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Rearing Tobacco Budworm and Bollworm for Host Plant Resistance Research

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Contents

Abstract

Research to develop cotton germplasm resistant to tobacco budworm, *Heliothis virescens* (Fab.), and cotton bollworm, *Helicoverpa zea* (Boddie), requires insects in large numbers and of uniform age. Natural populations world be desirable, but, because of variability in densities of natural populations, these requirements are best met by rearing insects in the laboratory while maintaining genetic diversity representative of natural field populations of insects. Over the past several years, we have developed procedures to rear these two species. Moths are housed in large cages in a separate facility from larvae. Procedures for the rearing operations are described with detailed explanations of adult handling and egg production, collection, and hatching into first-stage larvae. The techniques are time- and cost-efficient compared to the best published systems. One worker can handle up to 14 cages, each with 1,800 to 2,000 adults, in a safe, clean, scale-free work environment. Using a mechanical egg harvester, the same worker collected an average of 376,580 tobacco budworm and 618,806 bollworm eggs per day during July 1995. These were hatched to first-stage larvae and used to infest plants within field plots. Larvae are reared to pupae in a separate facility using semi-automated techniques.

Introduction

Research in breeding plants for resistance to insects utilizes field plot and laboratory techniques, which require uniform infestations with larvae of specific ages and numbers. Natural infestation of these insects cannot be relied upon to produce timely and uniform infestation of plants within field plots for evaluating germplasm. Even when insects are naturally present in field plots, they are often not uniform in distribution and density. This requirement can best be met by laboratory rearing of large numbers of insects that adequately represent the genetic diversity of field populations.

Cotton, *Gossypium hirsutum* L., is an economically important crop. Tobacco budworm, *Heliothis virescens* (Fab), and cotton bollworm, *Helicoverpa zea* (Boddie), are major economic pests of cotton. An important, effective, and economical way to control these pests is to develop varieties of cotton with genetic resistance to these pests.

Scientists in the United States Department of Agriculture, Agricultural Research Service, Cotton Host Plant Resistance Research Unit of the Crop Science Research Laboratory at Mississippi State University annually evaluate cotton breeding lines for resistance to tobacco budworm or bollworm. Over the years, procedures for screening these lines in the field by exposing them to these insects under artificial infestation required that we

rear these Helothines in large numbers for field and laboratory use.

The procedures that have been developed to rear large numbers of tabacco budworm and bollworm for field infestation are described in this technical bulletin. They should be useful to other plant resistance programs and entomological research that requires large numbers of insects for field testing.

Adults to Neonate Larvae

House for Moths

Adults moths are housed in a separate building from the larvae and pupae. This building, which is referred to as the moth house, is a room 7 by 6.3 meters with controlled temperature and humidity (Figure 1). The room is housed inside a metal quonset building. Temperature is maintained at 27 ±2 °C and humidity remains above 60% (Parrott et al., 1986). Six light banks on each side of the room are arranged and mounted on the walls to form stalls surrounding each cage. One 15-watt light is mounted on either side and on top of each cage. Light intensity of these lamps is kept to a slight glow at night. These light sources are used to simulate dusk or moonlight, attracting the moths to oviposition screens, which form the sides of the cages. An even distribution of eggs results.

An independent scale collecting unit is installed on two sides of the moth house (Davis and Jenkins, 1995). Each scale collecting unit has six scale-collecting prefilters (one per cage) installed in the lower portions of each set of light stalls (Figure 1).

As the adult moths move, particularly during the night, they shed many scales, which exit the cages and are trapped as a thick layer of scales on the filters each day. These scales are removed daily from these prefilters using a vacuum system with the scale-collecting container mounted outside the moth house. This scale-collecting container is emptied and cleaned once or twice a month depending upon the number of moths present in the moth house.

Special environmental and safety precautions are adopted while cleaning the scale-collecting container because inhaling scales can result in serious health problems. The final filter in the system is a Viledon MF90 pocketfilter (Fruedenberg Nonwovens L.P., Chelmsford, MA), which removes more than 95% of the particles 0.5 micron and larger.

This entire system involving the cages and air handling system we call ALERT (for **A**dvanced **L**epidoptera **E**nvironmental **R**earing **T**echnology) is described in detail by Davis and Jenkins (1995). The moth house floor is mopped daily with a germicidal detergent (ProQuat 256, 1-CHEM Laboratories Inc., 6474 Russell, Detriot, MI 48211) as a general disinfectant.

