Protocols for maintaining a colony of *Manduca sexta* (tobacco hornworm) suitable for studies of innate immunity and other investigations of a model insect species

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Abstract

Manduca sexta, the tobacco hornworm, is a lepidopteran species often used as a model organism for biological research. It reaches a large size (~10 g) as a last instar larva, useful for biochemical studies, and its development can be synchronized for studies of endocrinology and developmental regulation. We describe here our detailed protocols for rearing a continuous colony of *M. sexta* in the laboratory. We feed larvae an artificial diet based on wheat germ and rear larvae at 26°C in individual plastic cups with clean procedures to produce larvae suitable for studies of immune responses. Wandering larvae at the end of larval development are placed in plastic boxes containing vermiculite for pupation, and then pupae are transferred to clean plastic boxes in the incubator. Prior to adult emergence, pupae are placed in a cage with a tobacco plant. Moths mate and oviposit on the leaves, and eggs are collected for continuation of the colony.

Introduction

Manduca sexta, the tobacco hornworm (Order Sphingidae), is used by many research groups as a model species for research in insect biology. This lepidopteran insect is useful in experimental research because its large size benefits biochemical and anatomic studies, and it is possible to synchronize development of cohorts of larvae for investigations of development and hormone functions. It has become over the last 50 years an important experimental organism for studies of neurobiology and behavior (Heinbockel et al., 2013; Martin et al., 2011; Fandino et al., 2019), development, endocrinology and metamorphosis (Nijhout and Williams, 1974; Gilbert et al., 2002; Hiruma and Riddiford, 2010; Nijhout et al., 2014; Truman et al., 2006; Truman and Riddiford, 2007; Wilmsen et al., 2023), plant/insect interactions (Schuman et al., 2015; DeFino et al., 2024), biochemistry and physiology (Canavoso et al., 2001; Wieczorek et al., 2003; Windfelter et al. 2024), mechanisms of *Bacillus thuringiensis* Cry toxins (Soberon et al., 2010), and immunity and host-parasite interactions (Kanost and Nardi, 2010; Amaya et al., 2005; Chevignon et al., 2015; Windfelter et al., 2022; Shan et al., 2023; von Bredow et al. 2023; Miao et al., 2024). In addition its preference to feed on plants from Solanaceae that contain chemicals toxic to many insects provides opportunities to investigate mechanisms of tolerance or detoxification of plant xenobiotic defenses (Kanost et al, 2017).

A protocol for continuous rearing of the tobacco hornworm (then named *Protoparce sexta*) was described by Waldbauer et al., (1964), and large scale rearing methods were described by Yamamoto (1969). Reinecke et al., (1980) used time-lapse cinematography to describe growth, development, and behavior of laboratory-reared *M. sexta* larvae. There have been additional papers describing methods for rearing *M. sexta* (Hussa and Goodrich-Blair, 2012; Spencer et al., 2024). Most laboratories rearing *M. sexta* appear to use methods stemming from a detailed description by Bell and Joachim (1976). Our methods are also based in part on the procedures described in that paper, but with modifications. We have cultured *M. sexta* in our laboratory for 33 years, beginning with *M. sexta* eggs from Carolina Biological Supply, with occasional supplementation of eggs from Carolina Biological.

General rearing information

The protocols described here are for maintaining a colony (30 larvae/week). The number of larvae reared can be scaled up as needed for use of insects in experiments. The cost of supplies for rearing to the pupal stage by our methods (excluding labor and overhead) is estimated at $1.50 each. We maintain our colony with work of a paid undergraduate student, working approximately 8 hours/week. When lab members need larvae for experiments, this protocol provides enough eggs to rear hundreds of additional larvae/week, and typically the researcher cares for the experimental larvae, using the same protocols described below.

We use an artificial diet that is based on wheat germ, casein, and vitamins, with agar as a solidifying agent (recipe below). We pour this diet into glass cake pans and store covered at 4°C (for up to approximately three weeks). To feed larvae, we use a lab spatula to cut pieces of diet of the desired size for different larval stages and place the diet pieces into plastic cups. In recent years (after methyl bromide was banned for stored grain fumigation in USA), it appears that most commercial wheat germ in the USA is from wheat that was treated with methoprene or some other juvenile hormone analog, as a grain protectant. Larvae fed diet made with this wheat germ develop normally until the fifth instar, but never wander or pupate, eventually having watery feces, and die. Methoprene is not certified for use as organic, so wheat germ labeled "organic" should not have been exposed to JH analogs; however, it has become increasingly difficult to find sources of organic wheat germ. We currently purchase raw wheat germ from Frontier Agricultural Sciences.

