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Great Lakes Forestry Centre
Insect Production Services

STANDARD OPERATING PROCEDURE

Number: IPS/012/002

Rearing Choristoneura occidentalis

Effective Date: 5 March 2015

Canada

TITLE: Rearing <u>Choristoneura occidentalis (Co)</u>

APPROVING OFFICIAL:

DD / MM / YY

Manager, Insect Production Services _____ ___/___/_____

SIGNIFICANT CHANGES FROM PREVIOUS VERSION:

N/A

1.0 INTRODUCTION

1.1 Purpose

This Standard Operating Procedure (SOP) has been established to ensure that procedures used for the rearing of the *Co* (western spruce budworm) are implemented consistently among Insect Production Unit (IPU) personnel and to minimize the spread of pathogens and microbial contaminants within and between insect colonies.

1.2 Scope

This SOP shall be followed by all IPU personnel for the rearing Co.

1.3 Definitions

Biological Safety Cabinet (BSC) – A class 2 containment cabinet designed for both worker and sample protection; room air is drawn into the front of the unit; the unit is designed in such a way that room air is HEPA filtered before blowing over the work area; air-borne hazardous particles coming off samples in the work area are pulled away from the worker and the air is vented back into the room after HEPA filtration; this type of unit is not suitable for worker protection from chemical fumes.

Chemical Fume Hood – Safety cabinet designed for worker protection but not sample protection; room air is drawn into the front of the unit, chemical fumes or air-borne hazardous particles are pulled away from the worker and are vented to the outside of the building.

Controlled Copy – A copy of an SOP distributed to select GLFC personnel having a unique copy number and dated signature of the IPS manager. Controlled copies are intended to ensure that GLFC personnel follow the most recent version of the SOP.

Effective Date – The date from which the procedures given in an SOP are to be implemented.

Insect Production Services (IPS) Manager – The individual who has overall responsibility for activities of the IPS team.

Insect Production Supervisor – A member of IPS having supervisory authority over the daily operation of the insectary.

Insect Production Unit (IPU) – A work unit of IPS consisting of personnel who perform insect rearing, diet making and methods development activities at GLFC.

Laminar Flow Hood – A safety cabinet designed for sample protection but not worker protection; the unit is designed in such a way that room air is HEPA filtered before blowing over the work area back into the room (i.e., air blows directly at the worker); this type of unit is not suitable for working with hazardous materials.

Material Safety Data Sheet (MSDS) – A summary description of a chemical, reagent or substance prepared by the manufacturer or supplier and required by WHMIS legislation to inform workers about procedures required to safely work with the material.

Methods Development (MD) Lab – A research facility under the control of IPS used exclusively by the IPU for developing new rearing methods and for establishing new insect colonies.

Quality Control (QC) Lab – An analytical laboratory under the control of IPS used by the QCU for monitoring production, process and product control for all IPU insect colonies, and for developing new QC methods and procedures.

Quality Control Unit (QCU) – A work unit of IPS consisting of personnel who conduct routine production, process and product control testing and develop new QC methodology in support of IPU activities.

Standard Operating Procedures (SOPs) – Directives describing routine administrative or technical procedures conducted by IPS personnel or users of the IQ facility.

1.4 Safety

1.4.1 Personal protective safety equipment (i.e., lab coat and disposable chemical protective gloves) shall be worn for the conduct of sections ***2.3*** through ***2.13***.
1.4.2 Adults shall be handled within a BSC or chemical fume hood to provide worker protection.
1.4.3 Safety precautions identified in the referenced SOPs shall be followed.
1.4.4 Personnel shall have access to, and be familiar with, the MSDS for formaldehyde and for sodium hypochlorite (i.e., bleach).

1.5 Materials

1.5.1 Safety equipment:
- (a) class 2 BSC
- (b) lab coat
- (c) disposable chemical protective gloves
- (d) chemical fume hood

1.5.2 Supplies and equipment:
- (a) sanitation supplies/equipment specified in the current version of SOP Number IPS/009, *IPU Personnel Responsibilities*
- (b) consumables, including: creamer cups, un-waxed lids, regular diet, autoclaved gauze, parafilm, sterile paper towel, Cut-Rite® waxed paper, autoclaved tap water, paper trays, clear plastic bags (2, 6 and 40 lb), 50 ml screw cap vials, petri dishes, twist ties, plastic garbage bags, paper bags, 1% Javex® solution, 10% Javex® solution.
- (c) analytical balance
- (d) mist bottle
- (e) perforated metal trays
- (f) funnels
- (g) vented plastic pupa boxes with covered vents
- (h) vented plastic mating boxes with covered vents
- (i) hatching pans with covers
- (k) sterile scissors
- (l) sieve

