6. Diet Preparation

6.1. INTRODUCTION

The procedures for preparing codling moth diets are not uniform from diet to diet, partly because the diets have different ingredients and partly because each laboratory has developed its own unique procedures including the exact sequence of adding the ingredients. The detailed preparation procedures used for diets listed in Tables 3 and 4 can be found in the published papers. Even though there may be some flexibility in exactly how a diet is prepared, there are some common practices in preparation. This section summarizes these common procedural practices.

Singh (1977) reviewed the subject of insect diets — content and preparation, and summarized the preparation procedures of 15 diets for the codling moth.

According to Moore (1985):

- "Good laboratory and chemical techniques should be used including accurate weighing of ingredients and complete mixing, especially of the smaller quantities. These small quantities may be incorporated by trituration (grinding to a fine powder) with sucrose or an inert material such as cellulose, or by dissolving in an organic solvent such as alcohol or ether, applying to the solid components and evaporating the solvent.
- Most diets require heating to dissolve the agar, and the diet may be sterilized as a part of the process. Sterilization may cause the breakdown of some ingredients, but Vanderzant (1969) suggests that there may be some desirable effects also — heating stops enzyme action in plant products, ruptures cells and affects solubility of ingredients. Heating should be at the lowest practical temperature and the shortest time to minimize any detrimental changes in the diet. Flash sterilization (Sikorowski and Goodwin 1985) heats the diet to an elevated temperature for a short time, and produces a better quality diet than the conventional autoclave (Sikorowski and Lawrence 1994a).
- Care should be taken to insure that condensation of moisture from the diet does not occur and drown the young larvae or induce contamination in the diet."

Some common practices in diet preparation:

- Follow aseptic procedures in the laboratory. The diet preparation room should be scrupulously clean (Reed and Tromley 1985). Working areas and equipment must be cleaned regularly with a disinfectant, e.g. NaOCl, or anti-microbial agent. Glassware should be washed with hot soapy water, rinsed with distilled water and covered until used (section 22.7).
- Some ingredients require overnight soaking prior to preparing the diet, e.g. whole beans.

- Diet trays must be washed with strong detergent (e.g. trisodium phosphate) and water, sterilized with steam and dipped in a NaOCl (bleach) solution.
- Ingredients should be weighed and measured ahead of the actual diet preparation (**Figure 3**).
- Some ingredients are needed in very small quantities and therefore these are often mixed with other larger-volume ingredients before diet preparation. This premix permits a thorough distribution of each ingredient, ensuring a good mixing of the ingredients with other materials in the final diet. A premix can be stored and used for several batches of diet over time.

FIGURE 3
Augers, hopper and balance to deliver high-volume diet ingredients from storage room to cooking room and kettles below. (OKSIR facility, Osoyoos, Canada)

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The same principle applies to liquid ingredients that can be diluted and bulked in water (e.g. ascorbic acid) or other solvent.

- Some ingredients should be dissolved in water before being added to the diet.
- Cholesterol and lipids should be dissolved in acetone, hot ethyl alcohol or methylene chloride (dichloromethane). In a fume hood allow the solvent to evaporate; then add the fatty ingredients to the diet (Moore 1985).
- Carboxy methyl cellulose (CMC), a thickening agent, must be wetted with alcohol or other wetting agent to prevent lumping (Howell 1972a, Howell and Clift 1972).
- Tween 80 may be added to act as an emulsifier for fat-soluble ingredients.

FIGURE 4
Steam-jacketed kettles for cooking the diet, each with two counter-rotating paddles to mix and stir the diet

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Note blue ducts from ceiling to each kettle which automatically deliver via augers weighed amounts of high-volume ingredients. (OKSIR facility, Osoyoos, Canada)

FIGURE 5
Diet is dispensed on to trays and then smoothed with a wide spatula. (OKSIR facility, Osoyoos, Canada)

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- Acetone or ethyl alcohol may be used as solvents for mould inhibitors, e.g. methyl *p*-hydroxybenzoate, potassium sorbate (Moore 1985).
- Mix ingredients thoroughly in a blender, or for large volumes in a steam-jacketed kettle that has mixing paddles (**Figure 4**). Heat according to the type of diet being prepared. In some laboratories the diet is autoclaved (L. Neven, pers. comm.). Heating sterilizes the diet, and some ingredients may need to be cooked to make them edible.
- Some ingredients can be sterilized without heating, e.g. liquids passed through a membrane filter, e.g. Millipore (Rock 1967).
- Adjust the pH of the diet by adding an acid or a base.
- Add ascorbic acid and other ingredients that are heat sensitive after the diet has cooled to 60°C.
- During diet preparation, workers must wear face masks to protect them from hazardous dusts and fumes, and rubber gloves to protect them from corrosive liquids. Diet handling and preparation rooms must be well-ventilated to remove hazardous fumes and dusts (section 21).

FIGURE 6
Two parallel diet-dispensing lines with belts showing enclosed aluminium portions (with blue units) that include paraffin wax sprayers and steam scarifiers. (OKSIR facility, Osoyoos, Canada)

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- Mechanizing and automating procedures (Harrell and Gantt 1984) can reduce labour costs and also accelerate the processes, e.g. an auger and hopper connected to a balance so as to deliver a predetermined amount of an ingredient, water pump to deliver a predetermined amount of water, diet pump, automated tray-filling system and line, automated paraffin wax sprayer and scarifier (**Figures 5** and **6**).
- Hot paraffin wax can be sprayed onto the surface of freshly dispensed diet in a tray using a hot pressure system (Howell 1967; Mani et al. 1978), spread onto the diet with a brush (Ashby et al. 1985), or sprinkled onto the diet using a modified 'salt shaker'. The film of wax must be somewhat porous and be about 0.75 mm thick (Ashby et al. 1985; Howell 1967). The wax layer slows dehydration and discourages mould growth (Howell 1967). Immediately after applying wax, scarify or roughen the surface with hot steam jets (preferable) (**Figure 6**) or with a sterile fork (depth about 0.4 cm) (Brinton et al. 1969). A rough surface encourages neonate larvae to enter the diet (Beck and Chippendale 1968, Bathon et al. 1991).

- To avoid infection by microbes (section 15), Batiste and Olson (1973) encased newly prepared trays of diet in polyethylene bags which were blown up, each having an air inlet and exhaust. Regulated clean air with a constant RH was blown into the bag throughout the growth period of the larvae. In South Africa, brown paper bags are used to cover trays of diet to minimize contamination and keep the RH high (Addison and Henrico 2005; D. Stenekamp, pers. comm.).