We have not had problems with fungal contamination, perhaps because of inclusion of methyl paraben in the diet, but occasionally there has been bacterial growth on the diet. Because our experiments involve immune responses of the larvae, we need insects that have had minimal microbial exposure, so that they are

immunologically “naïve.” Therefore, we typically include antibiotics in the diet, currently tetracycline and kanamycin. We have also used ampicillin in the past, but have had problems with ampicillin-resistant bacteria. Our experiments often measure responses to injected dead bacteria or to microbial cell wall materials. For experiments involving challenge by live bacteria, it may be necessary to use diet lacking antibiotics, to avoid interference with the infection results.

Eggs are collected from tobacco leaves from a plant that has been placed in a cage with moths overnight. The eggs are decontaminated by a brief treatment with diluted bleach and then placed in a small plastic container, which is placed in an incubator. We keep the incubator at 26°C with a light cycle of 16 h on and 8 h off. This long day length prevents the pupae from entering diapause. If humidity is low, a sponge placed in a plastic box containing water can be kept in the incubator to increase humidity. After two days, pieces of artificial diet are added to the cup with eggs, for hatchling larvae to feed on. Newly hatched larvae are transferred to a small piece of diet in a small plastic cup (two larvae/cup). After three days, larvae are separated to one per cup, with fresh diet. We maintain the larvae individually for the rest of larval development, because they tend to bite each other, a potential source of infection through wounds. Frass is removed, and fresh diet is added every two days. On the day larvae molt to the fifth instar, they are transferred with fresh diet to a larger plastic container. Fifth instar larvae are checked each day, frass is removed and fresh diet is added to the cup. When larvae stop feeding and enter the wandering stage, they are rinsed briefly in tap water and placed in groups into plastic boxes containing vermiculite and kept at 26°C. The larvae burrow into the vermiculite and pupate. After the pupal molt, the tanned pupae are transferred to plastic food storage boxes and kept in the 26°C incubator for approximately 20 days. When pupal cuticle is softened and darkened before adult emergence, the pupae are transferred to the floor of the adult cage. The cage includes a tobacco plant for oviposition and a source of sucrose solution for adult feeding. Moths emerge and are able to fly and mate in the cage, and females deposit eggs on the tobacco leaves.

Manduca sexta Rearing Protocols

For the following procedures, we use two sizes of plastic deli containers. The smaller cups are 1.25 oz. and air exchange is achieved by poking three holes in each lid with an 18 gauge needle. The larger cups are 8 oz, and air exchange is accomplished by poking about 10 holes in each cup with an 18 gauge needle (for eggs) or by drilling two holes in each cup with a drill (for fifth instar larvae).

EGGS:

Moths in the cage will deposit eggs mostly on the undersides of the tobacco leaves during the night. Collect eggs during the day when the moths are not active. On days that eggs need to be collected (Monday, Thursday, and Saturday for the colony maintenance protocol), roll eggs off of tobacco leaves with fingers, and collect into a small strainer.

Make sure to remove all of the eggs from the plant, the pot and saucer, and the soil (because missed eggs will hatch and larvae will begin to eat the leaves). If the egg batch is large, discard extra eggs. (With too many eggs, the cup will be too crowded when the larvae hatch.) Note that with the current schedule, eggs are removed from the plant on Wednesdays and discarded; therefore, the Thursday egg batch will have eggs from only one night. On Mondays and Saturdays, the collected egg batches will contain eggs from two nights.

Eggs are next soaked briefly in bleach solution as a decontamination step to kill external viruses or bacteria. Transfer about 100-200 eggs into a small cup and add about 10 ml 3.5% bleach (30 mL Clorox bleach in 820 mL deionized water). Dip the strainer in a container of 3.5% Clorox to disinfect it (to prevent re-contamination of eggs).


