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Principles of mass culture of the gypsy moth parasitoid *Aphantorhaphopsis samarensis* (Villeneuve) (Diptera: Tachinidae: Siphoninae)

F. Wolfgang Quednau and Karl Lamontagne

Laurentian Forestry Centre
Information Report LAU-X-121
(revised version 2003)

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ABSTRACT

Techniques for rearing the gypsy moth parasitoid *Aphantorhaphopsis samarensis* (Villeneuve) are described. The biological characteristics of the host plant, the host insect, and the parasitoid are outlined and data on productivity are presented. The necessary working equipment and facilities are illustrated.

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RÉSUMÉ

Les auteurs décrivent des techniques d'élevage de masse applicables au parasitoïde *Aphantorhaphopsis samarensis* (Villeneuve) et présentent les caractéristiques biologiques de la plante-hôte, de l'insecte-hôte et du parasitoïde, ainsi que des données sur la productivité. Des illustrations du matériel et des installations d'élevage sont fournies.

INTRODUCTION

A cooperative research program in biological control between the International Institute of Biological Control and the Canadian Forest Service has led to the discovery and importation of the gypsy moth parasitoid *Aphantorhaphopsis samarensis*[1] (Diptera: Tachinidae). This parasitoid appears to be both univoltine in nature and virtually host-specific to gypsy moth, giving it desirable attributes for biological control. It is associated with low-density populations of gypsy moth in central Europe and has been credited with the capacity of maintaining gypsy moth populations at relatively low-density levels and even preventing an outbreak of the host insect (Mills *et al.* 1986, 1989; Mills and Nealis 1992). After having been attacked by *A. samarensis*, the gypsy moth larva succumbs during an early instar before major defoliation occurs in the field.

This association of *A. samarensis* with low-density levels of its host makes it difficult to secure sufficient material for release directly from field collections. In 1989, a colony of the parasitoid was established at the Laurentian Forestry Centre of the Canadian Forest Service in Sainte-Foy, Quebec, with the objective of identifying the biological requisites of the species for mass culture (Quednau 1993) and of providing material for initial field releases (Nealis and Quednau 1996). Since 1991, thousands of puparia of *A. samarensis* have been reared and European wild populations of this fly have been infused to improve the genetic quality of stock. The purpose of this report is to outline in more detail the methods of rearing this beneficial insect as part of a novel approach for biological control of the gypsy moth in North America.

Aphantorhaphopsis samarensis is a difficult insect to rear in captivity. Techniques are complex and labour-intensive. Critical innovations developed in our laboratory and described in detail in this report are:

- Provision of natural host-finding stimuli. Second- and third-instar host larvae are exposed to the parasitoid while feeding on tender oak foliage.

- Continuous supply of host plants. Young oak trees grown from acorns are stored, once tender foliage has been grown, at 7°C for a maximum of 2 months.

- Continuous supply of host larvae. Egg masses of the gypsy moth are incubated on artificial diet. The ensuing caterpillars, once they attain second instar, are stored at 7°C for a maximum of 40 days, during which time they feed very little.

- Rapid exchange of host material. A new type of cage with a detachable bottom was developed, allowing for quick rotation of host insects.

- Separation of free parasitoids from unparasitized host larvae. A new type of rearing container was developed to separate the free, mature parasitoid larvae from unparasitized caterpillars.

- Manipulation of facultative diapause response. Diapause in *A. samarensis* is prevented when puparia, once formed, are held at 20-25°C. If diapausing puparia for hibernation are needed, rearing rooms are set at fluctuating day and night temperatures and the puparia, once formed, are stored at 13.5°C for 1-2 months until the insects have attained the pharate adult stage, then cooled down further.

REARING METHODS

Three components must be provided for the perfect interaction of the parasitoid with its host: plants on which the host insects are exposed to the ovolarvipositing fly, hosts of suitable instar, and the parasitoid ready to produce progeny (Fig. 1).

The host plant

Red oak (*Quercus rubra* L.) is the most suitable species because of the development of large, tender leaves; also European oak (*Quercus robur* L.) can be used, but foliage hardens sooner. Mature seeds (acorns) are collected in large quantities in fall from suitable sites where older, healthy trees produce plenty of seeds. As many as 20,000 acorns (ten 20 L buckets) may be needed for one year's *A. samarensis* rearing. A careful selection of the acorns should be made. When immersed in water for 24 h, the healthy seeds fall to the bottom of the container. The acorns are held for a few days on newsprint paper at room temperature until they look dry. During this time curculionid (weevil) larvae may exit infested seeds.

[1] Formerly known as *Ceranthia samarensis*. As pointed out by Anderson (1983, 1996) and O'Hara (1989, 1998), the genus *Ceranthia* was split off as a monophyletic taxon from another group including *Aphantorhaphopsis* Townsend, 1926, which is heterogenous (polyphyletic) and which contains the species *samarensis*, originally described as *Actia samarensis* Villeneuve, 1921.

For stratification at 2-4°C and 95% relative humidity, storage containers must allow gas exchange with the atmosphere while maintaining high acorn moisture levels. Polyethylene bags 0.006 mm (0.004") thick or less (size 45 x 100 cm) are recommended (Bonner 1992) to allow aeration and to release CO_2 by the acorns during stratification. The acorns can be stored in this condition for at least 9 months. Minimum hibernation time is 2 months. As the storage period is extended for 1 year or longer, percentage germination may be reduced. However, Bonner (1992) reports viability without critical losses up to 3 years.

For germination, the acorns are brought to room temperature (20-22°C) in the greenhouse and mixed with an equal volume of moist PRO-MIX soil. Light is excluded and humidity retained by pulling a black plastic bag over the container. Depending on the length of cold storage, it may take from 2 weeks to 2 months until the roots begin to show (Fig. 2). The acorns are planted 2 cm deep, horizontally with roots pointing down, in styrofoam drinking cups (7 cm upper diameter, 9 cm high) with four holes in the bottom. The soil composition is black top soil (2.5 parts), PRO-MIX (2 parts) and peat moss (0.5 parts); the soil should be neither too heavy nor too light. The plants must be watered at least twice a week, never allowing the soil to dry out. In order to maintain the roots in a humid atmosphere, a plastic basin measuring 50 x 38 x 12 cm (Rubbermaid Canada) with metal screening on a wooden frame is used to hold the plants 2 cm from the bottom of the basin, which is filled with water up to the level of the screening (Fig. 3). Approximately 30 cups are placed upright on the screen and the roots eventually penetrate the holes in the cups. Losses of up to 40% may occur after planting the hibernated acorns. Surplus sprouted acorns in moist PRO-MIX can be returned to the cold room and planted later.

For optimal growth, conditions are 22°C and ordinary fluorescent lighting at 17:7 (L:D) h photoperiod. The greenhouse must be painted white in summer to reduce heat radiation from the glass. Light intensity should be moderate and sodium lamps should be avoided because they dry out the air. Foliage will develop after 3-6 weeks, but may take longer if the night temperature is not maintained at 20°C. If a greenhouse is not available, office space equipped with fluorescent lights giving an intensity of about 20 $\mu mol \cdot s^{-1} m^{-2}$ at the level of the plants may be used instead (Fig. 4). After tender foliage is grown, the plants are stored in a humidified cold room (7°C) under the same lighting conditions, and can be used for up to 2 months after which the foliage hardens and becomes unsuitable for the gypsy moth larvae, which prefer succulent leaves as food.

Having plenty of fresh foliage available for the exposure of the gypsy moth larvae to the parasitoid is the basis of the rearing. Acorns must be available and germinated well ahead of time.

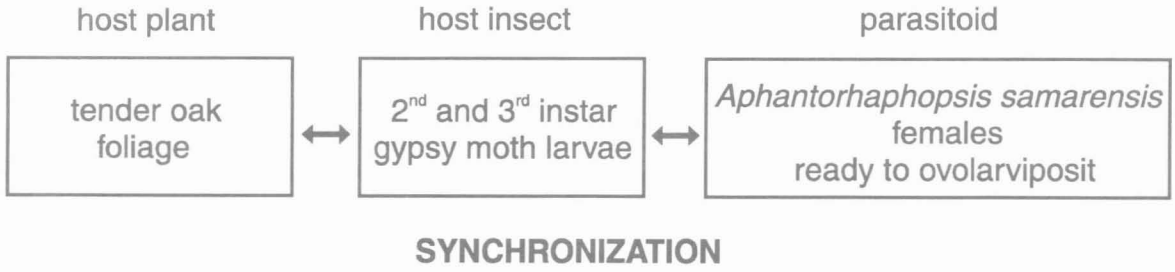

Figure 1. Concept of host-parasite interaction.

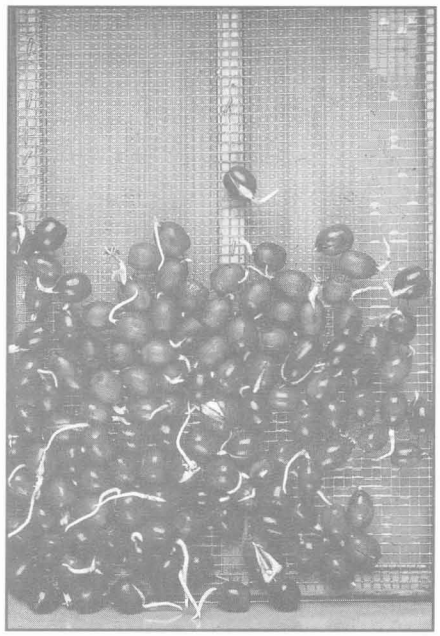

Figure 2. Sprouted acorns of red oak (*Quercus rubra*).

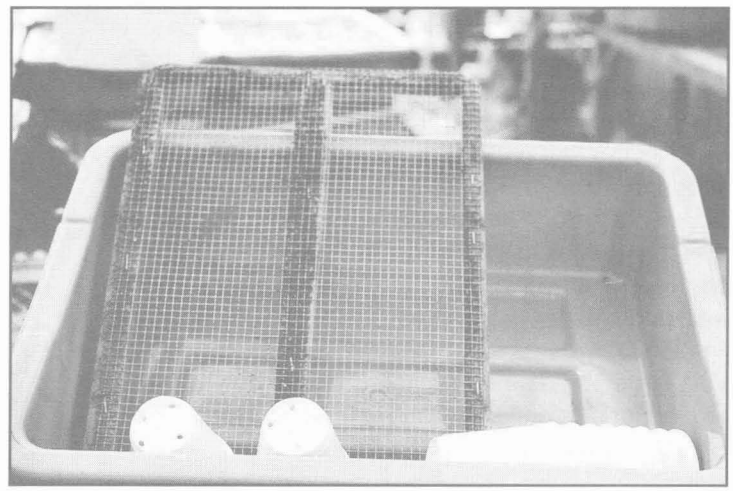

Figure 3. Plastic basin and metal screening to hold cups with small oak trees.