1.5.3 Forms:
- (a) *Co Distribution* (IPS Form Number 0127/002, Appendix 1)
- (b) *Co Bag Label* (IPS Form Number 0129/002, Appendix 2)
- (c) *Co Tracking* (IPS Form Number 0120/002, Appendix 3)
- (d) *Co Discards* (IPS Form Number 0130/002, Appendix 4)
- (e) *Co L2 Count* (IPS Form Number 0128/002, Appendix 5)
- (f) *Co Rearing Schedule* (IPS Form Number 0131/002; Appendix 6)

2.0 PROCEDURES

2.1 Facility Sanitation Procedures

Procedures identified in the current version of SOP Number IPS/009, *IPU Personnel Responsibilities*, shall be followed.

2.2 Records for Distribution of Insects

2.2.1 No insects shall be distributed until results of QC analysis have been received by the IPU and identified as suitable for use (refer to section ***2.13***).

2.2.2 Orders for *Co* shall only be accepted from internal and external clients through the IPS web-based storefront (i.e., requests submitted through standard email or telephone are not to be accepted).

2.2.3 The IPU email account shall be reviewed daily (excluding weekends and holidays) for the receipt of orders from the storefront. Orders shall be printed, stamped as "Received" and shall be signed/dated by the technician receiving the order. The electronic copy shall be filed in the electronic archive for either internal or external clients, as applicable. The printed copy shall be placed into the in-box (labelled as "outstanding orders") for either internal or external clients, as applicable. In-boxes shall be reviewed by each IPU technician on a daily basis (excluding weekends and holidays) and shipping performed by applicable personnel on the requested shipping date (subject to availability of eggs). Upon completion of the shipment, the printed order shall be stamped as "Completed", signed and dated by the applicable technician, then placed into the out-box labelled as "completed orders" for either internal or external clients, as applicable. At the end of each month, IPU personnel shall photocopy completed external orders (i.e., those not already paid by credit card) and deliver them to the GLFC finance department for billing according to the current fee schedule. All printed copies of completed orders (internal and external) shall be maintained with IPU records. All distribution of *Co* to clients internal and external to GLFC shall be documented on the *Co Distribution* form (IPS Form Number 0127/002, Appendix 1). A separate form shall be used for each cohort of every generation and shall be maintained with IPU records.

2.2.4 Upon the initiation of each Co *Distribution* form, the following records shall be documented:

(a) ID code for the cohort
(b) total # larvae in diapause for the cohort [this number is derived from the *Co Tracking* form (IPS Form Number 0120/002, Appendix 3) for the previous generation; total includes progeny that may have been discarded for QC reasons, as provided by the QCU]
(c) # kept for colony maintenance (this will have been provided by the QCU)
(d) # discarded for QC reasons (this will have been provided by the QCU)
(e) # available for distribution (this number is derived by subtracting the # kept for colony maintenance and the # discarded for QC reasons from the total # larvae in diapause)

2.2.5 Larvae destined for distribution to clients *external* to GLFC shall be shipped during diapause (i.e., the IPU will not conduct larval rearing for external clients); all shipments shall be documented on the lower portion of the *Co Distribution* form; records shall include:

(a) Date distributed
(b) # L2 requested

(c) # L2 distributed (minimum 5% surplus shall be provided to account for mortality during diapause)
(d) Recipient name and affiliation

2.2.6 Larvae destined for distribution to clients *internal* to GLFC may be distributed during diapause or set up on diet (as identified in **2.3**) and distributed prior to thinning (i.e., the IPU will not conduct diet changes for clients); all distributions shall be documented as identified in 2.2.7.