Navon and Moore (1971) minimized desiccation by putting the diet into polyethylene bags and then punching holes in the bags to permit larval penetration. These authors also tested polyethylene film instead of the bags.

6.2. DIETS FOR LEPIDOPTERA

The preparation of the Ivaldi-Sender general-purpose diet is described in Table 1 and that of the Singh general-purpose diet in Table 2. Consult Ivaldi-Sender (1974), Singh (1983, 1985) and Bathon et al. (1991) for details.

6.3. DIETS THAT STAY MOIST AND SOFT

At an early stage of diet preparation, dissolve agar powder in hot water and boil until the agar solution thickens and, when removed from the container, forms a gel. When the agar solution cools to about 60°C, add other diet ingredients.

Note items discussed in section 6.1.

6.4. DIETS THAT DRY OUT AND HARDEN

On the day before diet preparation, shred the sheets of paper pulp and soak in water overnight (this encourages the rapid disintegration of the pulp during diet preparation). Also sift and weigh (or measure) dry sawdust.

The exact amount of water added to the diet during preparation can be determined only through experience. Brinton et al. (1969) stated that "It is most important to get the correct consistency, and this can be achieved only through experience." Ashby et al. (1985) noted that "sterile water can be added if the finished diet is too dry".

Note items discussed in section 6.1.

6.5. EQUIPMENT FOR PREPARING DIETS

Standard laboratory equipment includes: glassware, Petri dishes, pipettes, holding and mixing containers, plastic bottles, mixing tools, fume hood, laminar-flow hood, balances (several kinds), magnetic-stirring hot plate, oven, microwave oven, thermometers, pH meter, timer, graduated cylinders, refrigerator, freezer and water distiller or water-filtration system. Equipment (Annex 1) that is somewhat unique to preparing insect diets includes: sawdust sifter, paper-pulp chopper (hammer mill), grinder, shaker/mixer (dry diet ingredients), blender/mixer, steam-jacketed kettle with counter-rotating paddles or autoclave or flash sterilizer, vat mixer, heated pressurized sprayer (paraffin wax), steam-jet system (scarify diet in tray), augers, holding/cooling tanks, diet dispensing system with tray-handling

equipment, trays, carts (hold trays), diet pumps, water pump, tray washer and cart washer.

Papers in the book edited by Singh and Moore (1985) included lists of relevant equipment. Chapter 12 in Cohen (2004) provided information on equipment used for processing insect diets. In Annex 2, Butt (1975) listed the mixers used to prepare various diets. Edwards et al. (1996) and Miller et al. (1996) described the extrusion technique used in processing the diet of the pink bollworm.

7. Disposing of Spent Diet and Liquid Wastes

7.1. ENVIRONMENTAL CONCERNS

Mass-rearing facilities (**Figure 7**) using an artificial diet produce a large quantity of solid and liquid waste, i.e. spent diet (**Figure 8**) and cleaning and washing liquids. When such a facility is constructed, arrangements must be made for the disposal of these wastes in an environmentally acceptable way. If this is not done properly, then environmental pollution will occur followed by community problems (IAEA 2008).

Liquid wastes must be treated before being released into nearby bodies of water. Solid wastes after treatment may be buried in a sanitary landfill, burned, or sold as fertilizer.

If Calco Red dye is present in the spent diet, the dye might remain non-degraded in a landfill for many years; this matter must be considered before such diet is buried.

7.2. TEMPERATURE TREATMENT

Treatment by heating is a common method of disposing of spent diet; heat destroys any living organisms in the diet. In the OKSIR facility, spent diet is placed into

FIGURE 7
OKSIR codling moth mass-rearing facility at Osoyoos, Canada,

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FIGURE 8
Tray of spent diet, showing empty pupal cases projecting out of diet. (OKSIR facility, Osoyoos, Canada)

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a special 'heat treatment room', held at 60ºC for three days (Cossentine et al. 2005) (section 15.3) and then taken to the community landfill.

Toba and Howell (1991) suggested that paper trays and cardboard strips be held in a room at about 49ºC for 12 h to kill any insects present before discarding them. They also suggested that spent diet be frozen at about –18ºC for two days to kill any insects present; the same treatment is applied to used diet trays before cleaning and reuse.

Adults from oviposition cages are killed by freezing for several hours.

7.3. GRINDING

Grinding the spent diet to a powder destroys any living insects in the diet, and then this ground diet could be buried in a landfill. However, Cossentine et al. (2005) pointed out that grinding the spent diet creates dust in the immediate vicinity of the rearing facility. This dust could contain the CpGV (section 15.3), and it could be drawn into the facility through air-intake ducts (sections 22.6 and 22.7) (Gast 1968). This disposal method is no longer recommended.

7.4. LANDFILL

It is relatively easy and inexpensive to dispose of solid wastes in a sanitary landfill providing the environmental and legal issues have been addressed. As already noted (section 7.1), if Calco Red dye is present in the spent diet, the dye might remain non-degraded in a landfill for many years.

Moths that have been killed by freezing should be placed into degradable containers and buried in a landfill.

Workers handling spent diet and moths must wear face masks to protect themselves from dust and moth scales.

7.5. LIQUID WASTES

Liquid wastes must be treated before being released into nearby bodies of water. If a facility can not use the local sewer system and local treatment plant for the disposal of liquids, then an independent treatment operation must be built and operated by the facility (Wyss 2002). Liquid waste must not be dumped in an untreated form into a nearby body of water. Special arrangements may be needed to dispose of chemicals.

8. Seeding the Diet with Eggs or Larvae

The next sections on rearing provide information from Singh (1977), Reed and Tromley (1985) and Ashby et al. (1985), but also from personal experience in rearing the codling moth using the Brinton et al. (1969) diet, modified as per Proverbs et al. (1982), at the Pacific Agri-food Research Centre (PARC 2007) in Canada during 1982–1993.

8.1. EGGS

8.1.1. Eggs on Waxed Paper Sheets

Codling moth females lay circular, thin, flat eggs on any smooth surface. Waxed paper has been used to collect eggs since 1952 (Dickson et al. 1952) and it is certainly the most common oviposition substrate (**Figure 9**). Paper waxed on both sides is more sturdy and durable (especially after surface sterilization) than paper waxed on only one side. Other surfaces tested include plastic sheets (White and

FIGURE 9
Trays of freshly prepared diet on a cart

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Egg sheets are placed on wire mesh racks above the diet. Wall of rearing room (behind cart) has small holes at many levels through which regulated/conditioned air enters the room from an air plenum (vertical fluorescent lights inside air plenum) to pass over the trays (OKSIR facility, Osoyoos, Canada).