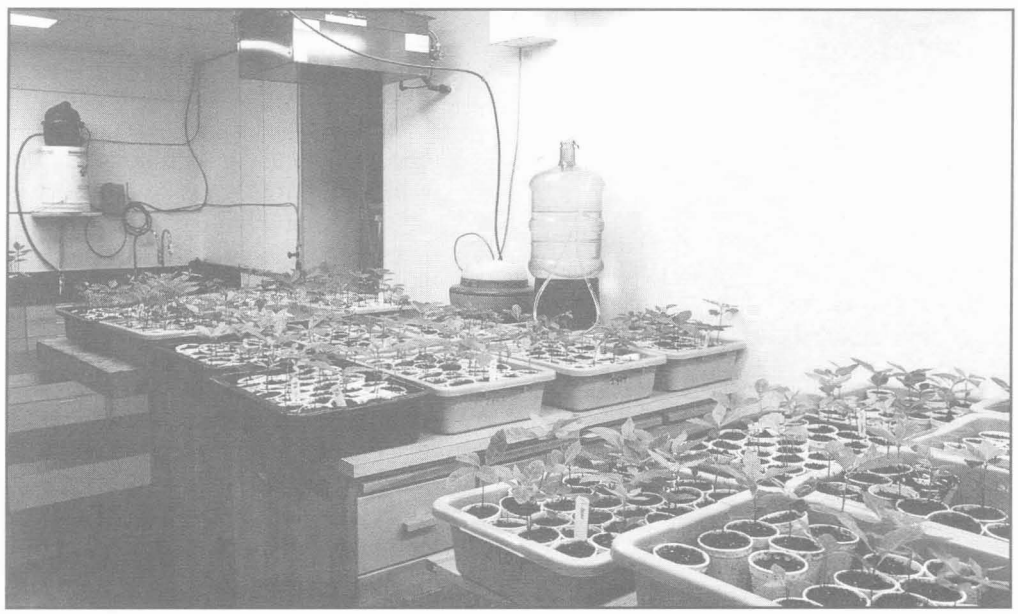

Figure 4. Office space equipped with fluorescent lights to grow small oak trees.

The host insect

Gypsy moth (*Lymantria dispar* L.) eggs can be collected in the fall at sites where the insect occurs naturally at fairly high density. Egg masses are stored at 2-4°C (humidified) for at least 4 months (diapause) before the eggs are ready to eclose at 25°C; otherwise, incubation time will be prolonged. A disadvantage of using egg masses collected in the wild is the considerable mortality caused by severe frost if the eggs collected in late winter or early spring were not protected by snow. At the Laurentian Forestry Centre, laboratory-reared (supplied as a courtesy of USDA Otis Methods Development Center, Otis ANGB, MA, USA) gypsy moth egg masses were used. These egg masses (Fig. 5) were laid on brown kraft paper and stored in the cold for 6-7 months; under these conditions, the eggs hatch in 2 days at 25°C.

Very large numbers of 2nd- and 3rd-instar host larvae are needed for efficient production of the parasitoid. The following points have to be observed:

- Storage of post-diapause egg masses. Egg masses about 175-250 days in diapause can be stored at 5-7°C (humidified) for a period of 1½ months. During the latter half of this period, percentage eclosion is progressively reduced.

- Surface sterilization of egg masses. In order to reduce the incidence of nuclear polyhedrosis virus, the egg masses are dipped in 10% v/v formaldehyde solution (10 cc of commercial 37% w/w HCHO diluted in water until 100 cc) for 1 h and rinsed for 2 h in flowing water (Odell *et al.* 1985) before incubation. The egg masses are removed from the paper with a spatula, immersed, 100 egg masses at a time, in a beaker with wire screening slightly below the surface of the solution to keep the eggs submerged (Fig. 6, left). To rinse out the formaldehyde, egg masses are placed in plastic cylinders (10 x 5 cm) with wire screening on the bottom and a snap-on lid (Fig. 6, right). The cylinders are held immersed in the water tank by pieces of heavy angle iron. Rapid exchange of tap water is ensured by pieces of plastic hose fitted on opposite sides of the tank (Fig. 7). After washing, the egg masses are placed on diet and incubated.

- Preparation of diet. Formulation of diet (Bell *et al.* 1981) is as follows:

Water	1200 mL
Agar	22.5 g
Casein	37.5 g
Salt mix (Wesson)	12.0 g
Sorbic acid	3.0 g
Methyl paraben	1.5 g
Wheat germ	180.0 g
Vitamin premix (Vanderzant)	15.0 g

This recipe makes about 1400 mL of diet. Boil water in a 3 L saucepan; add the agar and return to a boil. Add casein, salt mix, sorbic acid and methyl paraben; stir well to dissolve the methyl paraben. Return to a boil and add wheat germ; if the mixture starts to foam add the remainder of the wheat germ to stop it. Before adding the vitamins, the mixture should be boiled for 10-30 s to destroy the enzymes (lipases) that break down important nutrients for the caterpillars. Pour the mixture into a blender (Waring commercial blender, 2 L capacity) and add vitamins, stirring to dissolve them. Blend for 2 min at high speed; pour into appropriate rearing cups.

- Rearing cups. Three different types of rearing cups were used:

Type 1.
Plastic ERA containers (made by Eraware, Ville Saint-Laurent, QC, square, 908 mL, with tight-fitting covers. The covers are perforated on the periphery with about 100 holes of 0.68 mm diameter, small enough that neonate gypsy moth larvae cannot escape. The inner side of the cover or the bottom of the container is filled with diet 0.5 cm deep. These units are used to incubate egg masses and feed the ensuing larvae until 2nd instar. One recipe of diet is sufficient for 20 containers (Fig. 8). Unfortunately, when these containers are washed and reused, the covers no longer close hermetically and neonate gypsy moth larvae may escape. To prevent this, cloth tape may be used for sealing the cracks or the containers must be replaced by new ones.

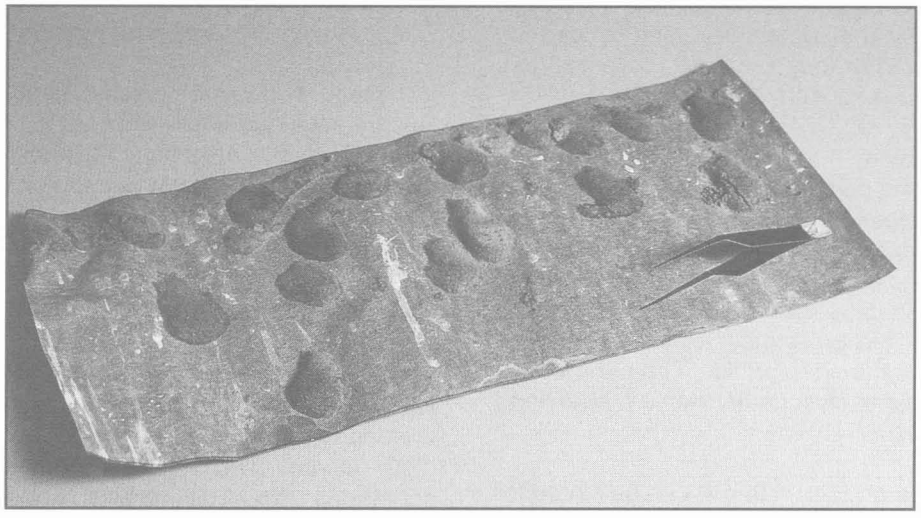

Figure 5. Egg masses of gypsy moth (*Lymantria dispar*) laid on paper.

Figure 6. Left: egg masses submerged in formaldehyde solution. Right: plastic cylinder to retain egg masses during washing.

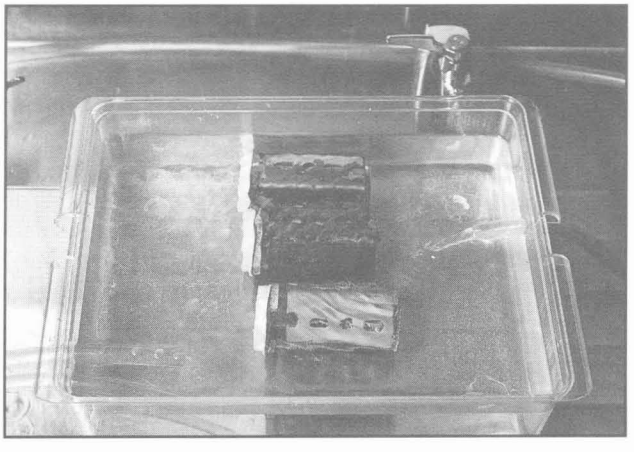

Figure 7. Rapid water exchange system for washing out formaldehyde.

Type 2. (disposable)
Semi-transparent, 163 mL soufflé cups (No. P550) with plastic snap-on lids (No. PL5) (made by SOLO Cup Company, Urbana, IL, USA) filled 0.5 cm deep with diet (Fig. 9). These cups are used to feed the smaller host larvae as they have been collected from foliage in the host exposure cages.

Type 3. (disposable)
Compound rearing container, a SOLO cup as above with about 15 holes (0.25 cm diameter) in the bottom to allow the mature fly maggots to fall through and collect on the bottom of a styrofoam drinking cup that is firmly pushed over the SOLO cup for hermetic sealing. Diet for the gypsy moth larvae is placed on the inner side of the snap-on lid. The parts of this container are held firmly together with a rubber band (Fig. 10).

The rearing cups and lids with diet can be stored for at least 2 weeks at 2-8°C, so that a supply is always available.

- Rearing host larvae on diet. One to three surface-sterilized egg masses are placed in a type 1 container and incubated at 25°C and 35-60% relative humidity. About 40 containers are needed to accommodate 100 egg masses. After eclosion, it takes another 7 days at this temperature to obtain the 2nd-instar larvae, recognizable by their pitch black colour. Before the larvae have reached 2nd instar, it is advisable to redistribute the material of each unit into two new containers with fresh diet. This will reduce contamination and the possibility that the insects will feed on their excrement. Once the host insects have reached 2nd instar, the containers are stored at 7°C; at this temperature the larvae feed very little and a continuous supply is available over 1-1½ months. However, rapid utilization of the larvae in the host exposure cages is recommended, so that no molds will develop during this time, and if they occur, the cultures should be discarded. Due to periodic exposure to room temperature during the collection of larvae needed for exposure to the parasitoids, 2nd instars will eventually molt to the 3rd instar. This is not a disadvantage, because the latter are equally suitable as hosts for *A. samarensis*.

  The good condition of the host larvae is of utmost importance for obtaining attacks by the parasitoid and the production of healthy puparia of *A. samarensis*.

The parasitoid

Biology of *A. samarensis*. *Aphantorhaphopsis samarensis* is an ovolarviparous endoparasitoid. Eggs are deposited on the bristles of 2nd- and 3rd-instar gypsy moth larvae. The neonate parasitoid larva quickly penetrates the host cuticle and develops in the hemocoele, forming a respiratory funnel that produces a dark circular scar on the caterpillar. There are three larval stages and moulting takes place in the respiratory funnel. Development is rapid (Fig. 11). Under laboratory conditions, 8-14 days after the host has been attacked, the mature parasitoid larva bores out of the host. Pupariation is completed within the next 12 h. In non-hibernating cohorts, total generation time from egg to adult is 20-26 days at 25°C, and 33-40 days at 20:15°C (12:12 h; day:night). In nature, *A. samarensis* is univoltine with the possibility of a partial second generation in unusually hot summers. In the laboratory, the dormant state is facultative and diapause does not occur if puparia, once formed, are stored at 20°C. The number of progeny (puparia) produced over the lifetime of a *A. samarensis* female is 30-100 puparia, and the average longevity of the parasitoid is about 40 days (Quednau 1993).

