m^2$. For each plant species along the transect, we record the identity of the plant species, its linear distance from the host plant in meters (noted from the transect tape), and if this plant has any FW webs on it. If the plant species is not known to us, we use a unique identifier to mark it in the data sheet (e.g., unknown 1), and collect a voucher that can be identified by a specialist later. This plant voucher should preferably have reproductive organs (flower or fruit), should be pressed as soon as it is collected, and should have a label with the same identifier used in the transect data sheet along with sample location data. We only include plant species in our transect data that are known to be used by FW and do not record any plants that are known to not be used by FW such as grasses, gymnosperms, forbs[12].

2.2.2 Deploying Caterpillars in the Field

When choosing a field site for FW deployment for experiments to test bottom-up and top-down effects in the field, it is important to avoid placing FW near heavily trafficked areas like parking lots, trailheads, and trails. Many times, the caterpillars we deploy into the field are hidden enough in the host plant that people will not notice them, but it is better to avoid any intentional or unintentional field sabotage by people recreating near the field sites.

Deployment strategies vary slightly depending on whether it is FW eggs or webs that are being placed into the field. In order to fasten egg clusters onto host plants, we place the eggs facing outwards, so the wax paper is flush against the underside of the leaf, and eggs are not sandwiched in between the wax paper and leaf. It is important to attach the eggs on the underside of the leaf as this is where FW females often lay their eggs and also so that the eggs do not desiccate in the sun. We use metal hair clips (Sally Beauty Supply) to fasten the wax paper containing the egg cluster to the leaf, being careful that the hair clip does not touch or crush any eggs, but also making sure that the wax paper is held firmly in place on the leaf. We individually number the metal hair clips using white lab tape and a medium point Sharpie so that we can follow and monitor individual clips easily in the field. We use one or two clips to anchor the wax paper with an egg cluster to the host plant, often with one clip on each end of the wax paper along the longitudinal axis of the leaf (parallel to the major leaf vein). We fasten the webs to the host plant in a similar way (Figure 9A), but we attach the edge of the web to the petiole of the leaf, which is stronger than the leaf and can support the weight of a web. To do this, we clip the entire web that the FW caterpillars created in the lab (including any leaves they may currently be feeding upon) to the leaf petiole so that we disturb the FW caterpillars as little as possible. If the webs are large, we often use several hair clips and attach them to both the petiole and the tree branch to ensure the web is

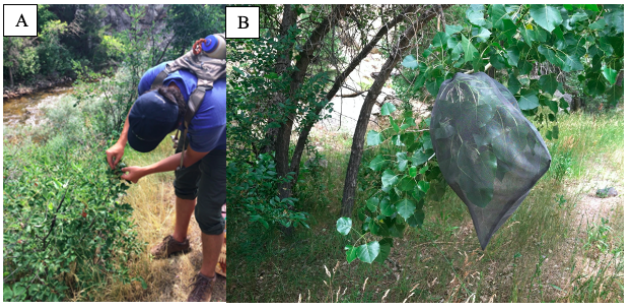

Figure 9. FW caterpillars deployed in the field for field experiments. **A)** A student attaching a FW web to a choke cherry plant with a hairclip; **B)** A mesh net bag on a broadleaf cottonwood with FW caterpillars housed inside that are protected from predators and parasitoids. The net bag is tied onto the branch tightly so caterpillars cannot escape.

secure on the host plant. For some experiments, we deploy FW caterpillars into the field and need to exclude parasitoids and predators so that we can measure the effects of host plant alone without the effect of natural enemies. We use net bags made of green netting (7 holes per *cm*; Barre Army Navy Store, Barre, VT) and secured with a nylon drawstring (Figure 9B).

We deploy FW eggs and webs in the field trying to mimic how they would be found in nature. For instance, FW webs are never found at the bottom of a plant and thus we place ours at about shoulder height or above, because this is how they are typically found in the field. Often FW webs in some canyons are found on the south-facing slope, so we try to deploy our webs similarly. FW webs are usually found on outer branches of the host plant; thus, we fasten our eggs and webs onto the leaves close to the very end of the branch, but some large webs have to be tucked further into the tree where there are thicker branches to support their extra weight.