Cages for Moths

Metal cages 64 cm on a side, mounted on legs with rollers that make the cages 145 cm high, are used to house adults (Parrott et al., 1986; Davis el al., 1985) (Figure 2). These cages are fitted on five sides with oviposition screens. Each of these screens is fitted into a frame, which can be slipped into the cage frame to form five sides of the cage.

The top screen is used for feeding, accomplished by placing sponges saturated with sugar water on top of the screen.

The bottom of the cage is an inverted pyramid with a small square door at the apex of the pyramid. This is used to introduce wire-mesh trays containing pupae and as a place to remove dead moths. The inverted pyramid is covered with 317-mm hardware cloth, which allows the scales shed by the moths to exit the cage and become entrapped in the multiple filter system.

Pupal trays are placed in the cages in a manner that leaves space between the cage walls and the pupal tray, allowing dead moths to fall beneath the pupal tray but letting live moths exit pupal cases and move upward into the cubic portion of the cage for feeding and oviposition.

The cage frames are made of 2.54-cm angle iron. Each piece of angle iron on the sides is fitted with a metal spring clip, which holds the oviposition screen tightly to the frame. This prevents moth escape and allows for transfer of oviposition screens without moth escape.

The oviposition screens are replaced daily. A new screen is placed on one side of the cage screen to be replaced and is moved horizontally to take the place of the screen with eggs. Oviposition screens on the sides of the cages are made with metal frames fitted with white organdy cloth, while the top screen is fitted with 14-mesh nylon screen, which allows moths to feed through the top screen.

One of these cages houses 1,500-2,000 mixed sex adult moths. It is not necessary to sort pupae by sex. We place a random sample of pupae in the cage, which should give approximately 50% females.

Feeding of Moths

Proper feeding of moths is essential for maximum egg production. Moths are fed a 10% solution of sucrose. Common synthetic sponges (12 x 7.5 x 2 cm), available from a grocery store, are used to hold the sugar solution and present it to the moths for feeding. Sponges soaked in the sugar solution are placed on top screen of the cages. By trial and error, we determined that four sponges should be used to feed a cage of tabocco budworms and five sponges should be used to feed a cage of bollworms since they are larger adults and require more food.

During the time we have cages at capacity (1,500 to 2,000 moths per cage) and large numbers of eggs are needed, it is important to feed the moths twice a day. The first feeding is about 9:00 a.m. and the second feeding at about 9:00 p.m.

In order to avoid excessive evaporation of the sugar solution, each sponge is covered with a square plastic petri dish slightly larger than the sponge. The lower surfaces of the sponges are on the top screen and the moths feed on the sugar solution through the screen. Sugar solution is prepared daily in one-gallon glass jars. If only a few cages of moths are being reared, this solution can be prepared on alternate days.

Before use, the sponges are washed in hot water, squeezed, then soaked in sugar solution and placed on the screen. Sponges are used continuously for 3 to 4 days after which they must be sterilized in an autoclave for 30 minutes and dried for 25 minutes before reuse. This keeps the sponges clean and helps prevent microbial contamination. When a sponge loses its shape and functional capacity, it is discarded.

Egg Harvesting

Each day, screens with eggs attached are removed from the oviposition cages by inserting a new screen against the existing one and forcing it horizontally through the metal spring clips of the angle iron sides, which moves the egg-laden screen away from the cage and inserts a new screen side on the cage. The screens with eggs attached are then taken to the wash room. The mechanical egg harvester (Parrott and Jenkins, 1992) (Figure 3)consists of a 113-liter nalgene holding tank for sodium hypochlorite solution (0.18%), which is prepared by mixing 0.8 liter of liquid bleach (Clorox 5.25% sodium hypochlorite) in 23 liters of water.

A stainless steel wash tank provides holding racks for 20 oviposition screens (all the screens from four cages). This allows all the eggs from 8,000 moths (four cages) to be collected at one time using the mechanical harvester. The wash solution from the nalgene tank is pumped through five fan spray nozzles mounted on a moveable carriage above the wash tank. The sodium hypochlorite solution dissolves the egg adhesive and releases the eggs from the screens. The wash solution plus the eggs flow out of the collection tank and through a 60-mesh sieve, which collects the eggs. The wash solution is pumped, by a second pump, back into the nalgene tank for reuse.