Swirl eggs in bleach solution to break up clumps of eggs to expose all egg surfaces to the bleach solution. After about 1 minute, pour eggs into the disinfected strainer and rinse well with tap water. Blot excess water from eggs by placing strainer on a paper towel. Disinfect the paint brush with 70% ethanol and wipe dry. Put a ~2 cm square of filter paper on the lid to a small cup and put the eggs on the filter paper with the paint brush.



Use an 18 guage needle to poke about 10 holes in a large cup. Place the lid with eggs in it into the cup with no food. Add a lid and put the eggs in the incubator. After 2 days, add pieces of diet to the cup. Eggs will hatch in about 4 days. (Artificial diet is not added until Day 2 so it will not dry out before the eggs hatch.) The egg cup is used as the source of hatchlings on Day 4, and is discarded on Day 5.

Put another plant in the cage (after checking it for eggs).

EGG/PLANT SCHEDULE:

Monday	Remove plant with eggs from cage and replace with another plant. **Collect** eggs.
Tuesday	Remove any missed eggs from used plants.
Wednesday	Remove plant with eggs from cage and replace with another plant. Discard eggs.
Thursday	Remove plant with eggs from cage and replace with another plant. **Collect** eggs.
Friday	Remove any missed eggs from used plants.
Saturday	Remove plant with eggs from cage and replace with another plant. **Collect** eggs.
Sunday	Remove any missed eggs from used plants.

FEEDING STAGE LARVAE:

For examples of how much diet to use, we keep clay models in the shape and size of diet pieces we typically use to feed different larval stages.

Pick up just-hatched larvae by the horn with flexible forceps from the Day 4 egg cup. Choose pale larvae, which are newly hatched (not green larvae, which are more than a day old). Pull gently until the larva lets go of the surface. Put two larvae per diet cube in a small cup. Poke 3 holes in lid with an 18 gauge needle. Place the cups in a tray, attach a record strip, and record the date. These hatchlings are called Day 0 larvae. Rear larvae at 26ºC. (Currently we are keeping 10 larvae three times a week, on Monday, Wednesday, and Friday.)



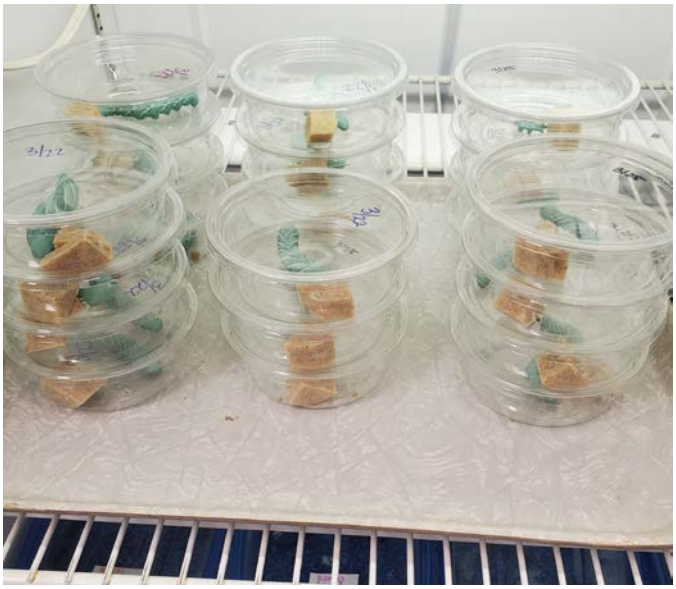


Three days later, separate the larvae so that there is one per small cup. Put one larva in a new cup with new diet, and put the other larva back in original cup with fresh diet. Poke holes in the new lids. (These are Day 3 larvae.) Record the date.


Tap out frass and replace diet cube every 2 days (Day 5, 7, 9, 11). Check all larvae each day, even if it is not their normal day to be fed, and if the diet is dry or there's not enough to last another day, replace the diet. Freeze any larvae/cups that contain fungi, bacteria, or dead or sick larvae. Record the date each time they are fed.