2.2.7 Larvae to be reared for distribution to *internal* GLFC clients may be taken out of diapause at the discretion of the IPU (i.e., after 20-37 weeks of diapause; quantity and day of week is also at the discretion of the IPU); larval rearing and subsequent distribution to *internal* clients shall be documented as identified on the upper portion of the *Co Distribution* form; records shall include:

(a) Date(s) out of diapause
(b) # larvae taken out at each date identified above; numbers taken out are at the discretion of the IPU pending actual or anticipated requests by clients
(c) # cups set up (30-larvae cup) at each date identified above
(d) ID of the diet used for setting up the larvae (e.g., Reg ¾c 27/09/05)
(e) Date(s) in which the insects were picked-up by the client(s).
(f) # cups picked up by the client(s) on that date
(g) recipient name(s)
(h) # cups discarded [i.e., cups set up at the date(s) identified in (a) above but not distributed]

Note: total # cups picked up by clients plus the total # cups discarded must equal the total # cups set up.

2.2.8 Larvae distributed to *internal* clients during diapause shall be documented on the lower portion of the *Co Distribution* form; records shall include:

(a) Date distributed
(b) # L2 requested
(c) # L2 distributed (a minimum of 5% surplus shall be provided to account for mortality during diapause)
(d) Recipient name

2.2.9 Larvae shall not be distributed after 37 weeks of diapause, unless specifically requested by the client; the number of larvae discarded from each cohort of every generation shall be documented (including date) on the bottom of the *Co Distribution* form.

2.2.10 Upon distributing or discarding all insects from each cohort, IPU personnel shall review the *Co Distribution* form to ensure that the sum of all distributions and discards is equal to the number that were available. IPU personnel shall check the boxes on the bottom of the form to confirm that the numbers balance, or make a notation of the

discrepancy when the difference cannot be resolved. The form shall be maintained with IPU records.

2.2.11 A determination of the number of larvae discarded from each generation shall be determined by maintenance of a *Co Discards* form (IPS Form Number 0130/002; Appendix 4). This shall be accomplished as follows:

(a) Each time a cohort of *Co* is taken out of diapause, the following information shall be determined and recorded on the *Co Discards* form:
   i) ID code
   ii) date into diapause
   iii) date out of diapause
   iv) total # of L2 in diapause
   v) # kept for colony maintenance
   vi) # discarded for QC reasons
   vii) # available for distribution
   viii) 37 week discard date

(b) Upon completion of the *Co Distribution* form for each cohort as stated in 2.2.1 through 2.2.10, the following information shall be determined and recorded on the *Co discards* form:
   i) # distributed during diapause
   ii) actual discard date
   iii) total # used
   iv) total # discarded
   v) % discarded

(c) A separate *Co Discards* form shall be used for each generation and shall be maintained with IPU records upon completion.

2.3 L2 Setup

2.3.1 Progeny from each mating chamber from each cohort will have been identified by the QCU for use in either *colony maintenance* or *distribution* to clients (refer to section **2.13**).

2.3.2 Progeny destined for *colony maintenance* shall be removed from the diapause cold room on the date specified on the *Co* rearing schedule (refer to section ***2.15***).

2.3.3 Progeny to be reared for *distribution* to internal GLFC clients may be taken out of diapause at the discretion of IPU personnel (i.e., after 20-37 weeks of diapause; quantity and day of week is also at the discretion of the IPU); larval rearing and distribution to *internal* clients shall be documented as identified in 2.2.7.

2.3.4 Larvae shall be set up on diet on the same day that they are removed from diapause (day 0; typically Monday).

2.3.5 Within a BSC, autoclaved scissors shall be used to cut the gauze sheets containing L2s into patches containing approximately 30 larvae; excess parafilm shall be cut away and discarded.

Cutting patches

2.3.6 Patches shall be placed into creamer cups containing artificial diet (¾ cup regular diet containing formaldehyde and anti-fungal spray; maximum 1-wk old) and fitted with un-waxed cardboard lids; larvae for *distribution* may be set up using diet specially prepared as per the end-user's request.

2.3.7 L2 patches shall be placed with the parafilm side facing the lid and gauze side facing the diet; cups shall be maintained lid side down on the rearing trays; re-usable perforated metal trays shall be used for *colony maintenance* and disposable paper trays shall be used for rearing larvae destined for *distribution*; metal trays shall not leave the IPU facility.

2.3.8 The upper portion of the Co *Tracking* form (IPS Form Number 0120/002; Appendix 3) shall be completed to indicate:
- (a) ID code of the cohort
- (b) date out of diapause
- (c) # weeks (+days) diapause
- (d) # larvae taken out of diapause
- (e) # cups set-up with approximately 30 larvae
- (f) ID of the batch of diet used for set-up (e.g., Reg ¾c 27/09/05)
- (g) initials of the individual who setup the patches

2.3.9 The tracking sheet shall be attached to a clipboard and shall be maintained with the associated cohort at all times.