It is important to take rigorous field notes when deploying FW so we can find them again in the field, and also so that we can communicate with others about web location. We always record GPS coordinates, but also note landmarks near the webs (e.g., a distinctive rock or street signs). We also sometimes take pictures of the FW in the field to better locate the branch where they were deployed. In addition to information to find them in the field, the notes also include which colonies were deployed, how many caterpillars were deployed in each web (these can be counted in the lab and marked on their deli container to save time in the field), the dates and locations for each deployment, and the dates on which they were monitored. It is important to monitor deployed FW webs at least every other day if not every day because FW sometimes move their webs short distances; these short distances can be tracked if FW webs are closely monitored, but if are left on their own for a few days it is easy to lose track of the webs that we have transferred. If a FW web moves, the individually numbered hair clip should also be moved with it, and we record that it moved in our field notes. On the day that FW eggs and webs are collected from the field, we put each one in its own individually-labeled deli container with moist filter paper and host plant leaves from its host in the field. Once the FW are safely back in the lab, we count how many caterpillars were recovered so that we can calculate percent survival.

3 DISCUSSION

Recently FW have emerged as a model study system for ecological and evolutionary studies related to diet breadth. When rearing FW in a colony it is very important to have a system of organization and a consistent set of protocols. We have learned over the past 10 years through a series of trial and error which methods lead to the highest survival of FW in our colony. We have learned that it is essential to have consistent labeling and data management systems so that everyone in the lab knows which caterpillars are being used for different experiments and how to feed and care for them. Unfortunately, we have also suffered through many errors in the past and our protocols presented here will help other labs to avoid the same pitfalls. For instance, we have found that if FW females are allowed to lay eggs on the plastic container with no wax paper, then it is almost impossible to remove these eggs without damaging them, and when the eggs hatch it is hard for them to find food which leads to starvation. We have also learned how sensitive FW are to ambient light conditions. For adult moths to mate, they need to be exposed to twilight and they cannot be left in a room with the lights on all of the time. Similarly, FW caterpillars are equally sensitive to lights being left on in the lab and if they receive this extended photoperiod cue, they will not enter diapause and will instead emerge as adults in autumn when it is too late to complete another generation before the leaves fall.

We found no statistical differences in the percentage of successful matings that led to fertile eggs across the three container sizes. However, while the differences were not statistically significant, there was a trend where moths that were mated in medium and large containers had 50% more fertile egg clusters compared to moths mated in the small containers. Thus, although our results are not statistically different, this is a good example of a situation in which biological relevance still matters. When rearing FW, every successful mating helps keep the colony going and using containers that result in 50% more fertile eggs is important when so much time, effort, and money is invested into rearing. Thus, we recommend that researchers invest their resources wisely and avoid the use of small containers for mating.

Our field protocols have been used by us and other

researchers for many years. Besides studies from our own research team in Colorado[5;12], the transect method to assess the plants available for FW was used by Mason and collaborators[27] on the east coast. The deployment of caterpillars in the wild to measure predation and parasitism has also been used for other systems[28], and we have also previously tested with FW (unpublished data). Although some of our protocols have recently been used for FW by our research group, we have adapted many of these methods from other systems and they are general methods used in entomological studies. The immunological methods are based on Carper et al.[29], but we have optimized these methods for the last few years. Thus, there is ample evidence that our protocols are generalized methods that work well for FW.

Our findings create opportunities for future FW researchers to improve and standardize their research, as well as propose ideas that refine our methods. As FW becomes more common as a model organism for ecological research, standardized rearing protocols reduce variables that cause disagreement between studies. For example, using different mating containers can affect the probability of reproductive success, which can have subsequent impact on experiments relying on fertilized FW eggs that yield caterpillars. Our results will benefit researchers that rely on manipulating numerous aspects of FW biology, including separating siblings, performing crosses between maternal lines, and conducting reciprocal transplants of FW between geographic locations.

An important trade-off in ecological work is found in the amount of time and effort spent performing detailed observations and experiments in a given locality compared to saving time and effort by using coarse methods to evaluate processes at the regional level. Increasing collaboration among research groups from different institutions allows for the expansion of detailed measurements across larger areas, facilitating a deeper understanding of regional processes. Notably, to compare experimental data gathered by multiple people in different groups requires methodological standardization. Detailed protocols like the one we present here are thus an indispensable tool to enable collaboration.

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5 EDITOR'S NOTES

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