About 10-11 days after hatching, larvae molt from the 4th to 5th instar. On the day that the 5th instar larva has finished molting, put it in a large cup (with 2 holes drilled in the sides) with a diet cube. To tell if a larva is in its 5th instar, compare its head capsule to a definite 5th instar larva (e.g., one already in a large cup in the incubator). If it is in the 5th instar, its head capsule will be the same size as the large 5th instar larvae, even though its body may be much smaller. On days 10 and following, check each larva every day to see if it has reached the 5th instar. Put the date on the lids of the large cups, and stack cups from the same day. Fifth instar larvae eat a lot, so every day tap out any remaining diet and frass, and add fresh diet.

4th instar on left (small head capsule)
5th instar on the right (large head capsule)



NOTES:
1. The larvae should never run out of food.
2. Put each larva back in its own cup with its own lid (to reduce disease transmission).

WANDERING STAGE LARVAE:

After about 4 days in the 5th instar, larvae stop eating and enter the wandering stage and begin to trample their food and frass, and the pumping dorsal vessel will be visible. This signifies the end of the feeding stage, and the larvae are preparing to pupate.

Rinse wandering larvae under a gentle stream of cool tap water. Lay them on a paper towel and then place then in a plastic container with vermiculite. Make sure that the lid, with several holes drilled in it, is on tight, date the container, and put it in the incubator.

PUPAE:

After about 6 days, larvae will pupate. Move brown pupae to a plastic container lined with a paper towel, and place in the incubator. (Don't pick up green pupae since they are still soft and delicate.) When a container becomes full, start a new container. Record the start and end dates on the containers of pupae.

After about 3 weeks, pupae will start to turn very dark and soft. Check the two oldest boxes of pupae daily for pupae that are ready to eclose as adults. Place them in a rack in the moth cage.

ADULTS:

Pupae nearing eclosion are placed in the moth cage, with a tobacco plant for collecting eggs, and a feeder for 10% sucrose solution as a food source for the moths. The cage we use is 66 cm tall, 53 cm wide, and 53 cm deep. The cage is kept in a chemical fume hood to decrease the spread of moth scales into the air of the insectory (The dimensions for our cage were designed to fit in a particular fume hood. The cage could be larger if desired.) The top, back, and two sides are made of metal screen. The bottom and the door are plexiglass. The edges of the box are made of metal support strips. The plexiglass door is attached with two hinges and closes with a magnetic latch. We place absorbant bench paper on the floor and the three walls of the cage. To the side of the cage is a fluorescent light, which is set on a timer to come on at about 6:00 a.m. and off at 6:00 p.m each day. A separate light with a 15 watt incandescent bulb is left on all the time and provides dim light at night that facilitates mating and oviposition on the plant.

We clean the adult cage Monday, Wednesday, and Friday. Remove dead adults, dead pupae, pupal casings, and bench paper. Put these in a plastic bag and freeze. A hand held vacuum cleaner is used to vacuum up eggs (on cage floor, ceiling, and on walls), scales, etc. Fresh bench paper is placed on the floor of the cage. Then place the plant, pupae, and sucrose feeder back in the cage. Clean the door of the cage by wiping it with a damp paper towel.

When cleaning the cage, also disinfect the sucrose feeder by rinsing the feeder and lid well, soaking the items in bleach solution for at least 5 minutes, and rinsing well with tap water. Fill the sucrose feeder with 10% sucrose, screw on cap with hole, and add a rolled-up 11 cm filter paper. Note: Make 10% sucrose on Fridays by dissolving 20 g of sucrose in 190 ml purified water, and store in the refrigerator for up to one week.

GENERAL NOTES:

- Throw out cups and lids after use.
- Freeze all eggs,larvae, pupae, and adults that will be discarded. Immediately freeze any larvae or pupae that look sick or dead, or if they are very small compared to other larvae of the same age. Freeze escaped larvae since they may have been exposed to pathogens. Freeze any larvae with watery feces. Larvae that have any areas that are black have probably been damaged and should be frozen. Each day the frozen items from the previous day should be discarded in the trash can.
- Lights in the incubator are set to be on for 16 h (6:00 am-10:00 pm). Try to limit the amount of light that larvae receive during the dark cycle (10:00 pm to 6:00 am).
- Check the temperature of insect incubators daily.
- Check the light bulbs in the insect incubators daily.
- Do not soak metal baskets in bleach solution for long periods or the bleach will damage the metal.