2.3.10 Diet for all larval rearing shall not be stored for more than 2 weeks prior to use.

2.3.11 Trays of larvae shall be identified with the ID code for the cohort and shall be maintained in an environmental chamber at 23±3ºC, 55±10%RH and 16L:8D; larvae for *colony maintenance* and for *distribution* shall be kept on separate shelves.

Larvae for colony and for distribution

2.3.12 Larvae for *distribution* shall be given out prior to thinning.

2.4 Thinning

2.4.1 Only larvae destined for *colony maintenance* shall be thinned. Larvae destined for *distribution* shall be given out prior to thinning.

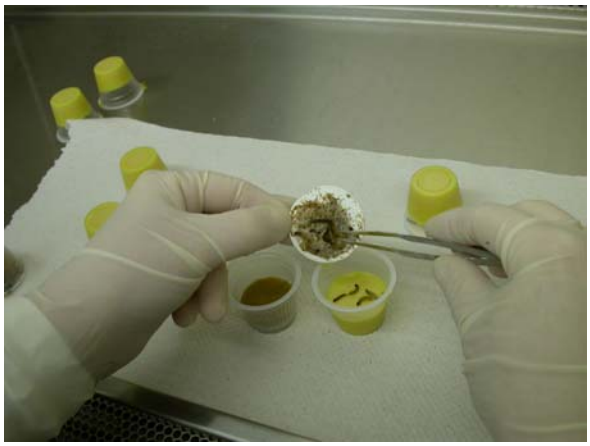
2.4.2 Thinning shall be conducted on day 10 or 11 (typically Thursday or Friday), pending operational requirements.

2.4.3 Gauze patches shall be removed at the time of thinning (within a BSC) from cups destined for *colony maintenance*; larvae that have not emerged shall be removed with the gauze; gauze patches shall not be removed from cups destined for *distribution*.

2.4.4 Gauze patches shall be maintained in a plastic bag, labeled with the ID code and frozen for QCU for analysis.

2.4.5 The box on the upper portion of the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3) shall be checked to indicate that the patches were placed in the freezer for subsequent QC analysis.

2.4.6 Larvae shall be thinned to 6 per cup (¾ cup regular diet containing formaldehyde and anti-fungal spray; maximum 2-wk old) within a BSC and shall be maintained lid side down; thinning may be delayed by one day if patch set-up was delayed by a day due to a statutory holiday; thinning may be conducted over 2 consecutive days when time is insufficient; larvae that are slow in development shall be discarded.



Thinning

2.4.7 If an entire weekly cohort is retarded in development, thinning may be delayed and the QC unit shall be notified.

2.4.8 Cups with dead larvae shall be removed from the rearing process (i.e., live larvae from these cups shall not be kept); after handling dead or contaminated larvae, forceps shall be exchanged for sterile ones and gloves shall be replaced.

2.4.9 When 10 or more cups with dead larvae are present, they shall be maintained in a plastic bag, labeled with the ID code and frozen for subsequent QC analysis; the box on the upper portion of the *Co Tracking* form shall be checked to indicate that the cups were placed in the freezer; when fewer than 10 cups with dead larvae are present, they shall be placed in a closed container (e.g., sealed plastic bag) for discard and no further documentation is required.

2.4.10 The upper portion of the tracking sheet shall also be completed to indicate the date thinned, number of cups attained for *colony maintenance*, identification of the batch of diet used (e.g., Reg ¾c 27/09/05), and initials of the individual(s) who did the thinning.

2.4.11 Cups with fungus shall be removed from the rearing process (i.e., live larvae from these cups shall not be kept) and a notation added anywhere on the *Co Tracking* form; the QC unit shall be notified when fungal contamination is problematic.

2.4.12 A larval collection from each cohort for the maintenance of a historical DNA record may be requested periodically (e.g., every 10 generations) by the QCU; instructions will be provided by the QCU at that time and shall be complied with by the IPU.

2.5 Pupa Harvest

2.5.1 Larvae shall be monitored daily for the onset of pupation; cups shall be flipped (i.e., lid side up) at pre-pupation; pupation will likely commence about day 22 (Tuesday).