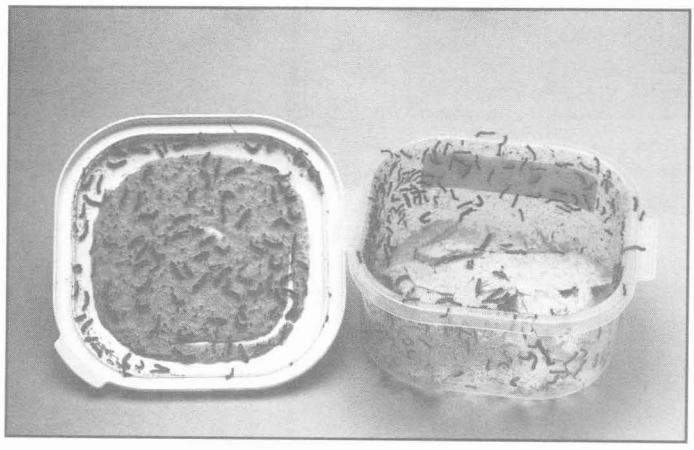

Figure 8. Plastic container (type 1) for rearing large numbers of gypsy moth larvae on diet.

Figure 9. SOLO cups in incubator to rear, on artificial diet, gypsy moth larvae that have been exposed to *A. samarensis*. Upper shelf: simple cups (type 2). Lower shelf: compound cups (type 3).

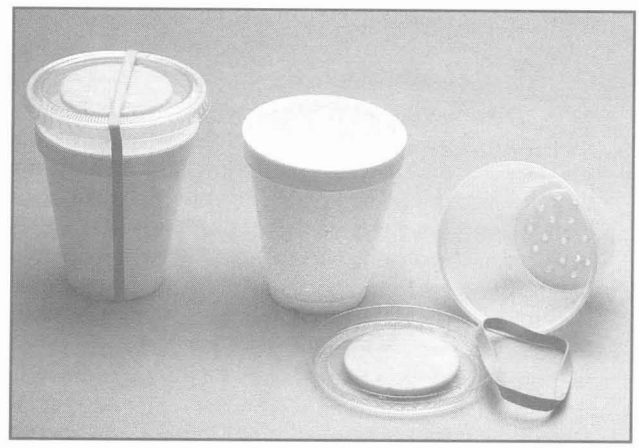

Figure 10. Parts of compound rearing cup (type 3) designed to separate mature parasitoid larvae from non-parasitized caterpillars. Note the artificial diet on inner side of lid. Left: assembled unit.

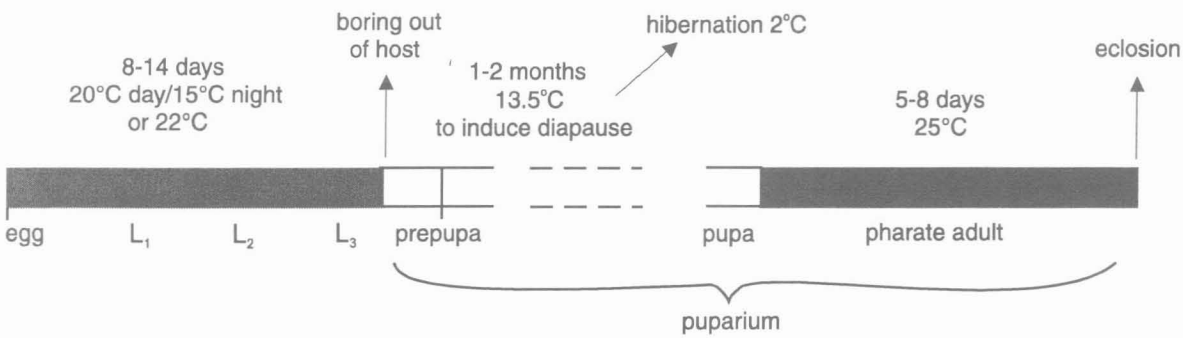

Figure 11. Life cycle of *A. samarensis* in the laboratory.

– Confinement cages for adults. *Aphantorhaphopsis samarensis* adults are frail and delicate, swift and elusive in flight. Their bodies dehydrate quickly, and they require very high humidity in the atmosphere to survive well. The flies drink water frequently. The basic design for the cages was taken from Odell and Godwin (1979). It was modified by a tulle covering to prevent damage to the flies' wings and permit better aeration.

Three types of cages were used:

1. Eclosion cage (Fig. 12). This cage ensures that adults eclose in a water-saturated atmosphere and that the flies alight on tulle and not on hard surfaces. Bottom part: same basin (Rubbermaid) as used to hold small oak trees, filled with water. Top part: plastic base cut from upper half of Rubbermaid basin, with tulle over wire hoops fitted in plastic (plexiglass) triangles held by screws in corners; the bottom is a piece of nylon screening (Nitex Nylon Monofilament Bolting Cloth, Fabric Number HC3-475, 14 strands/cm; SEFA Canada, Ville Saint-Laurent, QC) glued to the plastic frame with Bostic glue stick, and a foam rubber band is also glued over the edges for better sealing. Tulle can also be used instead of nylon screening, but smaller insects may fall through into the water. The cage measures 37 x 50 x 55 cm and a plastic bag is pulled over it and tightly fastened at the lower edge. An opening of 16 cm is provided to introduce the dish with the puparia and make it possible to reach in at arm's length to collect the insects; this opening is closed with a foam rubber plug. The cage is hermetically fastened over the water basin by means of four large Foldback 1414 clamps.

2. Cage for holding males and females after eclosion and during mating (Fig. 13). Made of tulle glued with Bostic glue stick over wire hoops with solid plastic (plexiglass) bottom, dimensions 25 x 25 x 18 cm. An opening of 4 cm in diameter is cut on one side and closed with a foam rubber plug; the opening allows access to the cage to introduce a small water basin with a dental cotton wick (Fig. 16, centre) and to introduce and recapture flies.

3. Gestation and host exposure cage (Fig. 14). The top part is composed of tulle over wire hoops attached to a plastic frame. The frame is made by cutting a 23 x 30 x 40 cm plastic box (T-295, Althor Products, Bethel, CT, USA) in thirds horizontally. The wires are glued with epoxy through holes in the corners of the frames or through 6-mm-thick plexiglass triangles glued at the corners of the cage. The detachable bottom is made of 38-mm-thick styrofoam (for best results use Cladmate XL Styrofoam, Dow Canada) with four openings of 5 cm in diameter to hold four small potted oak trees. The cage must fit tightly over the styrofoam to prevent insects from escaping. For that reason a foam rubber band is glued on the inner side of the plastic frame of the top part. Two openings are cut in the tulle at one side; one hole is cut 3 cm in diameter near the top to introduce the flies; the second opening, 10 cm in diameter, is cut lower down and is large enough for a person to reach in and remove the parasitoids. Both openings are closed with a foam rubber plug. A larger model of this cage, 45 x 32 x 55 cm, was also used and the frame is made of the same material as that of the eclosion cages (Fig. 15).

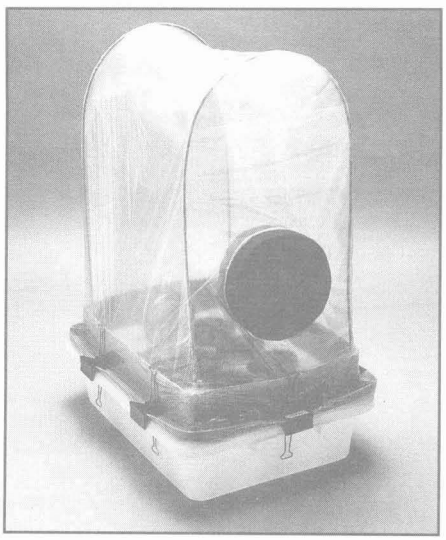

Figure 12. Eclosion cage.

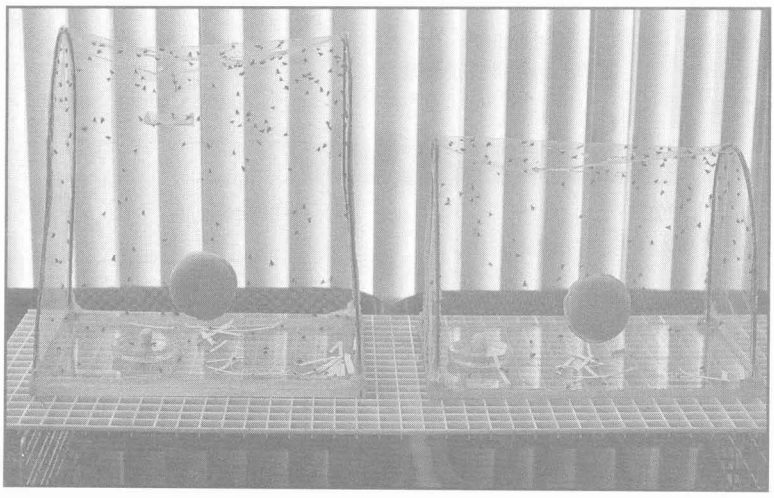

Figure 13. Cages for holding male and female parasitoids and for copulation.

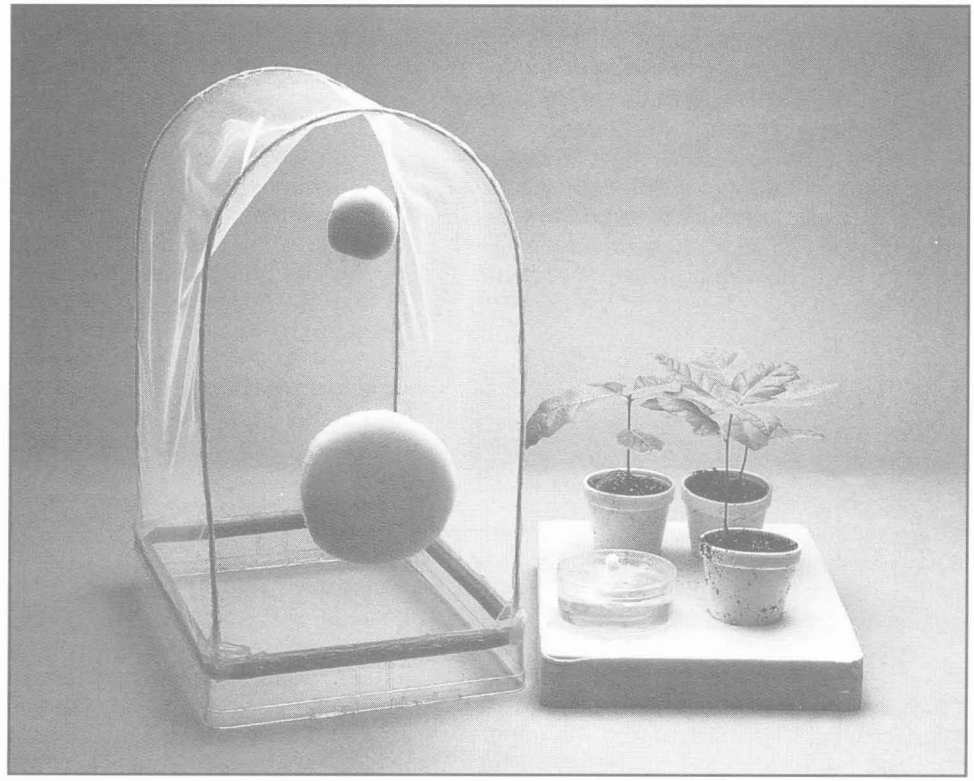

Figure 14. Gestation and host exposure cage. Left: top part made of tulle; note foam rubber band for hermetic closure. Right: bottom part made of styrofoam with small oak trees and dental cotton wick in water.