Hutt 1971; Mani et al. 1978; Bathon et al. 1991) and plastic pellets (Hathaway et al. 1972, 1973; Toba and Howell 1991).

Following microbial de-contamination (section 12.6), sheets with eggs are laid onto pieces of wire mesh over freshly prepared diet in trays (Bloem et al. 2000). Sheets laid directly onto diet encourage the growth of mould (Howell and Clift 1972). The exact size of the egg sheet will depend on the dimensions of the roll of waxed paper being used and how the paper has been cut, but it should cover almost the entire surface of the diet so that it becomes seeded uniformly throughout.

The number of eggs per sheet placed on a tray is important for rearing efficiency — too few eggs result in wastage of the diet and too many eggs result in wastage of insects due to competition for food (Howell 1970, 1971). Brinton et al. (1969) used an egg density of about 5 eggs/cm^3 of diet. Bloem et al. (1997, 2000) used 2500–3000 eggs on each tray of diet (about 3 L diet) as did Taret et al. (2007). Howell and Clift (1972) infested each tray with 1500–3000 eggs. In South Africa, 625 eggs/L diet are placed on freshly prepared diet (D. Stenekamp, pers. comm.). However, the appropriate number of eggs for each mL of diet is dependent on many factors and should be determined for each rearing situation.

Eggs on the egg sheets should be at the black-head stage and will begin hatching within the next day or two. When eggs hatch, the neonate larvae spin silken threads and lower themselves to the diet surface to enter the diet. The light intensity in the rearing room should be subdued (Brinton et al. 1969); bright light discourages larvae from entering the diet and they wander off (Batiste and Olson 1973; Reed and Tromley 1985).

Egg sheets should be removed from the trays when all viable eggs have hatched (2–7 days) (Brinton et al. 1969; Howell 1971; Howell and Clift 1972; Ashby et al. 1985; Toba and Howell 1991; Bloem et al. 2000). Removing the used egg sheets permits better air circulation over the diet surface and thus helps prevent the growth of mould.

8.1.2. Eggs in a Slurry

The pink bollworm produces eggs that are round, and this permits their easy removal from the oviposition substrate and accumulation in a liquid carrier, forming a slurry (Stewart 1984). The number of eggs per mL of slurry can easily be determined, and it enables the eggs to be delivered onto the diet surface volumetrically and mechanically and in known numbers. This system is used for the pink bollworm (Stewart 1984), cabbage looper (Leppla et al. 1974), boll weevil (Goodenough 1984, Roberson and Wright 1984, Smith 1999, Wood and Wendel 1999) and Mediterranean fruit fly (Schwarz et al. 1985). According to Roberson and Wright (1984), a pump and sprayer "deliver 4 mL of eggs (2100 eggs) in a furcellaran solution (0.5%) to the diet surface. The furcellaran solution is used to suspend the eggs, thus enabling uniform distribution with spraying."

8.2. LARVAE

For rearing on agar-based diets in small cups (Howell 1970) and on individual immature apples, the standard procedure is to place neonate larvae, one by one, onto the diet (Bathon et al. 1991). However, Howell (1971, 1972c) also placed neonate larvae individually onto diet in trays.

The procedure is as follows:

- Sterilize a small fine-tipped brush with alcohol, then with acetone and finally rinse in distilled water before picking up a larva with the brush (Howell 1971) or dip the brush in a 0.5% solution of NaOCl, then in distilled water (Hathaway et al. 1971).
- Pick up a larva from the egg sheet with the fine point of the wet brush and transfer it to the diet.
- Sterilize the brush **each** time a larva is transferred from the egg sheet to the diet.

This procedure is very time consuming and laborious, but in experimental situations and to prevent contamination with microbes, it is necessary to follow the procedure.

9. Rearing, Sexing and Collecting Larvae

9.1. ENVIRONMENTAL CONDITIONS

- **Temperature:** 25–28°C
  Hutt (1979), Proverbs (1982), Proverbs et al. (1982) and Dyck et al. (1993) suggested that the field competitiveness of mass-reared adults during cool weather might be improved if they were reared under fluctuating temperature simulating that occurring in the spring. Guennelon et al. (1981) used 25°C during the day, 20°C during twilight and 15°C during the night. Bloem and Bloem (1995, 2000) and Bloem et al. (1998a) used 21°C for 12 h and 33°C for 12 h, and found that dispersal activity in the field increased slightly. However, with this regime, moth production was reduced, the operational costs of making the temperature changes were high and the small benefits were not regarded as cost-efficient. However, in a non-peer reviewed document, Jallow and Judd (2007) concluded that "moths reared under fluctuating temperatures were significantly more competitive than moths reared under either constant temperature or through diapause. These effects were observed regardless of the sampling method (i.e. capture in pheromone-baited traps or in mating tables)". These results were unexpected (Bloem et al. 1998a) and indicate that an optimal rearing strategy has not yet been identified (sections 13, 14 and 19.4).

- **Relative Humidity:** 50–80%, usually 60–70%
  It is important to control the RH when mass-rearing in trays of diet, but the ambient RH is relatively unimportant when rearing in closed containers. For diets that dry out and harden, RH is critical. A reduction in RH as the diet dries out is required, e.g. from 75 to 55 or 50% within three weeks (Bloem et al. 2000; Taret et al. 2007).

- **Light:** Photophase:scotophase 16L:8D–18L:6D
  The main purpose in controlling light is to establish the photophase period to avoid diapause (long photophase, short scotophase) or to induce diapause (short photophase, long scotophase) in larvae (section 13). Also, dim or subdued light is required when neonatal larvae are entering diet in trays (section 8.1.1). The photophase that produces non-diapause larvae is not the same in all geographic locations and for all strains, therefore, local considerations are important. However, a photophase:scotophase of

18L:6D will avoid diapause development at all locations. Since in nature larvae develop inside fruit with low light intensity, they are quite sensitive to daylength, and light intensity in a rearing room can be quite low but still achieve daylength control for the larvae.

- **Air Movement**
  Air movement, both the direction and speed, must be regulated for diets held in open trays and especially diet that dries out and hardens. Horizontal (laminar) air flow between vertically stacked trays on carts is absolutely necessary to control the rate of drying of the diet and to suppress the growth of mould. Howell (1971) used two complete air exchanges per minute, but Brinton et al. (1969) found that three exchanges per minute were required. In the OKSIR facility, horizontal air flow is provided by air entering the room from many small holes in the side walls; each tray receives air from a row of holes just above it (**Figure 9**). Mani et al. (1978) described a similar system in which air was blown over trays of diet at a speed of 5–10 cm/sec.