The rinse cycle uses tap water to rinse the eggs and to remove sodium hypochlorite from the egg surfaces. Eggs are then removed from the collection sieve and washed with water a few times for further cleaning. Clean eggs are then taken to the laboratory for further processing.

To remove the eggs from the screens, they are washed for 1 minute, 45 seconds with sodium hypochlorite, followed by a 2-minute rinse with water. To completely remove all the eggs, screens are rotated 90 degrees and put through a second wash cycle.

Most of the eggs are collected from the first wash cycle. These are removed from the sieve before the start of the second cycle to prevent a second exposure to the sodium hypochorite solution. After the second wash the nalgene tank is drained of the sodium hypochlorite solution and new solution is prepared.

Production

Adults are kept in the cage for 7 days. Each time the cage is emptied of adults and readied for new pupae, it is thoroughly washed and dried. One to two cages are enough to maintain a moderate sized colony. During the months of June, July, and August, our research program requires large numbers of eggs to hatch for field infestations; therefore, the number of cages we maintain is increased to seven for each species, with five of these cages containing males and ovipositing females and two containing pupae and mating adults.

The numbers of eggs collected during June to August of 1995 are shown in Table 1. Mean daily egg production in July 1995 was 376,580 and 618,806 from tobacco budworm and bollworm, respectively. During these months, the number of cages with moths varied by species and week. These numbers illustrate the efficiency of the operation and the large number of eggs and first instar larvae that can be produced with our system. We achieved these egg numbers with one full-time technician handling adults and eggs.

Table 2 shows the typical egg production from a cage of 2,000 tobacco budworm moths. The first night after peak moth emergence, most of the moths are mating. Egg production peaks on the fourth night (not counting the night of peak emergence) and drops sharply thereafter. We normally keep a cage of adults 7 nights after peak emergence.

Egg Processing

Eggs that are allowed to hatch into larvae for use in bioassays with plant material in the laboratory or for placing onto plants in the field do not require surface sterilization. After these eggs are removed from the sieve of the egg harvester, they are rinsed three or four times in water to remove scales and debris. Eggs are then poured into a plastic rinse bottle, which is about three-fourths filled with water, to which a few drops of Tween 80 are added to prevent eggs from clumping.

Circular organdy cloths 36 cm in diameter, stretched onto hoops, are used for drying eggs. Eggs are evenly spread on these cloths, suspended on a rack, and a fan is placed nearby to aid in drying. The organdy cloths are wetted with water before the eggs are placed on them. This aids in spreading the eggs on the cloths. One cloth holds about 50,000 to 70,000 eggs. Placing more than this number of eggs on a cloth causes problems with eggs clumping, which interferes with even distribution of dried eggs in the grits. Eggs are allowed to dry for 3-4 hours before weighing and placing into corn cob grits (20/40-mesh in size). Grits are available from Anderson Cob Grit Company, Maumee, OH.

After drying, the eggs are gently brushed into a stainless steel pan. Eggs are then further concentrated by placing them into wide-mouth plastic cups. An electronic balance is used to weigh out the desired amount of eggs for each container of grits. There are 11,798$\pm$90 tobacco budworm eggs in one gram and 10,929$\pm$109 bollworm eggs in one gram. These data are based upon our counting and weighing 30 samples of 100 eggs.

The proper grits-to-egg ratio for delivering a desired number of larvae through the field inoculator can be calculated using expected egg hatch. We infest plants using the Davis inoculator (BioServ, P.O. Box 450, Frenchtown, NJ 08825) (Davis, 1989) and the techniques of Jenkins et al. (1982).

For example, with a 70% egg hatch and 8-10 larvae desired per stroke of the applicator lever, we place 4.5 grams of tobacco budworm or 5.0 grams of bollworm eggs in 775 grams of moistened cob grits in a 4-liter container. To ensure proper moisture in the grits, which are very dry in storage, we add 75 ml of water to 700 grams grits and mix thoroughly before the eggs are added to the grits. This adds moisture as well as reduces the dust in the grits and greatly improves larval survival.