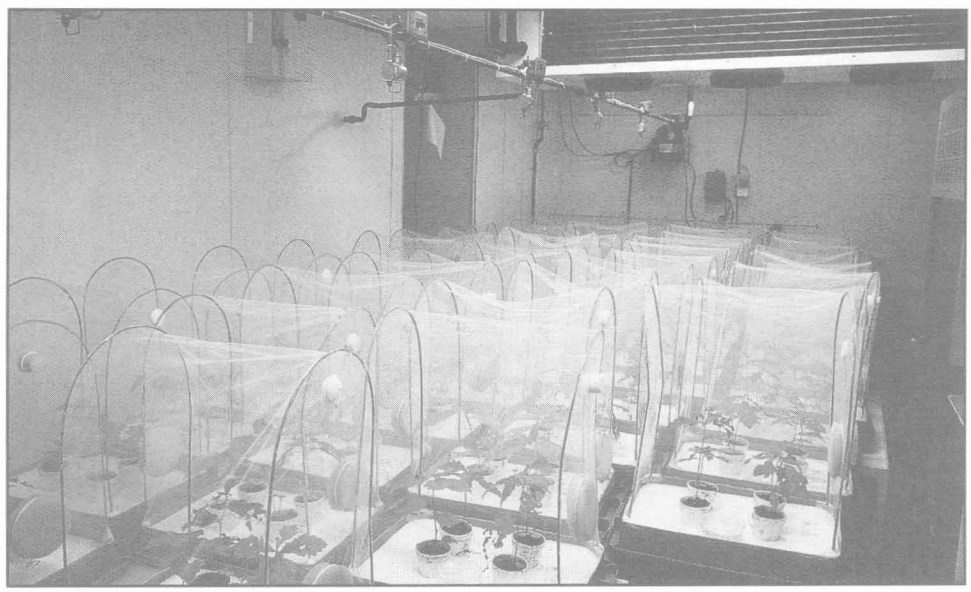

Figure 15. Large host exposure cages in rearing room equipped with automatic mist dispersion system.

- Food for the adult flies. A dental cotton wick soaked in distilled water contained in a small Petri dish (No. 1006 Falcon by Becton Dickinson Labware, Lincoln Park, NJ, USA) is placed in each type 2 cage (Fig. 16, centre). In the host exposure cage, a larger cylindrical container (8.25 x 8.25 cm, Althor Products No. R15) is used for the dental cotton wick (Fig. 16, upper row, left). In addition, honey-agar jelly is spread on top of the tulle cages. The composition of the jelly is as follows:

Water	130 mL
Honey	10 g
Sugar	20 g
Agar	0.55 g

Ingredients are added to cold water and under constant stirring the solution is slowly heated to the boiling point, then removed from the stove to prevent it from becoming too concentrated. It is poured into a large plastic dish (Falcon Plastics, 150 x 25 mm). After cooling in the refrigerator, the jelly can be distributed with a pipette which is kept clean in a shell vial glued to the petri dish cover (Fig. 16, upper row, centre). The medium should be stored in the refrigerator and prepared fresh every 2 weeks. Distilled water is atomized on the cages at least twice a day.

- Eclosion. Post-hibernation puparia are kept first for 5 days at 15°C; non-hibernated ones are transferred directly to 22-25°C and 18:6 h (D:L) photoperiod. Puparia in wet peat inside a plastic Petri dish (Falcon Plastics, 150 x 25 mm) are placed on the screening in the eclosion cage (Figs. 12 and 17). The peat is sufficiently wet when no more surplus water is dripping off. The humidity inside the eclosion cage should be near 100%. As eclosion commences, the insects are collected twice a day, individually, using a clean, dry 35 x 10 mm shell vial (1 fly per vial), which is stoppered with a foam plug (Fig. 18). After each collection, distilled water is sprayed on the puparia so the peat never dries out. In post-hibernation cohorts, a protandric pattern of eclosion is observed (Fig. 19). In non-hibernating cohorts, males and females eclose simultaneously (Fig. 20). In post-hibernation cohorts, especially in those that did not have uniform lengths of diapause at 2°C, the range between male and female eclosions is sufficiently large, so that later virtually all females can be mated. Minimum hibernation requirement at 2°C is 100 days. In this latter case, eclosions at 22°C may extend over a period of 30 days or more. The shorter the hibernation, the longer it takes for the pharate adults to eclose.

- Sexing. Insects are held in shell vials and sexed using a hand lens. The genital armature of the *A. samarensis* male is brown and sclerotized in a fold of the ventral posterior abdomen (claspers); the female's abdomen has a smooth, slightly tubular tip (Fig. 21). Males and females are placed in separate cages, 40-60 flies per cage. The males are held at 22:15°C (12:12 h; day:night) with ordinary fluorescent lighting of about 2 μmol· $s^{-1}m^{-2}$ intensity at the level of the cage for 3 days until maturity. The females are held at 15°C in dim light to reduce their activity until needed for mating. Eclosion date must be shown on the cages and new cages are used every day so the age of the flies is precisely known. To remove a female from its cage, a wire handle is fitted around a shell vial to serve as a "spoon" so the fly will fall in when touched gently (Fig. 16, lower row, centre).

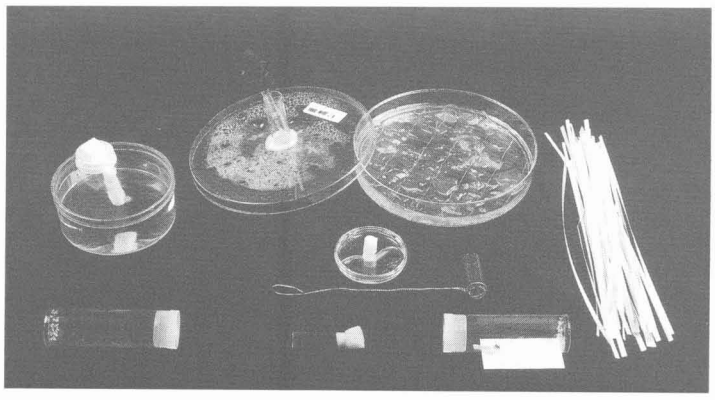

Figure 16. Utensils for feeding and capturing *A. samarensis* adults. Upper row, left: large container with water and dental cotton wick; centre: honey-agar jelly in Petri dish with distributor pipette; centre below: small Petri dish with water and dental cotton wick; right: cardboard paper strips. Lower row, left: butyrate vial with perforated bottom and polyethylene lid; centre: small shell vial with foam plug and "spoon" for capturing flies.

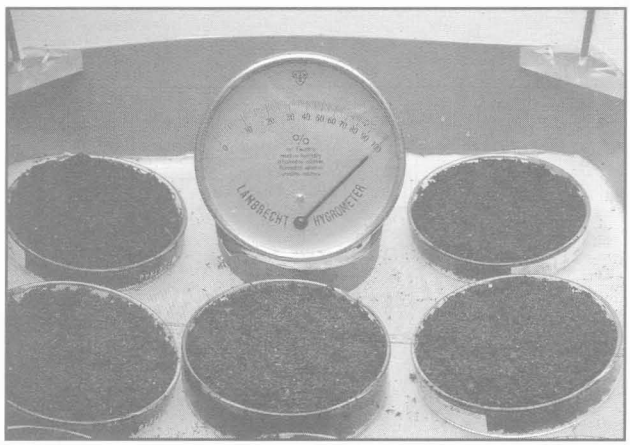

Figure 17. Puparia in wet peat in eclosion cage.

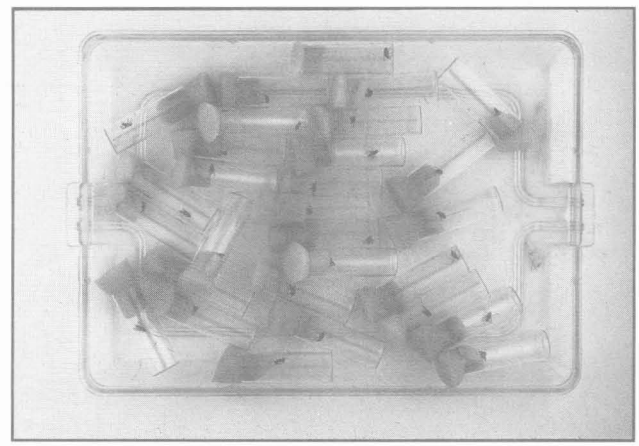

Figure 18. Shell vials, stoppered, containing *A. samarensis* flies.