2.5.2 Once significant numbers are present, pupae shall be harvested (within BSC) during 2 sessions approximately 4 days apart; the date of harvest sessions will be at the discretion of the observer, although the first harvest should normally occur on day 24 (typically Thursday) and the second harvest should normally occur on day 28 (typically Monday).

Pupa harvest

2.5.3 At each harvest session, cups with dead larvae, dead pupae, or fungal contamination shall be removed from the rearing process (i.e., live larvae/pupae from these cups shall not be kept); after handling dead or contaminated insects, forceps shall be exchanged for sterile ones and gloves shall be replaced; pupae harvested from each cup shall be placed into a temporary holding container so that mortality/fungal contamination within the cup can be ascertained before they are mixed with the rest of the cohort; when 10 or more cups with dead insects are present, they shall be maintained in a plastic bag, labeled with the ID code and frozen for subsequent QC analysis; when fewer than 10 cups with dead insects are present, they shall be placed in a closed container (e.g., sealed plastic bag) for discard and no further documentation is required.

2.5.4 When cups with fungus are detected/removed, a notation shall be added anywhere on the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3); the QC unit shall be notified when fungal contamination is problematic.

2.5.5 During the first harvest session, remaining larvae shall be transferred to fresh diet (six larvae per cup; ¾ cup regular diet containing formaldehyde and anti-fungal spray; maximum 2-wk old).

2.5.6 Larvae that do not pupate by the second harvest session shall be discarded (after destruction by freezing or autoclaving).

2.5.7 Pupae collected at each harvest date shall be separated by gender (males have 4 abdominal rings past the wing pads whereas females have 3); undersized or deformed pupae shall be discarded on the day of collection; pupae shall be observed visually and only those whose weights are in question need to be weighed individually and shall be

discarded if less than 70 mg for males and 100 mg for females; a BSC shall be used for the sorting of pupae, although weighing may need to be conducted on the lab bench; discarded pupae shall first be destroyed by freezing or autoclaving.

Weighing pupae

2.5.8 The middle portion of the *Co Tracking* form shall be completed after each harvest session to indicate:
(a) harvest date
(b) # pupae kept for each gender
(c) # pupae discarded for each gender
(d) # cups with dead larvae or pupae
(e) whether or not cups with dead pupae were placed in the freezer for subsequent QC analysis
(f) initials of the individual(s) who harvested the pupae

2.5.9 Upon completion of the final harvest session, the middle portion of the *Co Tracking* form shall be completed to indicate:
(a) total # males kept (2 harvest sessions combined)
(b) total # females kept (2 harvest sessions combined)
(c) calculation of 75% of males to be used for mating
(d) calculation of 75% of females to be used for mating

2.6 Surface Sterilization of Pupae

2.6.1 Pupae shall be surface sterilized within a BSC or chemical fume hood within two days of the second harvest session. Place pupae (separated by gender and by harvest session) into a 1L beaker 1/2 filled with 10% bleach (refer to 2.16.2). Using a gloved hand, pupae shall be pushed beneath the surface to ensure complete contact with

the Javex® solution. Pupae shall be allowed to soak for 10 minutes and shall be pushed beneath the surface at least twice during the 10-minute interval to dislodge trapped air bubbles and to ensure complete contact with the Javex® solution.

Surface sterilization of pupae

2.6.2 The contents of the beaker shall be poured through a sterile sieve to separate the pupae from the Javex® solution; decanting and subsequent rinsing may be conducted on the lab bench when space is limited or if there is no drain within the hood/cabinet.

Separating pupae from Javex® solution

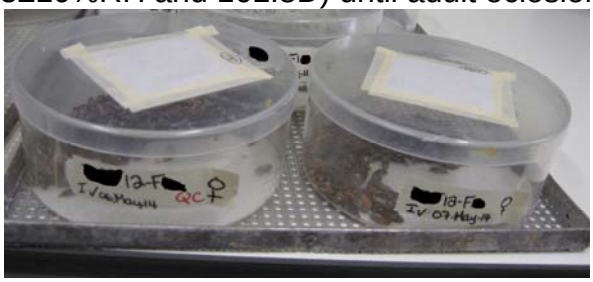
2.6.3 Pupae shall be placed back in the beaker along with 1 L autoclaved room temperature tap water; pupae shall be re-submerged in the water for about 10 seconds then separated using the sieve.