The insectary and all items used in insect culture must be kept clean!!

- Wipe down bench with 70% ethanol when you are done working.
- Rinse forceps and diet spatulas with water when you are finished with them. Disinfect with 70% ethanol before use.
- Put away clean dishes when they are dry.
- Wipe down the sink area daily with water and a paper towel. Otherwise scales and eggs will accumulate (and hatch) in this area.
- If you spill sucrose solution, wipe it up immediately and then rinse off the area with water. Otherwise the area stays sticky and collects dirt.
- All incubators should be checked at least once per month, and wiped out as needed.
- The insectary should be kept clean by dusting, sweeping and mopping as needed.

Critical steps for avoiding disease transmission

- Properly surface sterilizing eggs and avoiding re-infecting the eggs (e.g., with the strainer) after the bleach treatment.
- Put each larva back in its original cup, with its original lid.
- Keep the forceps (or your fingers, whichever you use) clean to avoid spreading feces from one larval cup to the next.
- Keeping the diet spatula free of feces.
- Freezing any sick larvae as soon as they are noticed.
- Disinfect everything that is re-used with bleach (pupae boxes/lids/etc), and do not reuse cups or lids.
- Make sure that the larvae never run out of food.
- Move 5th instar larvae to large cups within 24 hrs.
- Make sure bleach solutions are fresh (no more than one week old).

Bleach solutions: Make fresh bleach solutions each week. Discard old bleach solutions by rinsing them down the drain with a lot of water.

1. Bleach for tub. 1:50 dilution: pour ~140 ml bleach into ~7 liters tap water (~3 inches of water).
2. Bleach for Manduca eggs and egg strainer. 3.5% Clorox bleach: Mix 30 ml Clorox bleach with 820 ml deionized water. Keep some in a glass bottle (for disinfecting eggs) and put the rest into the plastic container for disinfecting the strainer.

Dry Diet Mix for *Manduca sexta* Larvae

5.67 kg (12.5 lb.) Raw Wheat Germ	Frontier Agricultural Sciences
1.13 kg (2.5 lb.) Casein	Frontier, Product # 1100
340g (0.75 lb.) Wesson Salt Mix	Frontier, Product # F8680
500g Vanderzant Vitamin Mix	Frontier, Product # F8045
100g Ascorbic Acid	Frontier, Product # 6015
50g Sorbic Acid (free acid) (2,4-hexadienoic acid)	Fisher/Acros, Product # AC12053-0010
50g Methyl Paraben (Methyl 4-hydroxybenzoate)	Sigma H5501
12.5 g Vitamin A Acetate (Retinyl Acetate)	Sigma # R3250-100G

Directions for Making Dry Diet Mix:
Weigh the above ingredients into a very large tub. Mix thoroughly.
Weigh out 670 grams of dry diet mix into Ziploc bags.
Label bags with the date and the weight (670 grams).
Store in the cold room until use.

The above recipe will make about 11 bags of dry diet mix; each bag will make 2 pans of prepared Manduca diet (each pan approx. 9x13").

Manduca Diet Preparation

Each batch makes two glass pans (approx. 9 x 13").
Prepared diet can be stored in refrigerator for about 3 weeks.

Equipment:
Metal pan (stock pot) with lid
Wooden spoon
Glass pans with lids
Disposable weigh dishes
2000 ml graduated cylinder
1000 ml graduated cylinder
Weigh scale
Hot plate
Gloves or hot pads

Ingredients:
Dry Manduca diet mix
Agar
Kanamycin
Tetracycline
Tap water

Instructions:
Weigh 40 grams of agar.
Add 2 liters of cool tap water to large metal stock pot.
Set heat to high setting, add agar to the water, and stir with a large wooden spoon.
While the water and agar mixture is heating, stir frequently to avoid scorching.

When the agar solution begins to boil, the agar should be completely melted.
Turn off the hot plate and remove pan from heat.

Immediately add 670 grams of dry diet mix. Stir to mix.
Add 550 ml of tap water. Stir to mix thoroughly.