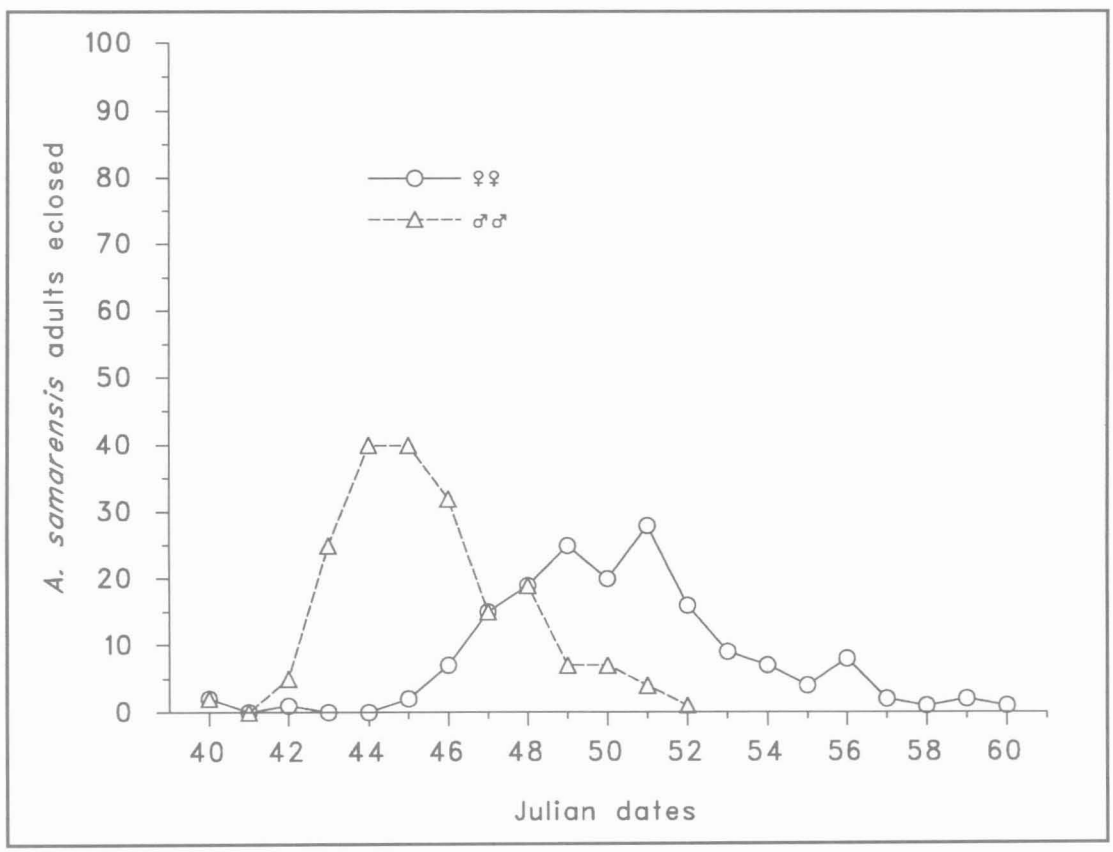

Figure 19. *A. samarensis*, eclosion pattern of 1119 post-hibernation (135-150 days at 2-4°C) puparia incubated at 15°C for 6 days and at 22°C thereafter.

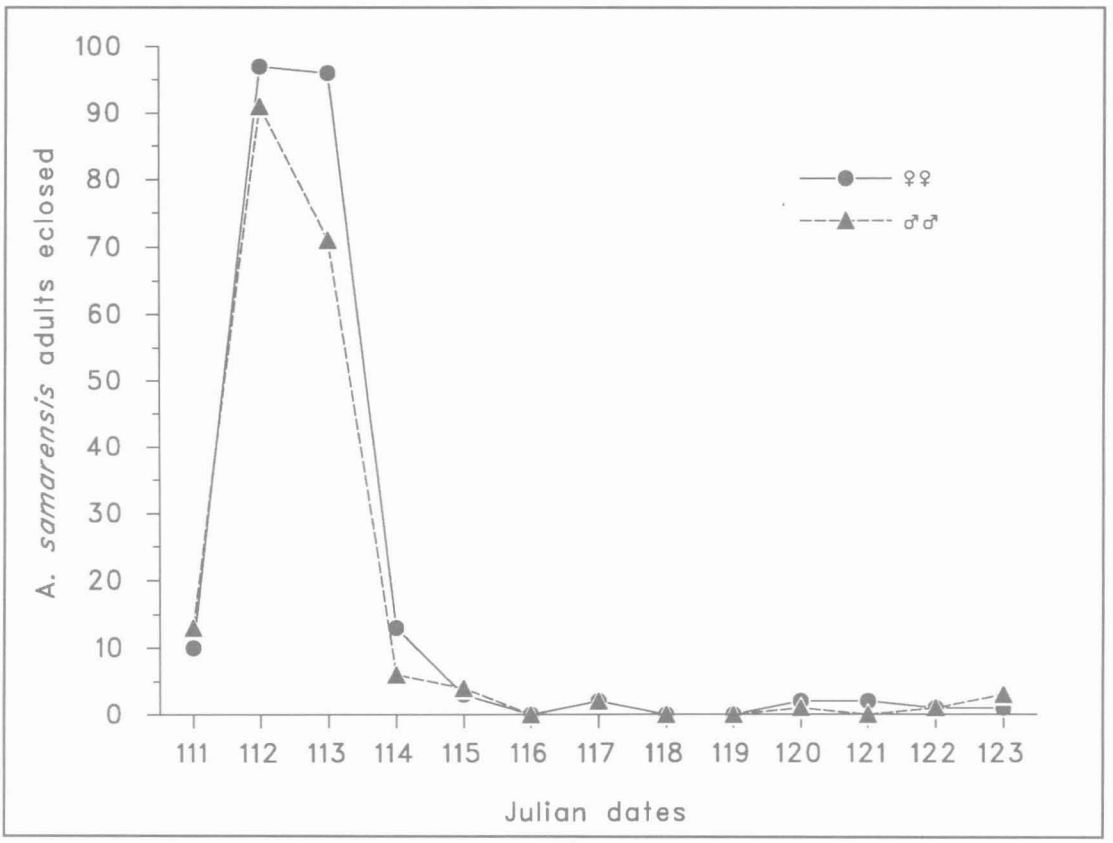

Figure 20. *A. samarensis*, eclosion pattern of 1074 non-hibernating, second-generation puparia at 25°C.

- Mating. Staggered age classes of males and females have to be available. Pairing of older (at least 3 days old) males with 1-4 (maximum 5) day old females is critical. Newly emerged females mate the most easily. Copulation lasts 2½-3 h. Mating success is 60-80% in the laboratory. The following steps should be taken using the arrangements shown in Figs. 13 and 22:

  - Mating cages are installed in front of a large window. The optimal light intensity for mating is from 20-100 (maximum 150) $\mu mol \cdot s^{-1} m^{-2}$ at cage level. Males ready to mate should swarm around in the cage. Mating is stimulated by varying cloudiness of the sky or a change in lighting in the laboratory in the order of 50 $\mu mol \cdot s^{-1} m^{-2}$. When there is an overcast sky or continued rain, the insects are often inactive. Fanning warm air with a ventilated electric heater or carrying the cages from dark corridors to well-lit rooms helps to stimulate the flies. Honey-agar jelly should be available to the insects on the cage screening.

  - To begin the mating, introduce five virgin females into a cage with males, thereafter one female for each copulation observed. The more males in a cage, the greater the chances of copulation (Fig. 23).

- The pairs are gently removed from the cage on a cardboard strip (Fig. 24) and each pair placed in a plastic tube (Althor Products butyrate vials 2.5 x 7.5 cm with snugly fitting polyethylene cover), the bottom of which is perforated (Fig. 16, lower row, left). The tubes with the insects are held in a humid atmosphere, which is ensured by using a piece of window glass to cover a plastic basin filled with water (Fig. 25). During copulation the flies are almost motionless; termination of coitus is easily seen as the insects begin to walk around in the tube.

- After separation the flies are sexed and the males returned to their original cage. The mated females are placed into gestation cages (Fig. 14). For easy separation of the sexes, one of the tubes is unstoppered and another unstoppered empty tube is held against it with the open ends together; after one of the flies has walked into the second tube, both tubes are quickly restoppered and the insects are sexed.

- All manipulations must be done under a large tulle cover over wire hoops to recapture any escaped flies (Fig. 26). The rapidity with which the males and females are rotated will greatly enhance mating success.

- As the mating process is time consuming, this type of work should be started early in the day. Mating frequency reaches a peak in the early afternoon and copulations may continue until 10:00 PM. After no more copulations are observed, the mating cages (with a few unmated females) are placed at 15:10°C (12:12 h; day:night), 95% relative humidity and moderate 12:12 L:D lighting to increase longevity of the insects. Some pairs may not have separated by the close of the work day. They may be left in the humid chamber until morning, but a small droplet of honey-agar jelly should be put in the tube.

- There is a fatigue period for males; males that have mated often during the first day will do so less often on subsequent days.

- More than 5 day old females should be removed from the mating cage since their presence may reduce mating activity of the males.

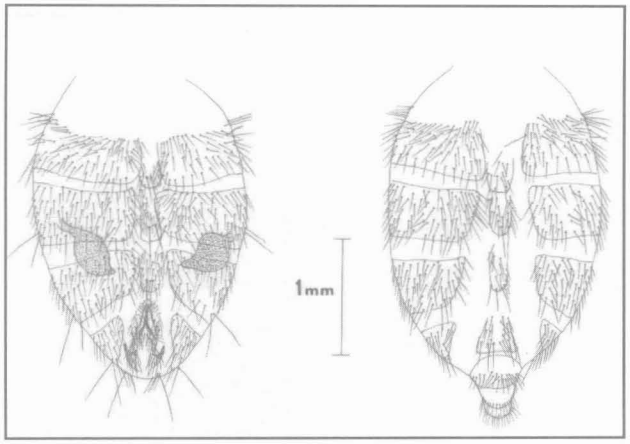

Figure 21. Ventral view of abdomen in *A. samarensis* to show genitalia. Left: male. Right: female.

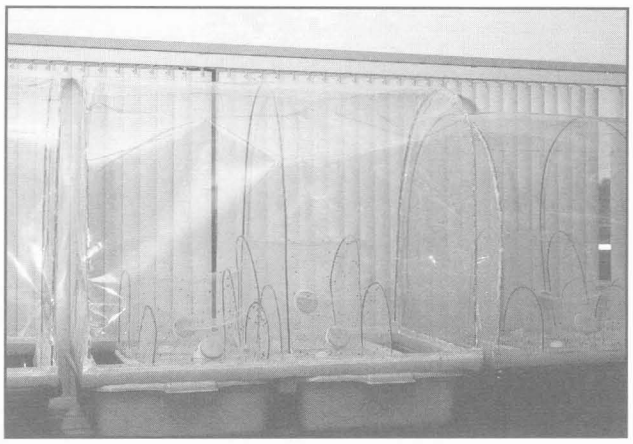

Figure 22. Mating cages installed over water basins with plastic coverings to retain high humidity.

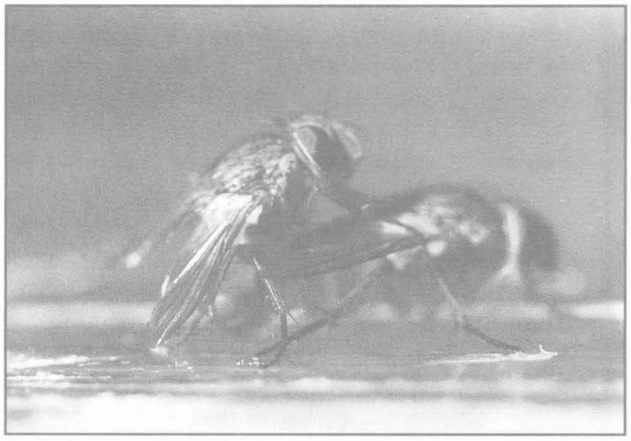

Figure 23. Copulation of *A. samarensis*.