9.2. MORTALITY

The highest mortality during the larval stage is for neonatal larvae between the time of eclosion and entry into and satisfactory feeding on diet in trays.

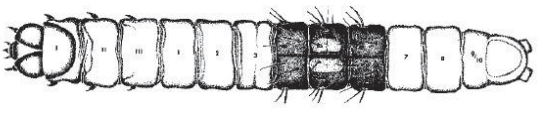
9.3. SEXING MALE AND FEMALE LARVAE

The identification of sex of mature (5th instar) larvae relies on the appearance of the testes as two dark elliptical bodies in the 5th abdominal segment, one on either side of the midline in male larvae (Proverbs and Newton 1962a; Hamilton and Hathaway 1966; Hansen and Harwood 1968; Reed and Tromley 1985; Beeke and de Jong 1991) (**Figures 10 and 11**).

9.4. COLLECTING LARVAE

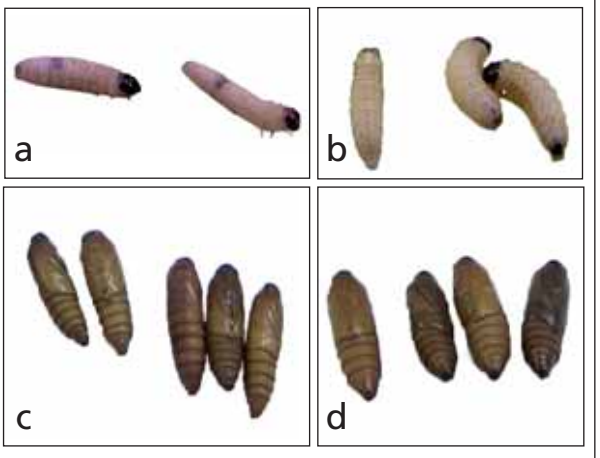
Collecting larvae is relatively easy if a soft diet is used, but it is still a laborious manual task when working with a large number of insects. However, the task is much easier if heat is used to drive the larvae out of the diet (Brassel 1978).

FIGURE 10
Mature male lepidopteran larva showing location of testes in 5th abdominal segment



Source: Beeke and de Jong (1991)

FIGURE 11
Codling moth larvae and pupae



A – male larvae, showing dark testes in abdomen,
B – female larvae,
C – male pupae,
D – female pupae

Source: Taret et al. 2006

10. Rearing, Sexing and Collecting Pupae

10.1. ENVIRONMENTAL CONDITIONS

- **Temperature:** 24–28ºC

- **Relative Humidity:** 20–70%, usually 50–60%
  It is very important to control RH when mass-rearing in trays, but the ambient RH is relatively unimportant when rearing in closed containers. For diets that dry out and harden, the RH may be quite low during pupal development and adult emergence (Bloem et al. 1998a, 2000).

- **Light:** Photophase:scotophase 16L:8D–18L:6D
  The main purpose in controlling light is to establish the photophase period, particularly for developing larvae. However, many mass-rearing systems keep pupae in constant darkness so that emerging adults are attracted to and move towards a UV light source. The adults are then trapped in a container which may be located in a cold room (0–2ºC) to inactivate them.

- **Air Movement**
  Air movement, both direction and speed, must be regulated for diets held in open trays and especially diet that dries out and hardens. Horizontal air flow between vertically stacked trays controls the rate of drying of the diet and suppresses fungal growth. Howell (1971) used two air exchanges/min, but Brinton et al. (1969) found that three exchanges/min were required. In the OKSIR facility, horizontal air flow is provided by air entering the room from many small holes in the side walls; each tray receives air from a row of holes just above it (**Figure 9**). Mani et al. (1978) described a similar system in which air was blown over trays of diet at a speed of 5–10 cm/sec.

10.2. MORTALITY

Pupal mortality is usually low.

10.3. SEXING MALE AND FEMALE PUPAE

Peterson (1965) described four dark segmental bands beyond the wing pad tips on the ventral surface of male pupae; female pupae have only three bands (Reed and Tromley 1985). Males have four freely articulated abdominal segments caudal

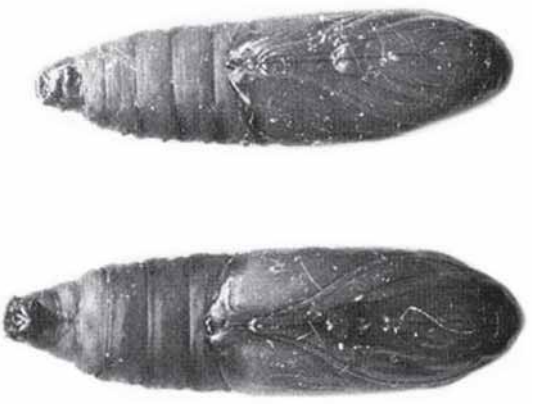
FIGURE 12
Ventral view of abdomen of female pupa of the codling moth

Source: Howell (1991)

FIGURE 13
Ventral view of abdomen of male pupa of the codling moth

Source: Howell (1991)

FIGURE 14
Ventral view of male (upper) and female (lower) pupae of the codling moth.



Source: Bathon et al. (1991)

to the wing pads, but females have only three. Bathon et al. (1991) describe this difference as five abdominal segments ventrally in the male and four in the female (**Figures 12–14**). Female pupae tend to be larger in diameter and heavier than male pupae (Reed and Tromley 1985, **Figure 14**). However, probably the easiest morphological feature is the location and appearance of the genital aperture (pore)

on the ventral side of the abdomen. The ostium bursae of the female pupa is found on the 8th and 9th abdominal segments (**Figure 12**), but the genital ostium of the male pupa is entirely within the 9th segment (**Figure 13**) (Bathon et al. 1991; Beeke and de Jong 1991; Howell 1991).

The sexes cannot be satisfactorily separated by machine. However, a sizing machine (Schoenleber et al. 1970) used rollers to divide pupae into 10 groups based on their diameter (Goodenough 1984). There is considerable overlap in size between the sexes; only in class 1 were there 100% males (10% of males, the smallest), and only in classes 9 and 10 (21% of females, the largest) were there 100% females (Schoenleber et al. 1970).

10.4. COLLECTING PUPAE

Collecting pupae from soft diet is done by allowing mature larvae to spin cocoons in corrugated cardboard strips placed on top of the diet (Dickson et al. 1952; Hamilton and Hathaway 1966; Hathaway et al. 1972; Howell and Clift 1972; Toba and Howell 1991). After pupation, the cardboard strips are torn open to break the cocoons and then the pupae are collected. Bathon et al. (1991) used rolls of polyethylene strips, and Proverbs and Newton (1962a) used slotted wooden strips held together with rubber bands.