2.6.4 Repeat 2.6.3 (i.e., total of two rinses). Separate the pupae by sieving, then dump them onto a sterilized metal tray (lined with sterilized paper towel) and allow them to dry within a BSC for approximately one hour.

Drying of pupae after surface sterilization

2.6.5 Transfer the pupae (separated by gender and by harvest session) to adult emergence boxes lined with sterile paper towel and having sterile paper towel taped over the screened vent (paper towel prevents the potential spread of microbial contaminants carried by wing scales which would otherwise pass through screens). To facilitate ease of handling and to minimize moisture buildup, a maximum of 150 pupae shall be held in each box.

2.6.6 Emergence boxes shall be labelled with the ID code, harvest session and gender; pupae shall be maintained in an environmental chamber (23±3ºC, 55±10%RH and 16L:8D) until adult eclosion.



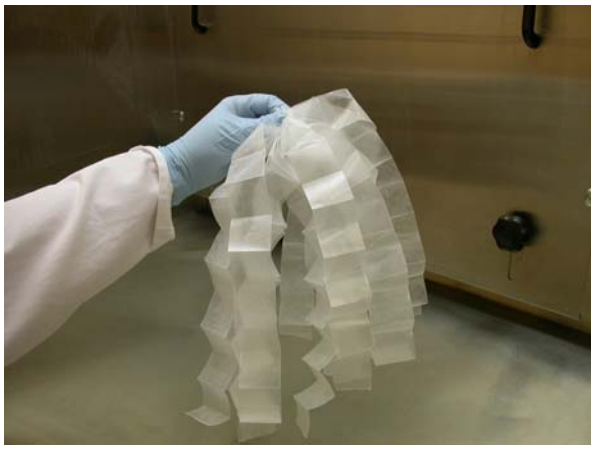
Adult emergence boxes

2.6.7 Emergence boxes shall be examined once daily for the appearance of adults; the paper towel liner shall be replaced if it becomes too soiled with meconium and wing scales.

2.7 Mating

2.7.1 Mating chambers shall be assembled on the day that they are required (oviposition substrate may be prepared ahead of time and stored); each chamber shall consist of a 40 lb clear plastic bag containing waxed paper oviposition substrate (lightly misted with autoclaved tap water), inflated with filtered air and sealed with a twist tie.

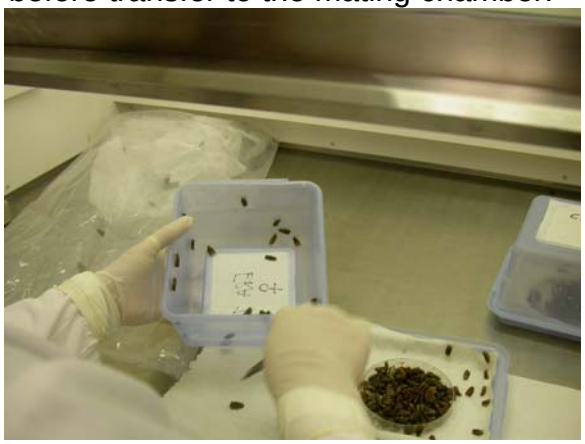
2.7.2 The oviposition substrate shall consist of 3 sets of 5 waxed paper strips (4cm x 40cm; Cut-Rite® brand) that are stapled together at one end, pleated (3cm pleats), and then partially straightened out.



Wax paper strips

2.7.3 As adults become available, they shall be transferred (within a chemical fume hood or BSC) to a mating chamber.

2.7.4 Adults shall be refrigerated at approximately 4ºC for 10 minutes to slow them down before transfer to the mating chamber.



Placing adults into lid of emergence box
for transfer to mating chamber

Transferring adults to mating chamber

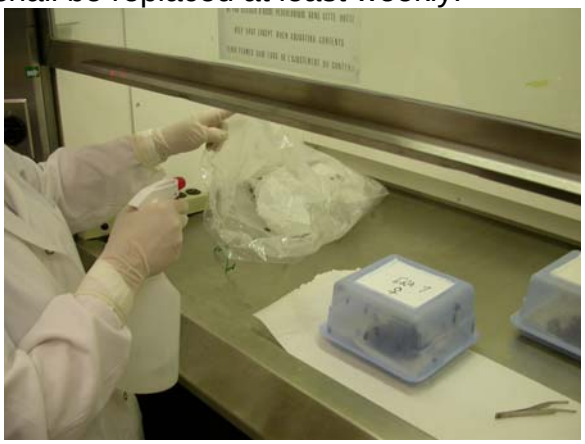
2.7.5 Adults used in each mating chamber may come from more than one pupa harvest date but only from one bi-weekly cohort; there shall be no mixing of adults between bi-weekly cohorts.