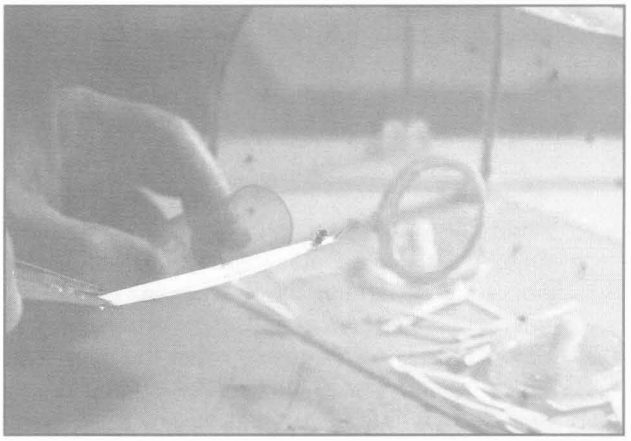

Figure 24. Removal of *A. samarensis* pair in copula from mating cage by means of a cardboard strip.

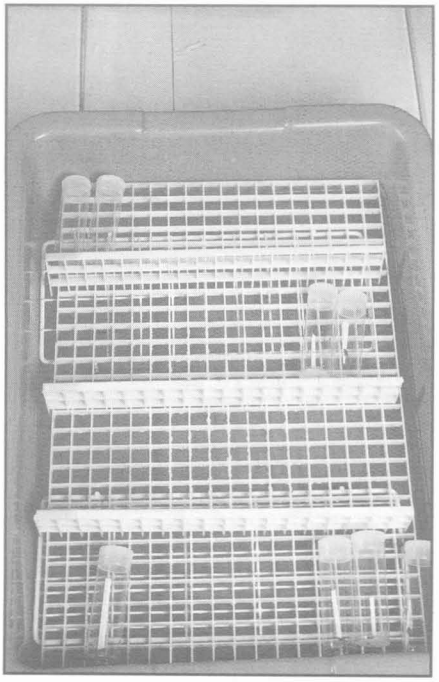

Figure 25. Water basin to accommodate flies from mating cages until the end of coitus in a humid atmosphere.

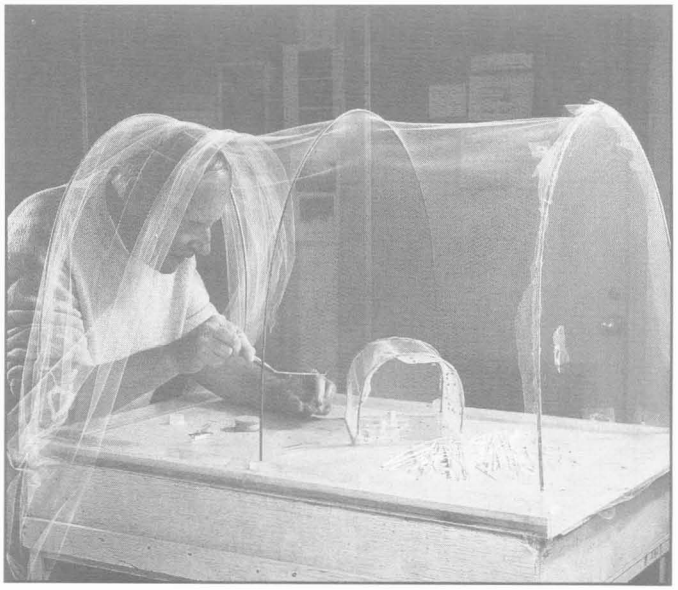

Figure 26. Large tulle cover over wire hoops to prevent flies from escaping.

- Gestation. For gestation, mated *A. samarensis* are held in groups of 20-40 in gestation cages (Fig. 14) with small oak plants. The larger cage model, 45 x 32 x 55 cm, can hold up to 100 flies. Honey-agar jelly is spread on top of the screening, and a container with water and a dental cotton wick is placed inside the cage. The cages must be replaced by new ones at least once a week for cleanliness and to ensure that the honey agar is not contaminated by microorganisms. Light must be low (dim light) to keep the flies quiet. Humidity of 95% should be maintained during this period. If humidified rearing rooms are not available, the same type of cover over water as used with the mating cages (Fig. 22) can be utilized. The cages are sprinkled with distilled water twice a day. Just before the end of the gestation period, about on the 10th day at 20:15°C (12:12 h; day:night) a few host larvae should be placed on the foliage. It is important that young flies ready to ovolarviposit are in contact with hosts as in nature, where perfect synchronization occurs. The gestation period can be lengthened to 17 days by placing the cages from 20:15°C to 15:10°C every second day. During gestation 10-50% mortality of the flies may occur. Some mechanical damage to individual flies seems inevitable if many flies are held in the same cage. Odell *et al.* (1979) report the death of 52% of *Blepharipa pratensis* (Meig.) before oviposition, due to bacterial infection. Therefore, the gestation cages must be scrupulously clean before use, preferably made of new materials or treated with ultraviolet rays after having been washed with 70% alcohol.

- Host exposure. At about the end of the gestation period, 1-2 *A. samarensis* females per host exposure cage are placed with 20-30 2nd-instar gypsy moth larvae on small oak trees (Fig. 14). It is important that the host larvae be of good quality and actively feeding on the foliage to stimulate attacks. To ensure that the gypsy moth larvae are attached to the foliage and do not fall to the cage bottom, they are first placed in Petri dishes and then directed, by means of a soft paint brush, towards foliage. All host exposure cages are numbered sequentially. Rotation of the gypsy moth larvae on foliage must be carefully planned. Host larvae to be exposed to the parasitoids can be prepared 1 day in advance, 20-30 larvae per Petri dish without food, and stored at 8°C. Exposure of hosts to the parasitoid is 2 or maximum 3 days with up to 100 cages rotated per day, depending on technical help. For the transfer of the flies, if they are undisturbed and sitting on the screening, the upper part of the cage is fitted over the newly prepared base with fresh plants and hosts. Alternatively, the flies can be captured singly using a small shell vial. The water basin with the wick is renewed with each transfer. Honey-agar jelly is reapplied. It is best to use new clean cage covers and replace the honey- agar jelly with each rotation. The old cage covers are dipped in hot water to remove the old jelly. Also the styrofoam bases must be wiped clean with 70% alcohol after each transfer of material. Spray the cage with distilled water after each transfer. In addition, all cages should be sprinkled with water twice a day.

  To avoid spraying the host exposure cages by hand, an irrigation system that disperses mists of water can be used. It consists of a central tube with solenoid valves and nozzles branching about every 90 cm. A solid-state precision timer with a 24-h cycle is set at 8-8-8 h or 12-12 h to mist the cages for 3-4 s each time. To ensure an even distribution of spray, the water pressure must be stable, the water free of deposits, and the nozzles properly cleaned (Fig. 15).

  For rearing *A. samarensis* on a large scale, it is possible to utilize up to five parasitoids per larger cage (45 x 32 x 55 cm) (Fig. 15). Fifty host larvae should then be exposed in each cage for a 2- or 3-day period. There is not much economy in space as compared to the small cages but labour is reduced considerably. Thermoperiod of 22:15°C (12:12 h; day:night), relative humidity of 95%, photoperiod of 12:12 h (L:D) and light intensity of 50 $\mu mol \cdot s^{-1} m^{-2}$ is recommended. The cooler night temperature increases longevity of the flies and slows down feeding of the host larvae at night. To ensure exactly 95% relative humidity in the rearing room, humidifiers should be stopped by timers for certain hours periodically to avoid spilling water on floor and tables.

  Host larvae are collected and replaced with unparasitized ones until parasitoids are dead or can no longer fly. Plants can be used until completely defoliated.

- Rearing parasitized host larvae. Exposed gypsy moth larvae are collected from the plants using flat stainless steel forceps and placed with diet in 163 mL SOLO cups (Fig. 28, left). Thirty larvae, maximum, can be reared in each cup, representing the host exchange of one cage. The cups should bear the date of the collection from the host exposure cages and be held at 22°C for 2-3 days to speed up development without effect on diapause. This is necessary because otherwise the smaller larvae will later fall through the holes of the compound rearing unit (Fig. 10) and starve. For

further rearing, if diapausing parasitoids are wanted, the cups must be transferred to a thermoperiod of 20:15°C (12:12 h; day:night), 50% relative humidity and 12:12 h (L:D) photoperiod. Otherwise, for continuous rearing, the temperature should be 22°C and photoperiod 18:6 h (L:D). About a week after ovolarviposition, before the mature fly maggots bore out of the host (Fig. 27), the gypsy moth larvae are transferred to the containers that ensure separation of the parasitoids from the caterpillars (Fig. 28, right). This is important because *A. samarensis* puparia may be damaged by the unparasitized hosts. In order to obtain large, healthy puparia, it is important that the parasitized gypsy moth larvae be well fed on diet until the parasitoids have bored out. When this transfer is made, the gypsy moth larvae should be freed of filaments, otherwise parasitoid larvae may become entangled in them.

The diet should be fresh and contamination-free. The application of the diet on the inner side of the cover affords a very economical use of the medium, it also prevents the diet from becoming contaminated with excrement. A larger supply of these covers should be kept ready in the refrigerator until needed.

- Collection of puparia. The compound rearing cups are examined every second day or at least twice a week. From the lower part of the container (styrofoam cup), any puparia and free maggots are collected and placed in separate dishes to avoid having the puparia disturbed by the wiggling fly larvae. After each examination, the old frass pellets must be discarded. The cover with the diet is replaced, if necessary. The diet must not cause obstruction of the bottom holes. Old 5th instar host larvae that are unlikely to be parasitized, and consume food unnecessarily, should be eliminated. Puparia may be found up to 21 days from the date marked on the cup, and the rearing unit can then be discarded.

- Handling of puparia and hibernation. Mature *A. samarensis* maggots become motionless within 3-4 h at 22°C and are still pale and soft. Pupariation is complete within 8-12 h. The hardened, brown puparia are washed with distilled water on a glass plate to remove adhering frass pellets and filaments. Godwin and Odell (1981) caution against unnecessary handling of puparia which caused 50-70% mortality in *B. pratensis* between pupariation and pupation. After cleaning and hardening, the puparia are spread on autoclaved, wet peat so that they do not touch each other (Fig. 29). The peat is placed 1 cm deep in the bottom part of a plastic Petri dish (Falcon Plastics, 150 x 25 mm). It is sufficiently wet if no more water is dripping from the dish. The puparia are then covered with another layer of wet peat for better protection against microorganisms. About 500 puparia can be placed in each plastic Petri dish. The Petri dish containing the puparia is placed over distilled water in a large plastic container with a tight-fitting cover, in which the dish is held on a modified rack for reagent tubes (Fig. 30). If the puparia are not covered by peat, which has antimicrobial properties, fungi (e.g. *Rhizopus* sp., *Pacilomyzus farinosus*) may develop on them. Also the cover of the water basin must be well closed to avoid contact of the material with the surrounding air, which often carries spores.