Larvae tend to remain in diets which dry out and harden, and they form cocoons and pupate inside the diet; adults emerge directly from the diet. Collection of pupae is quite difficult, and involves breaking up the diet and removing the pupae individually. Carpenter et al. (2004) attempted to find a method of extracting pupae efficiently. This included dissolving the silk cocoons by agitation in various concentrations of NaOCl and pressure washing the dry diet with water and NaOCl. Extracted mature pupae tolerated the treatment but only about 50% of adults emerged, and larvae and newly formed pupae were killed. Harvesting pupae of some lepidopteran species has been mechanized (Stewart 1984; Nordlund 1999). Pupae can be surface-sterilized by dipping in a 3% NaOCl solution (Bathon 1981; Taret et al. 2007). Sikorowski and Goodwin (1985) provided a method of surface sterilization of cabbage looper pupae.

11. Collecting, Holding, Marking and Sexing Adults

11.1. COLLECTING EMERGED MOTHS

Collecting adult moths is difficult due to the respiratory health hazard to workers from moth scales in the air (section 21.1). In the laboratory, emerging adults are attracted by light into transparent plastic bottles or screened cages (Hamilton and Hathaway 1966; Jermy and Nagy 1971; Ivaldi-Sender 1974; Mani et al. 1978; Guennelon et al. 1981).

At the Pacific Agri-food Research Centre (PARC 2007), trays of diet (from which adults were about to emerge) were placed on racks inside a large wooden box. Metal funnels were fixed onto one side of the box and a transparent plastic/screen cage placed over each funnel. Light in the room attracted the emerging adults into these cages (Brinton et al. 1969). Later, the adult-collection system was changed to reduce labour costs. The lights of a rearing room with open trays of diet on carts were turned off and UV lights below boxes (with screen bottoms) on the floor were turned on. Adults were attracted into the boxes by the light, and after 10–15 min a lid was placed on the box.

FIGURE 15

UV light inside opening of vacuum trap or suction device in ceiling of an emergence room. Duct leading away from this device channels adults to a cyclone. (OKSIR facility, Osoyoos, Canada)

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FIGURE 16
Upper part of cyclones where moths enter via flexible grey-coloured ducts. The cyclones extend down into the cold room. (OKSIR facility, Osoyoos, Canada)

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FIGURE 17
Lower part of a cyclone in a cold room in which adults collect (OKSIR facility, Osoyoos, Canada)

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Another small-scale system was used at the Yakima, USA, laboratory (Howell and Clift 1972). Corrugated cardboard strips with pupae were placed into an emergence box. A waxed paper bag was fixed over an opening on the top of the box to trap emerging moths.

To avoid the health hazard from moth scales, scientists at the Yakima Laboratory (Hutt et al. 1972; Moffitt and Hathaway 1973; Toba and Howell 1991) developed a system of collecting moths in a cold room. Subsequently Dyck et al. (1993) developed a similar system in which, with no human intervention (and no human exposure to scales in the air), flying adults in an emergence room were attracted to a UV light (Bloem and Bloem 1995, 2000) which was turned on for 50 min out of every hour (Bloem et al. 1998a, 2000). Emerged adults flew to the UV light (**Figure 15**) in the ceiling housed in a suction device or vacuum trap that transported adults through large ducts (**Figure 16**) into cyclones in a cold room (0–2°C) (**Figure 17**). Due to the sudden decrease in air speed in a cyclone, the insects fell to the bottom of the cyclone, were inactivated by the cold and were collected (Wolf and Stimmann 1971; Stewart 1984; Bloem et al. 1997, 1998a, 2000, 2004). This adult collection system has an advantage in that only adults that can fly are collected, helping to ensure that good flight ability is continuously selected for and maintained (Bloem and Bloem 2000) (sections 18.5.2 and 19.5). Mediouni and

Dhouibi (2007) used a similar adult collection system for mass-rearing the carob moth.

Batiste and Olson (1973) developed an adult emergence-collection device in which trays of diet were held in aerated darkened compartments at 28ºC. Emerged adults were attracted through stainless-steel tubes into collection tubes within a refrigerator (10ºC) by continuous UV light.

Moffitt and Hathaway (1973) showed that collecting adults using UV light and cold storage might have some negative effects on the response of males to a sex-attractant trap in the field.

11.2. HOLDING ADULTS IN THE COLD

Chilled adults are easily handled in a cold room (0–2ºC) for weighing, counting, sexing and irradiating. Chilling is superior to CO_2 as an immobilizing agent (White et al. 1970). However, Bloem et al. (1998a) discovered that chilling for too long can be detrimental to adults intended for field release; moths that had been held in the cold for 12 h performed better than those held for 36 h (Bloem and Bloem 2000). (Note sections 14.5 and 18.5.5 regarding the cold storage of adults.)

11.3. MARKING ADULTS USING FLUORESCENT DYES

In release and recapture studies, adults are marked with a fluorescent dye prior to release; usually the dye is a powder or dust (zinc 8-hydroxyquinoline) (Logan and Proverbs 1975). The powders are available in several colours (DayGlo 2007).

Moffitt and Albano (1972a, b), Proverbs et al. (1969, 1975, 1978) and Logan and Proverbs (1975) described how to apply and use such dyes. DayGlo dusts were applied by Bloem et al. (1998a, 2004) at the rate of 5 mg per 23–25 g of adults. Subsequent to the release, insects captured in traps are observed with long-wave UV light to detect the presence of dye.

The over-application of these dyes has been shown to have some negative effects on the behaviour of adults, e.g. olfactory response of males to calling females (Proverbs 1971, 1972; Logan and Proverbs 1975). Moffitt and Albano (1972b) reported that Blaze Orange and Rocket Red reduced fecundity, and Arc Yellow, Fire Orange and Neon Red reduced egg hatch. Helecon® 2200 and Rocket Red produced a reduced response of marked males to females, and these two markers were not used in field work on insect behaviour. Eosin-Y had no apparent effects on adult moths, but was harder to apply and more difficult to detect than were fluorescent powders. (Eosin-Y is a non-fluorescent red tissue stain).

Powder on marked insects may also contaminate non-marked wild insects that are trapped along with them in the field, causing misidentification (Proverbs 1982).

Hagler and Jackson (2001) and Parker (2005) reviewed the use of dyes for marking insects (also section 4.12).