The moistened grits are spread as a thin layer in an open pan. The eggs are spread evenly over the grits. The entire mixture is then thoroughly mixed and placed into plastic containers and kept at the appropriate temperature for hatching. Development of eggs into first-stage larvae requires 974 degree hours above a threshold of 12.6 °C.

The grit and egg mixture is allowed to stay in the incubator until eggs hatch. Hatching time is controlled by temperature so that hatching is completed between 1:00 a.m. and 7:00 a.m. on the day we expect to inoculate plants in the field. For example, eggs collected on Friday are placed into grits and held in an incubator set at 27 °C, which allows hatching on Monday morning. After the eggs have hatched, the container with the larvae and grits is gently rotated for 3-5 minutes to thoroughly mix the larvae and grits. The mixture of larvae grits is then poured into inoculation bottles available from BioServ. The 775-g mixture is evenly divided between three bottles. Each bottle is then gently rotated to mix the grits and larvae, and five strokes of the inoculator lever are made delivering grits and larvae onto a white surface; the number of larvae in each of the five strokes is counted to calibrate for field use.

Each bottle of grits (250 grams) and larvae will inoculate 220 m of row with one application of grits and larvae per 30-cm row (about every fourth plant). Bottles with the larvae/grits mixture are placed into a plastic cooler, and cool packs are placed on the top of the bottles for transport to the field. We try to inoculate all field plots within 2 hours of calibration of the bottles.

Infestation of cotton plants in the field is a straightforward operation. One stroke of the inoculator lever applies grits and larvae on a turgid leaf, preferably in the terminal. Larval applications are spaced about 30 cm apart. The operator can quickly move down the row while infesting plants. For routine evaluation of cotton lines for resistance, we normally infest plants with tabacco budworm the first week of squaring and each week for the next 4 weeks. From this, we obtain a 40-60% reduction in lint yield in a commercial cultivar of cotton. This represents a very severe infestation of tobacco budworm. The number of times and growth stage at which plants are infested depend upon the specific objectives of the experiment.

Maintenance of Genetic

Diversity in the Colony

To maintain genetic diversity and to maintain a colony that represents field insects, a minimum of 500 virgin laboratory females are mated with a minimum of 250 field-collected males in the fall of each year. Eggs from this cross are collected, and the larvae are reared under diapause-inducing conditions (Philips and Newson, 1966). These larvae are reared at 21.1 °C with a 10:14 L:D photoperiod. All larvae should pupate within 40 days under these conditions. These F_1 pupae are then in disapause.

Pupae are removed from diet, surface-sterilized with 10% Clorox for 3 minutes, rinsed for 15 minutes with cold water, and allowed to air dry. Pupae are placed one per cell into our 32-cell rearing tray and stored at 15.5 °C until the next spring, when the pupae are exposed to diapause breaking conditions.

Progeny from the emerging adults are the source of the new colony for the current year. We store 3,000-4,000 diapausing pupae of each species. In addition, during the winter months, we maintain a small colony of each species that has not been outcrossed. This provides insects for laboratory experiments during the winter and is considered a backup colony for use the next year.

In 1995, we changed these procedures slightly. We grew the F_1 larvae from the matings of laboratory

females with wild males under regular conditions and collected eggs for the second generation. Larvae from these were grown under diapausing conditions, and these pupae were stored to begin the colony for the next year.

In addition, we kept a small colony of the crossed insects as a backup colony rather than keeping the current year colony as the backup colony. We have had no difficulty with storing diapausing pupae of tobacco budworm. However, we have not been as successful in getting pupae of bollworm into a state of diapause that would hold them throughout the winter under our storage conditions. We are continuing to work on this problem with bollworm.

This outcrossing is very important in host plant resistance because we are selecting plants that should be resistant to field populations of the particular insect. Outcrossing of laboratory moths to field-collected moths improves insect vigor, more closely matches the genetic profiles of naturally occurring insects, and is a major aid in achieving this objective.

Precautions

Since both helothine species are reared in the same moth house, precautions are needed to prevent mixing of eggs between species. The following steps are taken in our program to prevent mixing of eggs between species.

1. Care is used to minimize escape of moths from cages of either species in the moth house. If escapes occur, these moths are captured by hand and killed. This prevents egg laying by escaped moths onto the outside of screens on cages of the other species.