  If diapausing puparia are wanted for hibernation, the puparia are stored at 13.5°C with a 12:12 h (L:D) photoperiod at low light intensity to avoid unwanted eclosion of adults. After 30 days at this temperature, red eyes begin to show on the pupae inside the puparium. It takes 42 days to arrive at the pharate adult stage (Fig. 31). They are then transferred to 8-10°C for 10 days and thereafter stored at 2-4°C for hibernation up to 8 months. If the puparia are put too soon to 10°C, i.e. before the pharate adults are formed, the prepupal stage will not survive hibernation and eventually die. The puparia should therefore be held for 1-2 months at 13.5°C for prehibernation. After a minimum hibernation of 3 months, puparia can be removed from cold storage and incubated, first at 15°C for 5 days and then in an eclosion cage at 22-25°C.

  For continuous rearing of the fly, diapause is prevented if puparia, once formed, are held at 20-25°C for pupation and eclosion. Any exposure of newly formed puparia to 15°C for 1 week or longer will induce diapause, in at least part of the material.

- Cleaning procedures. All equipment and cages must be scrupulously clean. Incubators and plant growth rooms must be washed on the walls and tables with Sporicidin disinfectant (Sporicidin International, Rockville, MD, USA; distributor in Canada: Canadawide Scientific Ltd., Ottawa) prior to use. Tulle cages are rinsed with hot tap water to remove the old honey-agar jelly, thoroughly wetted with 70% ethanol and let dry before the next use. Tulle covers that show mouldy spots must be replaced. Smaller tulle cages can be disinfected by exposure to ultraviolet light in a "Steril Gard" hood (Scientific Products ESBE, Ville Saint-Laurent, QC) for 20 min. For contamination-free storage, tulle cages can be deposited in large plastic bags that are hermetically sealed. All glassware (vials and petri dishes) and tubes for mating must be washed thoroughly in Sparkleen (Fisherbrand) solution. The cheaper

containers (SOLO cups and styrofoam drinking cups) should be discarded after use. For Eraware containers, immersion for 24 h in diluted hypochlorite solution (2% of commercial bleach) and subsequent drying is recommended. The unused gypsy moth larvae and old diet are autoclaved in Fisherbrand autoclave bags. To rapidly kill the insects, use cold rooms set at -15°C. The large old caterpillars from the compound rearing cups can be drowned in 50% alcohol. Foam plastic stoppers should be replaced when mouldy spots appear on them. Table surfaces and interior walls of rearing rooms should be washed frequently with Sporicidin disinfectant solution or with 70% ethanol.

- Parasitoid production. Under mass-rearing conditions, the average fecundity of an *A. samarensis* female is about 30 puparia produced per lifespan, although it may be 50 puparia per female, on the average, if the rearings are carried out very painstakingly with less material (Quednau 1993). As an example, the yield of puparia with 2-day interval collections in a post-hibernation rearing in 1996 is presented in Fig. 32. In the 100 cages used, there was a 14-day difference between the first and the last cages prepared for host exposures, because not all *A. samarensis* females had terminated gestation at the same time. There was a distinct peak in the number of puparia collected after the tenth day counted from when the first puparia were found, reflecting the synovigenic progeny disposal of the fly (Quednau 1993). The maximum number of puparia obtained during a single 2-day interval was 500, the total number of puparia produced 4597. In a more recent similar rearing, 6223 puparia were obtained.

Table 1 shows the production of *A. samarensis* at the Laurentian Forestry Centre in 1996 and their utilization. Table 2 gives the principal mortalities observed during incubation of puparia in post-hibernation and second-generation rearings. Considerable mortality by factors difficult to control occurs during the propagation of the flies and some of them are listed in Table 3. Recently *Leptomonas* sp. (Trypanosomidae) was found in puparia containing prepupae that would not transform into pharate adults and eventually decompose.

Carl and Kenis (1990) report 85-96% eclosion of *A. samarensis* puparia when field-collected, parasitized gypsy moth larvae were reared in the laboratory at 26°C and 18:6 h (L:D) photoperiod in Europe. Avoiding the diapause occurred when these conditions acted primarily between pupariation and pupation. It was not possible to obtain the same high eclosion rate at the Laurentian Forestry Centre. Also the exact environmental conditions for the hosts attacked in the field in Europe are not known and are likely to be different from those used in the laboratory in Canada.

Losses in the fly rearings can be counter-balanced by obtaining a high percentage of mating and excellent survival of flies during gestation and ovolarviposition.

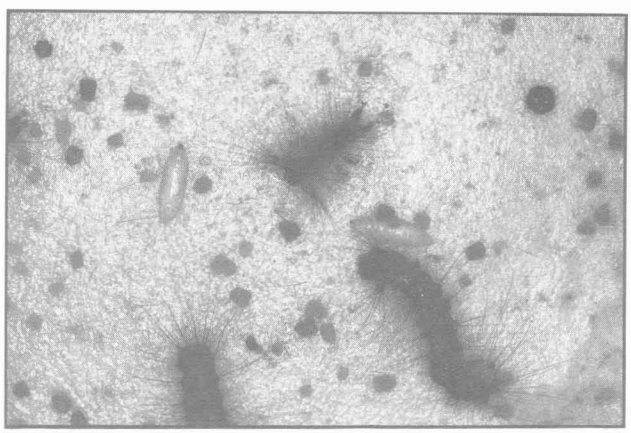

Figure 27. Mature *A. samarensis* maggots that have bored out of their hosts.

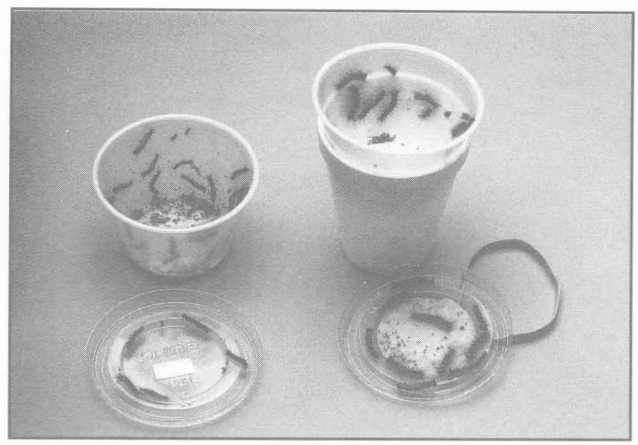

Figure 28. Rearing cups with parasitized gypsy moth larvae. Left: simple cup (type 2) with diet at the bottom. Right: compound cup (type 3) with diet on the cover.

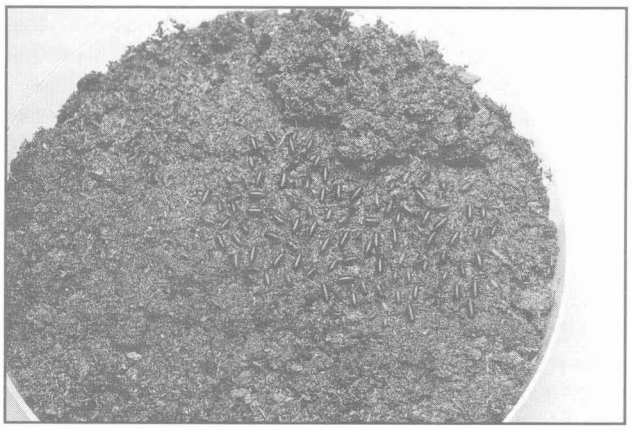

Figure 29. Puparia of *A. samarensis* on autoclaved peat in Petri dish.

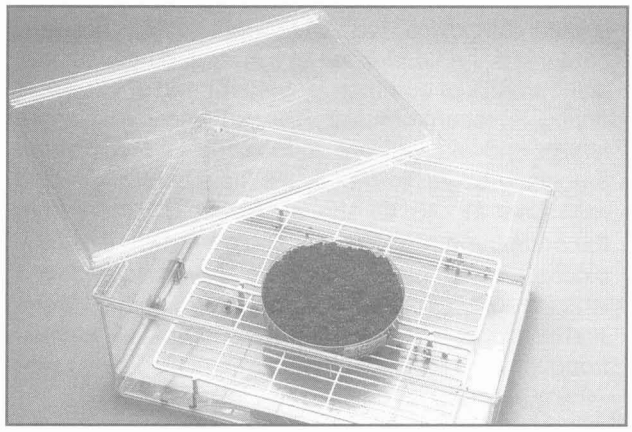

Figure 30. Plastic container to hold puparia of *A. samarensis* in a Petri dish with peat in water-saturated atmosphere for hibernation.

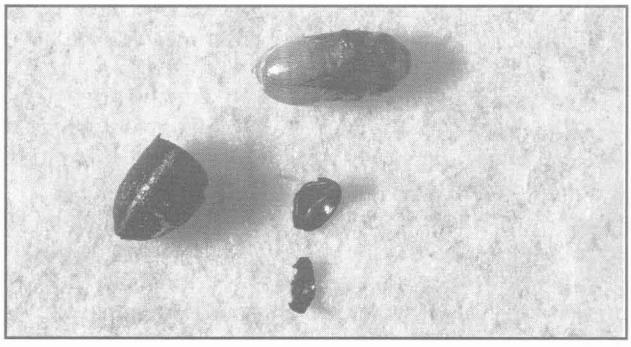

Figure 31. Pharate adult of *A. samarensis* taken out of its puparium.

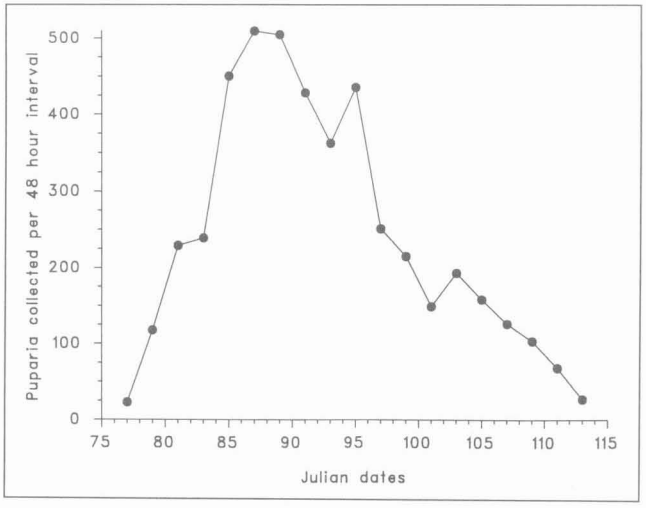

Figure 32. *A. samarensis*, yield of a second-generation rearing in 100 host exposure cages (two parasitoids per cage, rotation of 30 host larvae per cage every 3 days). Total yield: 4597 puparia.

REARING OPERATIONS

The different operational steps in rearing *A. samarensis* are summarized in Fig. 33, and the ambient conditions (temperature, humidity and light) required for each phase of the operation are listed. As in nature, the perfect synchronization of host plants, gypsy moth larvae and gestated (gravid) parasitoids is the key to the success of the whole rearing. Proper timing and planning the different operational steps is therefore imperative.

To help you to develop a plan for the implementation of the rearing, especially with a view to field releases, a timetable is presented in Fig. 34. One complete generation turnover for a larger cohort (about 200 gravid females) in a 22:15°C (12:12 h; day:night) thermoperiod will take 2½ months. It takes only 1½ months at 22°C with a 18:6 h (day:night) photoperiod.