11.4. SEXING MALE AND FEMALE ADULTS

When viewed from the ventral side, the sex of a codling moth adult is easily identified by overall size (female is usually larger in diameter and heavier than

FIGURE 18
Ventral view of abdomen of female adult of the codling moth

Source: Howell (1991)

FIGURE 19
Ventral view of abdomen of male adult of the codling moth

Source: Howell (1991)

the male) and structure of the external genitalia. Females have a large anal papilla (**Figure 18**) (a circular brown spot at the tip of the abdomen) and a dark ostium bursae in front of the papilla and males have a pair of claspers (**Figure 19**) (Howell 1991, Trécé 2007a, Wunderlich 2007). Also, males have a black spot in the centre of the underside of the forewing (Howell 1991).

Manual separation of the sexes can be done, at room temperature, by laboriously sucking up adults individually with a mouth aspirator (pooter) or low-suction portable vacuum aspirator (to protect the worker from breathing in moth scales) (Reed and Tromley 1985). Adults can be immobilized by a draft from a fan, enabling a worker to sex them visually and then suck them up individually (Hamilton and Hathaway 1966).

Moths can be sexed with a magnifier when immobilized in a cold room, e.g. 2–4ºC. However, working in a cold room is uncomfortable for staff, and a 'cold table' can be made in which a metal surface is chilled. Adults placed on the cold surface are chilled and immobilized, permitting the sorting to be done in comfort. Adults can also be sexed using a stream of cold air at 9ºC (air passed over ice) (Batiste and Olson 1973).

No satisfactory automated system to separate the sexes has been developed. However, it may be possible to separate the sexes by (1) collecting early-emerging adults (mainly one sex) and then collecting late-emerging adults (mainly the other sex) (Howell 1991), or (2) attracting emerging male adults to a sex-pheromone source and then trapping them.

Schoenleber et al. (1970) described five different mechanical devices designed to separate chilled (near 0ºC) adults:

- Perforated round-hole screen – uses differences in insect thickness

- Perforated slotted-hole screen – uses differences in insect breadth and length
- Air-gravity separator – uses differences in insect density
- Electrostatic separator – uses differences in charge of insects
- Vibrating inclined table separator – uses differences in insect surface texture and shape

The results showed that none was very good at separating the sexes, with the best being the slotted screen (about two-thirds of the moths in each of two 'separated groups' were of the same sex).

11.5. GENETIC SEXING

Research on a genetic sexing system is on-going (Marec et al. 2005, 2007; Vreysen and Hendrichs 2005; Vreysen et al. 2006; Makee and Tafesh 2007). However, this work has not yet produced a genetic sexing strain that can be used for field programmes (Franz 2005).

12. Mating and Feeding Adults, and Collecting, Incubating and Treating Eggs

12.1. ENVIRONMENTAL CONDITIONS

The following conditions are common for mating, oviposition and incubating eggs. (These activities are combined here since they all occur in an oviposition room.)

- **Temperature:** 24–28ºC
- **Relative Humidity:** 50–70%
  The RH must be between 50-70% during oviposition (Wildbolz and Mani 1971) to prevent the desiccation of eggs. However, once eggs have been laid and are being incubated in an airtight container, the RH can be maintained by enclosing a wet wick.
- **Light:** Photophase: scotophase 16L:8D–18L:6D
  Since mating and oviposition tend to occur at dusk (Bathon et al. 1991), lights should be dim or subdued; light intensies reported include 32–54, <150 and <538 lux. Some laboratories use natural daylight rather than artificial lighting, others constant light.
- **Air Movement**
  The presence of scales (from adults in cages) makes air circulation in the mating and oviposition room problematic, and filters in the air-handling system must be used to remove these scales. When in a mating and oviposition room, workers should wear face masks.

12.2. MATING ADULTS

Sexual activity peaks around dusk. In nature, females call by releasing a sex pheromone and males respond by finding the females, and then mating takes place. A successful mating lasts about one hour; the first mating tends to be shorter in duration than later matings (White et al. 1975; Howell et al. 1978; Howell 1991). After pushing the spermatophore into the bursa copulatrix, the male ejaculates into it a milky seminal fluid of mostly apyrene sperm followed by a more compact substance containing eupyrene sperm (Ferro and Akre 1975; White et al. 1975; Howell 1991). The spermatophore hardens in the female bursa copulatrix; it can even be felt when squeezing the abdomen of a mated female between two fingers. A spermatophore is shaped like a bilobed pear (Howell 1991; Trécé 2007a). Ferro

and Akre (1975) provided drawings of the female and male reproductive systems, and a drawing of the position of female and male genitalia during mating.

Males may copulate several times, but the size of the spermatophore transferred tends to decline after the first mating (Howell 1988). Males can produce a spermatophore about once a day, for 3–5 days. If the sex ratio favours females, males will mate more often than if the ratio is 1:1 (Howell et al. 1978).

On average female codling moths copulate once or twice. Mated females usually discontinue calling and do not remate, especially if the spermatophore received is large (Howell 1988). Howell et al. (1978) found that 37% of females mated more than once in laboratory cages. Increasing the sex ratio in favour of males increased the number of times a female mated (but decreased female longevity and fecundity), but increasing the sex ratio in favour of females decreased the number of times a female mated (Pristavko and Boreyko 1971).

No special mating cage is required; mating takes place within the oviposition cage. Nearly all males and females are sexually active on the first day following emergence (Howell 1988), and mating is virtually complete within 12–48 h (Howell et al. 1978) and females soon begin to lay fertile eggs.

12.3. FEEDING ADULTS

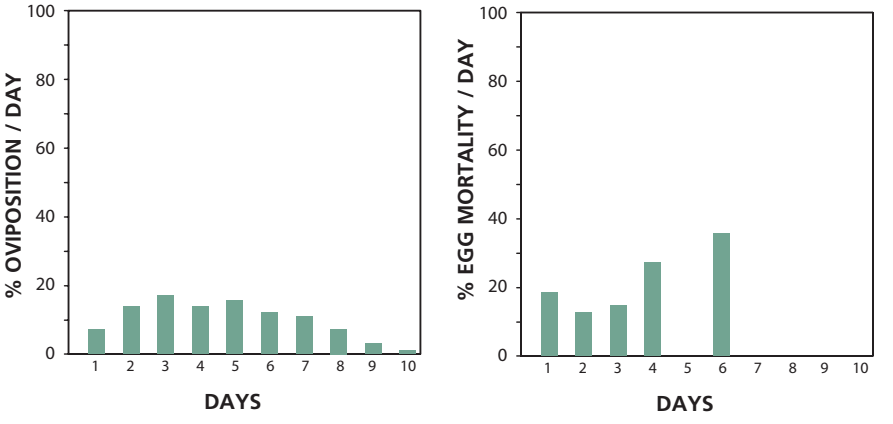
Feeding adults increases longevity. Some authors reported that post-emergence öogenesis was possible only when water was given to the moths (Howell 1981). Water is provided to adults during mating and oviposition, usually by wetting a cotton wick, cotton wool, sponge or piece of filter paper, or by providing a feeding bottle (Dickson et al. 1952; Hamilton and Hathaway 1966; Navon 1968; Proverbs and Logan 1970; Jermy and Nagy 1971; Navon and Moore 1971; Pristavko and Boreyko 1971; Wildbolz and Mani 1971; Huber et al. 1972; Reed and Tromley 1985; Bathon et al. 1991; Dyck et al. 1993). Sometimes sucrose or honey solution (3–10%) is provided (Jermy 1967; Sender 1969; Wildbolz and Mani 1971; Hatmosoewarno and Butt 1975; Guennelon et al. 1981; Ashby et al. 1985; Bathon et al. 1991; Neven et al. 2000). Nevertheless, feeding adults is time consuming and causes some mortality since adults may get trapped under wet pads (Howell 1981).