2. Oviposition screens used for each species are color coded and kept separate. Screens marked for one species are never used for the other species.

3. Sugar solutions used to feed each species are kept in separate jars. Sponges are soaked in sugar solution and placed on the top screens of each cage. Since moths also oviposit on these, sponges are used only with the specific sugar solution and cages assigned to the specific species.

4. The egg collecting sieve of the mechanical egg harvester is thoroughly cleaned after egg harvest of one species and before it is used with the other species. The tank of the mechanical egg harvester is thoroughly rinsed after harvesting eggs of one species and before harvesting eggs of the other species.

Production of Pupae for Colony Maintenance

Egg Sterilization

Eggs used for colony maintenance are surface-sterilized with formaldehyde (50 ml 37.5% formaldehyde in 120 ml distilled water). Eggs are soaked in this solution for 5 minutes, then washed in distilled water to which a few drops of Tween 80 have been added. The eggs are then spread on filter paper for drying after which the paper is placed in a sterile 500-ml glass jar for hatching. Surface sterilization of eggs with formaldehyde removes fungal and bacterial contaminants from the eggs used to produce larvae for colony maintenance.

At the beginning of 1995, we again saw the same condition. Upon the advice of ARS scientists at Tifton, Georgia, we decided to use only eggs from the first two nights of oviposition to maintain the colony. This procedure was successful in eliminating or minizing the agonadal disease from our colony. Moth health, as measured by egg population in 1995, was very good.

Rearing Pupae for Oviposition Cages

In a separate facility, tobacco budworms and bollworms are reared from neonate larvae to pupae in multicellular, plastic rearing trays. Trays are 15.24 x 27.94 cm 15-mil polyvinyl chloride, containing 32 individual cells of sufficient size to allow normal development of larvae of these two species (Davis et al., 1990).

The trays are sealed on top with a polyester perforated material, which has a heat sensitive adhesive on one side. These lids are sealed with a mechanical lid sealer. The entire system is described in detail by Davis et al. (1990). Trays are available from Dixon Paper Compay (4402 Locust Avenue, Lubbock, TX 79408) or Stephen Gould Corporation (91480 Deerecho Road, Luthervill, MD 21093) and lids and the automatic sealer for lids from Oliver Products Company (445 Sixth St., N.W., Grand Rapids, MI 49504).

Diet

The same diet is used for tobacco budworm and bollworm. The diet is purchased from BioServ (tobacco budworm diet product F9915 dry mix USDA, Vitamin premix USDA product 6265). Agar is USP 100-mesh and is purchased from AEP Colloids (106 Charleston Road, Ballston SPA, NY 12020). Diet is prepared in a 20-liter steam-jacketed Groen kettle (BioServ) with a variable speed Lightnin Mixer Series 20 (Mixing Equipment Company, Rochester, New York) attached to the side of the kettle for continuous stirring of the diet (Davis et al., 1990).

After mixing and cooking, the diet is pumped directly from the kettle through stainless steel pipe and rubber hose to a SEPCO Model 40A pipetting machine (Scientific Equipment Products, Baltimore, Maryland) (Davis, 1989) calibrated to dispense 6-7 grams of diet into each tray cell.

The trays containing diet are then placed in racks designed to hold 20 trays. These racks with trays are then held under a laminar flow hood for 1-2 hours of air drying before they are infected with larvae.

Surface-sterilized eggs from the colony are hatched in sterile jars into neonate larvae, which are dispensed into the 32-cell trays of diet by mixing autoclaved cob grits with larvae and using the Davis inoculator to dispense three larvae per cell (Davis et al., 1990). Immediately after infestation each tray is placed in the machine, which seals the cover on the tray. Inoculated and sealed trays are then returned to racks and taken to an insect rearing room maintained at 27.7$\pm$1 °C and 50 to 60% RH, and a 16:8-hour light/dark photoperiod.

Collection of Pupae

To permit maximum production, pupae are removed from trays the day before the first moth emergence is expected (21 days from inoculation). Pupae are collected by peeling the covers from the trays, inverting the trays on a plastic pan, and gently tapping the tray bottom to dislodge the pupae into the tray. Pupae are then collected by hand from the pan, washed in tap water, air dried in a plastic pan, and weighed. Tobacco budowrm pupae average 308 $\pm$2.4mg (n=329), and bollworm pupae average 511.8 $\pm$2.8 mg (n=316). These washed, clean pupae are placed in a clean moth cage in the moth house to begin another generation cycle.