(Optional addition of antibiotics: Wait 10 minutes before adding antibiotics. Antibiotics can become less effective if added when the food is too hot. Add 0.75 g each of kanamycin and tetracycline. Stir to mix completely.)

Pour into two glass pans. Let cool before covering with lid. Store at 4°C.

Manduca Colony Check List

	Mon	Tues	Wed	Thurs	Fri	Sat	Sun
Date:							
Wipe forceps and diet spatula with ethanol **before** use							
Throw out Manduca related items in freezer							
Check temperature in both incubators							
Check light bulbs in both incubators and hood							
Check timer settings							
Add water to humidity box in colony incubator and the other incubator if there are any insects in it							
Make fresh bleach solutions (in tub and for eggs)		X	X	X	X	X	X
Make 200 ml sucrose solution (20 g sucrose + 190 ml ddH2O), add dated label and store in refrigerator	X	X	X	X		X	X
Discard sucrose from previous Friday	X	X	X	X		X	X
Clean moth cage		X		X		X	X
Put fresh sucrose solution in moth cage		X		X		X	X
Put plant with no eggs in cage		X			X		X
Collect eggs (**thoroughly**!): don't forget to check soil; discard extra eggs to avoid hatchling crowding		X	collect & freeze		X		X
Remove any eggs/larvae from recently used plants							
Put food in 2 day old egg cup, freeze 5 day old cup							
Feed 10 just-hatched larvae from 4 day old egg cup (2 larvae per cup)		X		X		X	X
Separate Day 3 larvae to 1 larva/cup with new food		X	X		X		X
Check larvae 9 days and older for 5th instars and put them in large cups with food							
Feed 5, 7, 9 and 11 day old larvae							
Check other 1st → 4th instar larvae: feed if needed							
Feed non-wandering 5th instars in large cups							
Wash wandering larvae, put in vermiculite							
Move new pupae to correct box in the incubator							
Put dark pupae in cage							
Fertilize plants		X	X	X	X	X	X
Water all plants with damp or dry soil							
Wash insectary dishes							
Wash off forceps and diet spatula with water							
Wipe down counter with disinfectant							
Stock insectary with cups/lids, paper towels, 70% ethanol, Kimwipes, etc.	X	X	X		X	X	X

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Appendix: Growing tobacco plants for *M. sexta* oviposition

TOBACCO PLANTS:

Plants are grown under fluorescent lights positioned about two feet from the table surface. The lights are kept on 24 hours per day.

Planting seeds: Fill a pot with potting mix and soak with water and let drain. Sprinkle several seeds onto the top of the wet soil. (We use Tobacco Plant Seeds Green:Albino 3:1, from Carolina Biological.) Put plastic wrap over the pot, secured with a rubber band, and poke a few holes in it. Place pots under lights. The seeds will germinate in 4-5 days. Use a spray bottle of water to keep the top of the soil moist. Wait a couple of weeks to transplant the tobacco seedlings into their own pots.

Transplant seedlings into separate pots: In about 2 weeks, transplant the seedlings into separate plastic pots. Fill pot with potting mix to the top and soak with water (potting mix will pack down). Using a small spatula, make a hole in the center of the of the potting mix (1 in. deep and wide). Use the spatula to gently loosen the mix around seedling, lift seedling and carefully place in the hole. Pack down the potting mix carefully around the seedling. Place the pots under lights. Water as needed. (The transplants may wilt even if the potting mix is damp, but they will recover after new root growth.)

Maintenance of plants: Tobacco plants need to be pinched back, trimmed, and cut back to maintain the right size to use in the cage. Trim off leaves that start to develop from nodes, lower leaves that are close to the pot, and unneeded leaves that start to form at the growing tip. Trim off yellowing and overly large leaves. Plants can be cut back to just a few inches if they get too large. Water plants with cold tap water as needed. Drain excess water before placing the pots back in their saucers. When the leaves of the plant sustain significant damage from being used for egg laying, the plant is cut off just above a node about 2 inches above the soil line. Usually the plant will send out new leaves and can be cut back many times before the stem becomes too woody and has to be discarded. Use yellow sticky traps to reduce the white fly and fungus gnat population.

Fertilizing: Fertilize plants once per week with soluble fertilizer (e.g., Miracle Grow dissolved in tap water).