Fecundity can be increased by feeding a solution of 10% sucrose and 0.3% sorbic acid (Huber et al. 1972). Navon and Moore (1971) provided a complex diet (water, honey, ascorbic acid, thiamine, riboflavin, nicotinamide and choline chloride) to adults and found that this more than doubled fecundity when compared with water only (females lived up to 15 days). However, since the majority of the eggs were oviposited during the first half of a female's life, it would not be rational to keep cages longer than about 6 days.

Feeding adults with water, a carbohydrate or a carbohydrate-protein solution increases longevity (Howell 1981; Ashby et al 1985) but this does not significantly increase mating, oviposition or egg viability (Howell 1981).

Females lay 80–94% of their eggs within the first six days of life, regardless of the availability of water or food (Howell 1981, 1991; Gu et al. 2006) (**Figure**

FIGURE 20
Percentage oviposition per day by female codling moth adults and percentage mortality of eggs oviposited on a given day, in a laboratory



Source: Howell (1991)

20). The highest number of eggs laid is on days two and three (Bloem et al. 1998b) and 38% of the eggs oviposited on day six or later are sterile (Howell 1991) (**Figure 20**). Therefore, rearing programmes tend to maintain adults for only about 5 days, and feeding adults may not be worthwhile (Hamilton and Hathaway 1966; Howell 1970, 1981). Nevertheless, to provide insurance against high adult mortality from various environmental factors (e.g. low or high RH, high temperature), many rearing programmes continue to supply water to adults in mating and oviposition cages.

12.4. OVIPOSITION CAGES

To maintain a rearing colony, about 10% of produced adults are needed (Bloem et al. 1997, 2000); other authors report similar figures, 8% (Batiste and Olson 1973) and 15–20% (Mani et al. 1978).

Howell (1981) stated that "for economy in rearing massive numbers of codling moths, one must have an efficient means of obtaining the largest possible number of eggs per female in the shortest possible time with a minimum of labour and without sacrificing viability or vitality of the resulting insects. Also, with higher fecundity, fewer moths are needed to maintain a colony. Thus, over the years, a variety of oviposition cages has been developed, all of which provide a method of caging the moths and easy retrieval of eggs."

Oviposition in a laboratory cage proceeds without the need for special stimulation. However, in nature, female adults are stimulated to lay eggs when they detect apple odour (Wearing et al. 1973).

Types of cages and other oviposition substrates are described below:

FIGURE 21
Oviposition cage resting on slowly turning rollers. Constructed from aluminium and using brown paper waxed on both sides (OKSIR facility, Osoyoos, Canada)

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FIGURE 22
Rows of oviposition cages on slowly turning rollers (OKSIR facility, Osoyoos, Canada)

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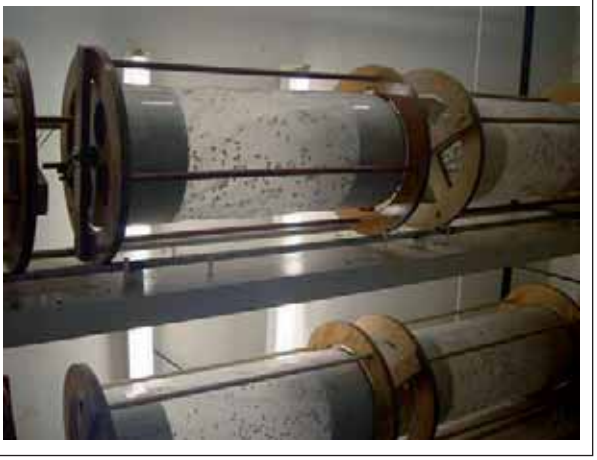
- Cylindrical plastic cages lined with waxed paper (Dickson et al. 1952; Hamilton and Hathaway 1966)
- Waxed paper or cellophane from roll threaded through a plastic-screen oviposition cage (Jermy and Nagy 1971)
- Cellophane paper; eggs removed by gently crinkling the paper (Rock 1967)
- Cellophane or polythene covering a beaker or tube (Huber et al. 1972; Bathon et al. 1991)
- Waxed paper bags or plastic bags (White and Hutt 1970, 1971; Hathaway et al. 1971, 1972, 1973; Howell and Clift 1972; Bathon et al. 1991; Toba and Howell 1991)
- Wire cages and plastic pellets (Hathaway et al. 1972, 1973; Toba and Howell 1991)

Proverbs and Logan (1970) developed a wooden cage, and each day a sheet of paper with eggs could be removed and a new sheet inserted, without removing the moths. A very similar all-metal cage was developed for the OKSIR facility, except that at the centre of one of the round side disks a water bottle and wick were inserted to provide water (Dyck et al. 1993) (**Figures 21–23**). Similar oviposition cages have also been constructed (Batiste and Olson 1973; Carlyle et al. 1975; Mani et al. 1978; Guennelon et al. 1981; Mediouni and Dhouibi 2007). Guennelon et al. (1981) claimed that their cage was more practical than that developed by Proverbs and Logan (1970).

Except for the waxed paper, other surfaces on the inside of the cage are screen wire mesh or roughened to discourage oviposition. To ensure a uniform distribution of eggs on the waxed paper sheet, the drum-like cage is slowly rotated/tumbled by two parallel rods (covered with rubber to grip the cage) rotating under

FIGURE 23
Oviposition cages on slowly turning rollers. (South Africa in 2007)



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the cage (Proverbs and Logan 1970, about four revolutions of the cage/h; Batiste and Olson 1973, about one revolution of the cage/h).