For our use, we produce 2,000 pupae of each species daily for 5 days per week during the season when we are using five to seven cages of each species, requiring 14 liters of diet for each species. Cost and time estimates for rearing these species from egg to pupae are given by Davis (1994). This is a very cost-efficient operation as shown by Davis (1994).

Time and Labor Requirments

The times required by one person to handle seven cages of tobacco budworm and seven cages of bollworm are shown in Table 3. The operation is very efficient.

One person handles the operation, which begins with pupae being placed into oviposition cages. This same

person colects the eggs and places them in grits for hatching into neonates for future field use. In our operation, one person can handle seven cages of tobacco budworm and seven cages of bollworm. From these cages we obtained 376,580 tobacco budworm eggs and 618,806 bollworm eggs per day in July 1995 (Table 1). We normally maintain five to seven cages of 1,800 to 2,000 moths each for each species. This requires enough larvae developing on diet to provide 2,000 pupae of each species five times each week for as many weeks as we need five to seven cages of adult moths.

The efficiency of the system can also be illustrated by comparing it to the system for bollworm described by Burton and Perkins (1989) for the operation at Tifton, Georgia. Their adult cages are 3.8-liter cylindrical paper cartons with six to eight paires of moths each. One of our large cages with 1,800 moths is equivalent to 113 of the 3.8-liter cages. When we have seven cages of each species in operation, we have the equivalent of 1,582 of the 3.8-liter cages.

One technician handles our entire 14-cage operation. Compare this to the number of technicians to handle the equivalent 1,582 small cages.

Effectiveness of Plant Inoculations in the Field

We have inoculated plants using these or similar techniques for several years and have been very successful in creating fairly uniform infestations of tobacco budworm or bollworm in field plots (Jenkins et al., 1982; McCarty el at., 1986). We have used these techniques in the development of several germplasms of cotton that are tolerant to tobacco budworm, (Jenkins et al., 1988abc) and to evaluate transgenic cotton germplasm for resistance to tobacco budworm (Jenkins, 1993; Jenkins et al., 1995).

The effectiveness of the field infestation techniques can be shown in a 1994 experiment in which we grew DP 5415 (susceptible cultivar) and NuCotn 33^B (a resistant transgenic cultivar) in inoculated and noninoculated plots. Lint yield of the commercial cultivar DP 5415 was 1,435 and 654 pounds lint per acre from noninoculated and inoculated plots, respectively, for a yield loss due to infestation with tobacco budworm of 781 pounds per acre. In contrast, NuCotn 33^B produced lint yields of 1,715 and 1,698 pounds of lint per acre from noninoculated and inoculated plots, respectively (Jenkins et al., 1995). This shows the effectiveness of the techniques in establishing field infestations of tobacco budworm and demonstrates their usefulness for measuring the resistance of a resistant cultivar of cotton.

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Table 1. Production of tobacco budworm and bollworm eggs from April 1995 through August 18, 1995 (one normal year use cycle for field infestation).

	Tobacco Budworm		Bollworm	
Month	Eggs	Mixed Sex Adults	Eggs	Mixed Sex Adults
	numbers			
April	2,871,000	8,261	-	-
May	2,747,000	17,395	-	-

June	7,414,000	29,441	12,870,000	29,692
July	11,674,000	33,804	19,183,000	26,716
August	4,307,000	9,950	7,029,000	7,351

Table 2. Daily egg production from one cage of tobacco budworm containing 2,066 unsexed pupae. This illustrates expected oviposition over age ofadult moths.

Day	Number of eggs
mating night	-
1	10,000
2	58,000
3	132,000
4	142,000
5	120,000
6	90,000
7	66,000
8	30,000
9	29,000
10	10,000
11	4,000

Table 3. Approximate time required for one worker to manage seven cages of tobacco budworms and seven cages of bollworms.

Task	Time in minutes
Change screens	60
Harvest eggs with mechanical harvester	60
Prepare diet for moths	20
Feed moths two times daily	60
Sterilize and prepare eggs for colony maintenance	30
Dry and weigh eggs for field use	60
Process eggs into grits	90
Cleaning of moth house and scale removal	60

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