FACILITIES

The following are different types of rooms needed for the efficient rearing of *A. samarensis*:

Room No. 1. Humidified. Fluorescent lighting. Thermoperiod: 20:15°C (12:12 h; day:night). Hold males after eclosion. Expose host insects in exposure cages to parasitoids.

Room No. 2. Humidified. Fluorescent lighting (dim light). Thermoperiod: 20:15°C (12:12 h; day:night). Hold mated females during gestation.

Room No. 3. Humidified. Weak fluorescent lighting. Thermoperiod: 15:10°C (12:12 h; day:night). Hold males that have already copulated.

Room No. 4. Not humidified. Fluorescent lighting. Thermoperiod: 20:15°C (12:12 h; day:night). Hold rearing cups (both types) with parasitized host larvae, if diapausing puparia are needed.

Room No. 5. Not humidified. 22-25°C. Fluorescent lighting. Use for eclosion cages and for containers holding parasitized host larvae in accelerated operation.

Incubator. 25°C, not humidified. Darkness. Incubate gypsy moth egg masses.

Incubator. 15°C, humidified. Fluorescent lighting (18:6 h; day:night). Hold puparia temporarily for second-generation rearing.

Incubator. 13.5°C, humidified. Fluorescent lighting (12:12 h; day:night). Hold puparia prior to hibernation.

Cold room. (2°C, humidified). Darkness. Hold hibernating fly puparia and oak seeds.

Cold room. (7°C, humidified). Fluorescent lighting. Hold small oak trees with tender foliage. Hold gypsy moth egg masses.

Cold room. (7°C, not humidified). Fluorescent lighting. Hold 2nd-instar gypsy moth larvae on diet.

Greenhouse.

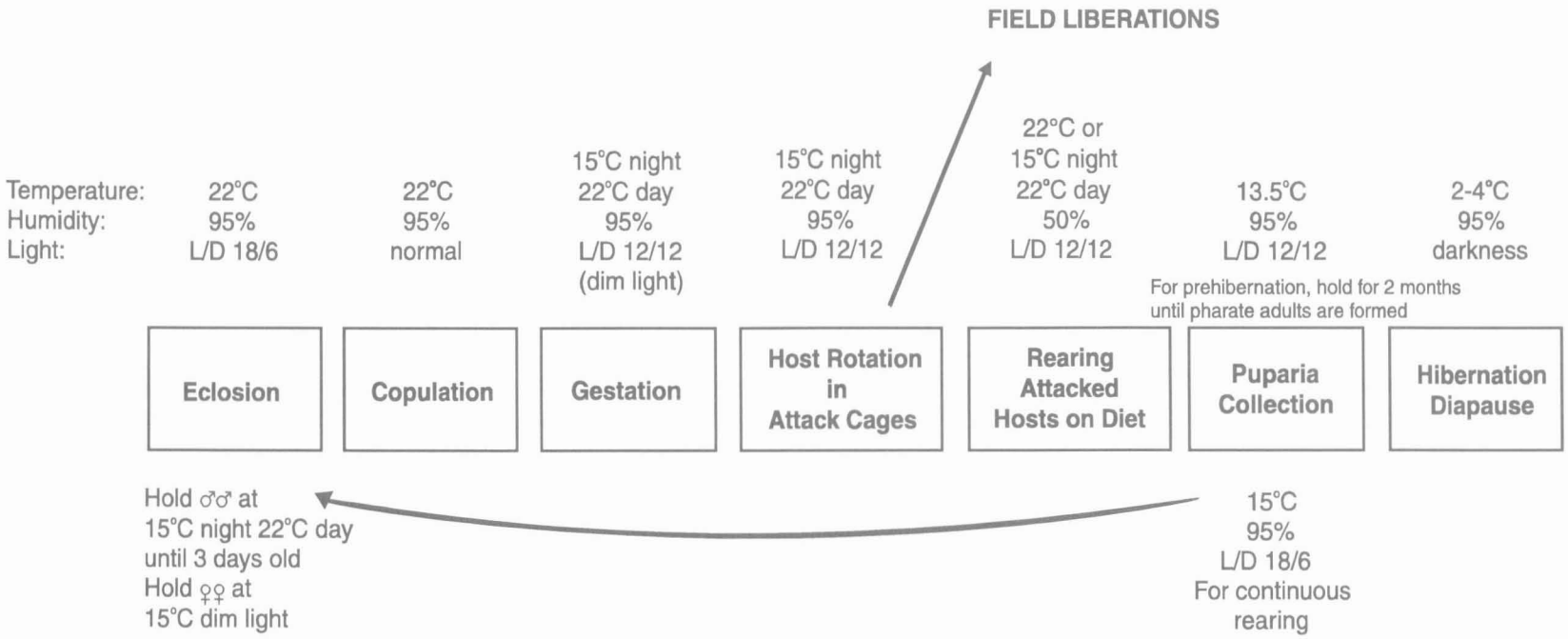

Figure 33. *A. samarensis* rearing operations and abiotic environmental requirements.

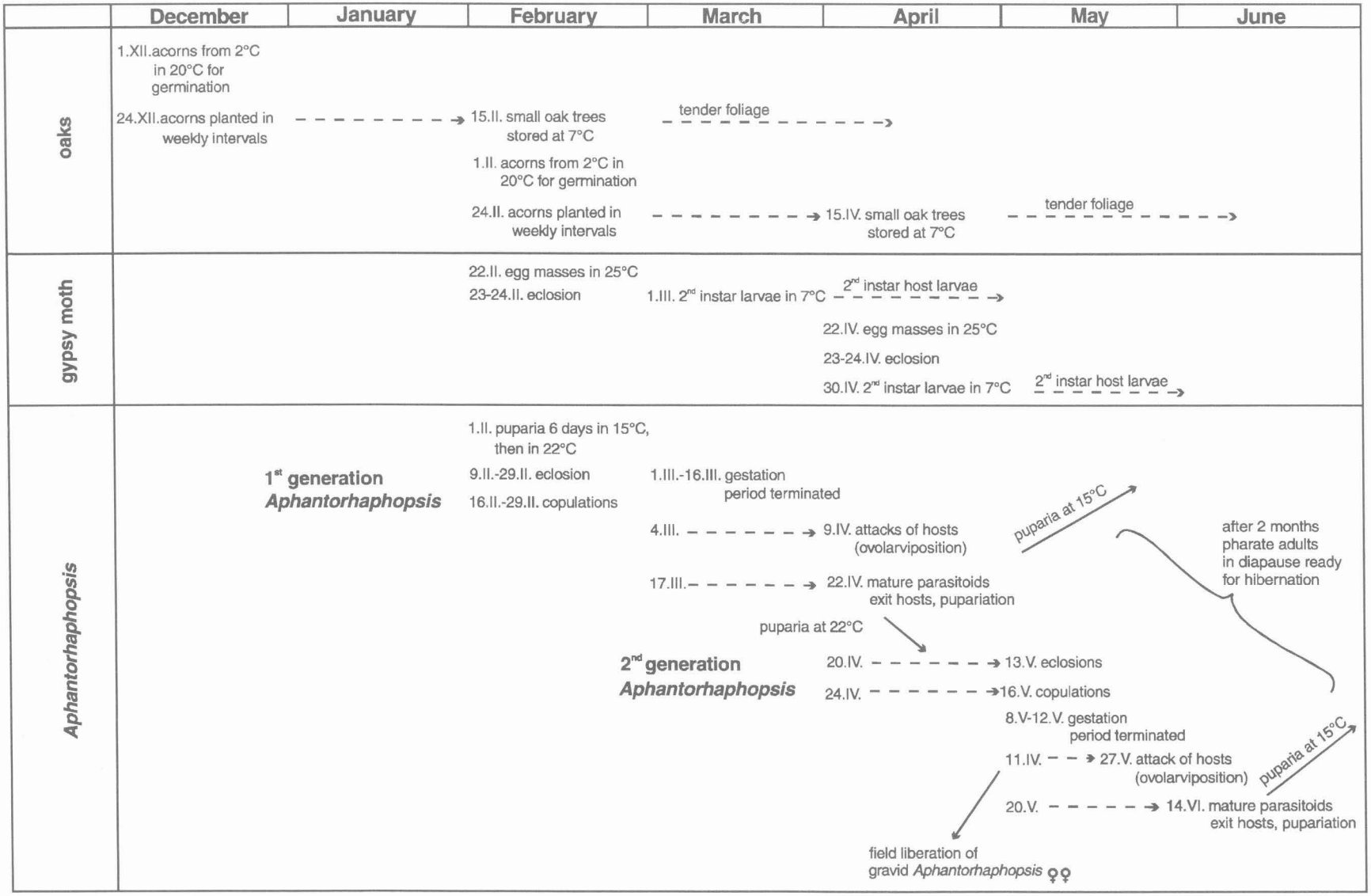

Figure 34. Timetable for events during *A. samarensis* rearing in the laboratory.

Table 1. *A. samarensis* rearings in 1996.

Rearing after hibernation (puparia 5 months at 2°C)	**Rearing 2nd generation** (puparia 1 month or less at 15°C)		**Rearing 3rd generation** (puparia 12 days or less at 15°C)	
1700 puparia	4587 puparia		2000 puparia	
↓	↓		↓	
707 eclosions (41%) at 22°C	2485 eclosions (55%) at 25°C		951 eclosions (48%) at 25°C	
↓	↓		↓	
275 copulations (80%)	1063 copulations (83%)		401 copulations (85%)	
↓	↓		↓	
survived 200 ♀♀ after gestation (70%)	survived 500 ♀♀ after gestation (47%)		survived 182 ♀♀ after gestation (45%)	
↓	↙	↘	↙	↘
4587 puparia	2030 puparia	200 ♀♀ liberated	132 ♀♀ retained	50 ♀♀ to USDA
			↙	↘
			500 puparia retained	1000 puparia to USDA

Table 2. Mortality of *A. samarensis* puparia.

Pharate adults not eclosed	Killed by fungi	Dead during eclosion	Pupae not formed	Empty puparia (decomposed)	Healthy, eclosed flies	Total number of puparia examined
			Post-hibernation rearing in 1996[1]			
274 (15%)	551	16	88	74	707 (41%)	1714
			Second-generation rearing in 1995[2]			
150 (11%)	42	25	181	0	945 (70%)	1343
			Second-generation rearing in 1996[2]			
120 (8%)	42	48	274	83	861 (60%)	1428

[1] Hibernation at 2°C was 5 months. Diapause apparently not terminated in 15% of individuals.
[2] Puparia held at 15°C for 2-3 weeks prior to incubation. Diapause induced in 8-11% of individuals.

Table 3. Principal mortalities in *A. samarensis* rearings.

Event	% mortality	Reason
Post-hibernation puparia (before eclosion)	50-60	Fungi, pharate adults fail to eclose
Non-hibernating puparia (before eclosion)	25-40	Pathogens, pupae not formed inside puparium, pharate adults fail to eclose
Females during gestation	10-50	Bacteria, starvation
Ovolarvipositing (gravid) females	20-50	Weakness, wing damage

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