The number of moths per cage, that each day will produce the desired number of eggs/cm^2 of egg sheet, is not a constant; it depends on several factors, especially the size of the cage and area of egg sheet, fecundity of the females and temperature (section 19.8). Each laboratory must determine the optimum adult density for its oviposition cage.

The number of males in a mating/oviposition cage can be reduced by one-third to one-half without decreasing fecundity and the quantity of hatched eggs. Therefore, some male adults can be used for other purposes, e.g. release in the field (Pristavko and Boreyko 1971; Hathaway et al. 1973).

12.5. INCUBATING EGGS

The daily removal of egg sheets from oviposition cages, and incubating them at the appropriate temperature and in a systematic way, will provide the needed number of eggs in the blackhead stage (just prior to hatching) on the days when fresh diet should be infested. To prevent desiccation, sheets with eggs should be placed in plastic bags along with wet wicks to keep the humidity high during incubation (Huber 1972).

Egg development can be synchronized with the diet preparation schedule by altering the temperature. The eggs can be stored for up to seven days at 13°C (Huber et al. 1972) and up to two weeks at 11°C (Reed and Tromley 1985).

Egg mortality is about 15–30%, even under apparently suitable conditions for mating, oviposition and egg incubation (**Figure 20**).

12.6. TREATING EGGS

To control microbial contaminants, it is necessary to surface-sterilize the eggs (Theron 1947). This permits larvae to eclose in a sterile environment before seeding the diet (Howell 1970, Proverbs 1982, Taret et al. 2007). Egg treatment is common in insect rearing (Sikorowski and Goodwin 1985; Sikorowski and Lawrence 1994a, b; Sikorowski et al. 2001).

Egg sheets for seeding fresh diet need to be cut to fit the length and width of the diet tray (**Figure 24**).

For immersion and surface-sterilizing, egg sheets can be placed into a custom-made 'book' consisting of wire-mesh 'pages', one sheet between two opposing pages. The wire mesh between each egg sheet permits the liquid to penetrate the stack of sheets and thoroughly wet both sides of each sheet (Fisher 1984a) (**Figure 25**).

In general, the procedure is as follows:

- The book of egg sheets is immersed into the disinfecting solution for several minutes in a stainless-steel tank (**Figure 26**), then taken out and rinsed in water. After a second rinse in water, the book of sheets is placed on a rack to allow the water to drain.

FIGURE 24
Cutting egg sheets into a size suitable for placement over trays of diet (OKSIR facility, Osoyoos, Canada)

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FIGURE 25
Egg sheets being placed between wire mesh (OKSIR facility, Osoyoos, Canada)

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FIGURE 26
Stainless-steel tanks (3) for immersion of egg sheets in a disinfecting solution and then rinsing in water (OKSIR facility, Osoyoos, Canada)

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FIGURE 27
Stainless-steel cart, specially designed to hold disinfected egg sheets, for transporting egg sheets from the egg-sheet preparation room to the diet-dispensing room. (OKSIR facility, Osoyoos, Canada)

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- A specially constructed cart with doors, developed at the OKSIR facility, is used to hold the clean egg sheets in the books and transport them to a sterile holding room (**Figure 27**).

Procedures used to treat egg sheets:

- Place egg sheets in a solution of 0.1% NaOCl for 2 min, rinse with sterile water for 30 sec and dry in air at 27ºC (Hamilton and Hathaway 1966; Hathaway et al. 1972; Toba and Howell 1991).
- Place egg sheets in a solution of 6.5% formalin for 30 min, rinse three times in distilled water and dry in air (Rock 1967).
- Place egg sheets in an aqueous solution of 3% NaOCl (commercial bleach that contains 5.3% available chlorine) and 0.1% wetting agent (e.g. Triton X-100) for 3 min, rinse in water for 1 min and dry in air at 27ºC (Brinton et al. 1969; Batiste and Olson 1973).
- Place egg sheets for 5 min, with periodic agitation, in 0.164% NaOCl solution (13.83 mL of 12% commercial bleach in each litre of water) and a few drops of liquid soap. Rinse in tap water for 5 min with periodic agitation and dry in air (PARC 2007).
- Place egg sheets in 3% NaOCl solution for 3–4 min, wash in tap water for 10 min and dry in air between layers of paper towelling (Bathon 1981).

- Place egg sheets in 1.3% NaOCl and a wetting agent for 1 min, rinse under running water and dry in air.
- Place egg sheets in a 4% solution of formaldehyde for 5 min, rinse with sterile water (Howell 1970) (section 21.2). Surface sterilization in formaldehyde dislodged a few eggs from the sheet (Toba and Howell 1991).
- Place egg sheets in a solution of 4% formalin, rinse twice in distilled water and dry in air (Hathaway et al. 1971).
- Place egg sheets in formalin (1 part 37% formaldehyde to 10 parts water) for 5 min, rinse in 70% isopropyl alcohol to facilitate rapid drying and dry in air (Howell and Clift 1972).
- Place egg sheets in a 10% formalin solution for 2–3 min, rinse in distilled water and dry in air (Huber et al. 1972).
- Place egg sheets for 5 min in 4% aqueous formaldehyde, rinse in water and dry in air (Hatmosoewarno and Butt 1975).
- In a fume hood, place egg sheets in a tray with 5% formalin (or 2% formaldehyde) and agitate gently for 10–15 min with forceps, rinse sheets in running tap water for 10 min, rinse sheets again in sterile water for 5 min and dry sheets at 25ºC between layers of soft tissue paper (Ashby et al. 1985).
- Place polythene strips with eggs into a nylon gauze bag and submerge in a 5% formaldehyde solution for 20 min. Rinse the bag in sterile water for 20 min and then dry the strips in a fume hood (Bathon et al. 1991).
- Fumigate egg sheets in formaldehyde vapour for 90 min at 23ºC (Toba and Howell 1991) (section 21.2). Fumigation with formaldehyde is an excellent treatment to control CpGV.
- Place egg sheets in a container for 6–8 h with formaldehyde vapour arising from one drop of 5% formaldehyde in each 200 mL air (Bathon 1981; Reiser et al. 1993).
- Place egg sheets in a 1 L closed jar containing a filter paper, moistened with five drops of 5% formaldehyde, at 22–23ºC for 6–8 h (Bathon et al. 1991).

Fumigating eggs of the cabbage moth *Mamestra brassicae* (L.) with formaldehyde vapour for 6 h prevents trans-ovum transmission of nuclear-polyhedrosis virus (Bathon and Gröner 1977).