2.7.6 Each mating chamber shall be set up using 100 newly emerged females and 100 one to two–day-old males.

2.7.7 Mating chambers (maximum 10) shall be set up until approximately the first 75% of each gender are used (i.e., only early emerging adults are to be used for mating, since developmentally slow individuals may be diseased); chamber set-up will normally occur over a period of 3-4 days due to the staggered eclosion of the pupae available.

2.7.8 When insufficient numbers of adults are available to complete a mating chamber during one set-up session, the bag may be completed on the following day (i.e., max. 2 day set-up time for each bag, although 1 day is preferred); partially completed mating chambers shall be held in the walk-in environmental chamber (23±3ºC, 55±10%RH and 16L:8D) for a maximum of 24h until sufficient numbers are available to complete the bag.

2.7.9 The interior of each mating chamber shall be misted lightly with autoclaved tap water upon completion of set-up and again on day 3. Spray bottles shall be dated immediately after filling with autoclaved water and shall be replaced at least weekly.



Misting the mating chamber

2.7.10 Each mating chamber shall be labeled with:
- (a) ID code
- (b) sequential bag number
- (c) date that the bag is started
- (d) date that subsequent adults are added
- (e) number of each gender added at each date

2.7.11 Completed mating chambers shall be maintained in an environmental chamber (23±3ºC, 55±10%RH and 16L:8D).


Completed mating chamber

2.7.12 As each mating chamber is prepared, the lower portion of the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3) shall be completed to indicate:
(a) date each bag is initiated
(b) initials of individual initiating the bag
(c) date each bag is completed
(d) initials of individual completing the bag

2.7.13 The date of completion of each mating chamber shall be considered as *day 0* for the purpose of egg mass harvest.

2.7.14 Upon completion of all mating chambers for the bi-weekly cohort, the empty pupa cases from the first adult emergence box of each gender shall be transferred to separate 50 ml screw cap vials, labeled with the ID code and gender, and maintained for subsequent QC analysis; when there are more pupa cases than what can fit in a vial, the leftovers can be discarded; the bottom portion of the *Co Tracking* form shall be completed to indicate that pupa cases have been frozen.

2.8 Egg Mass Harvest

2.8.1 Egg mass harvest shall be conducted in a BSC.

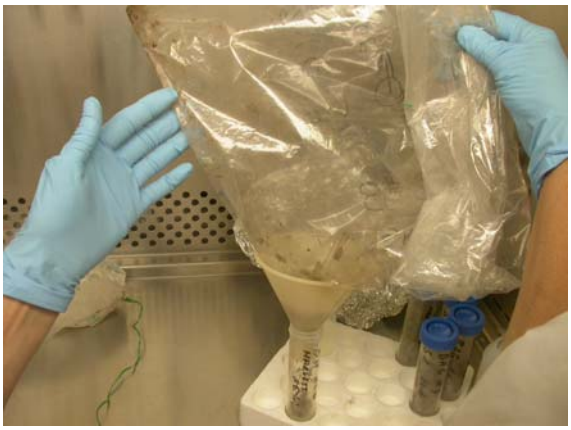
2.8.2 Waxed paper strips containing egg masses shall be harvested only once, 5 days after the mating chamber is set up (i.e., 5 days after a full 100 pairs have been inserted); because the 10 mating chambers may have been be set up over a period of 3-4 days, egg mass harvesting and subsequent surface sterilization may occur over a 3-4 day period; to minimize weekend work, egg masses may be harvested on day 4 or day 6, although day 5 is optimum.

2.8.3 Within the BSC (cleaned before and after handling each chamber), wax paper strips shall be removed from the mating chamber and set aside momentarily while the bag is resealed with a twist tie.

Harvesting eggs

2.8.4 Adults shall be harvested from each mating chamber and maintained separately for QC analysis; adults shall be removed by gently shaking them to a bottom corner of the bag, cutting the corner and dumping them through a large sterile funnel and into a labeled (ID code and bag number) 50 ml screw top vial (alternatively, adults may be dumped directly out of the top of the bag and into the funnel).

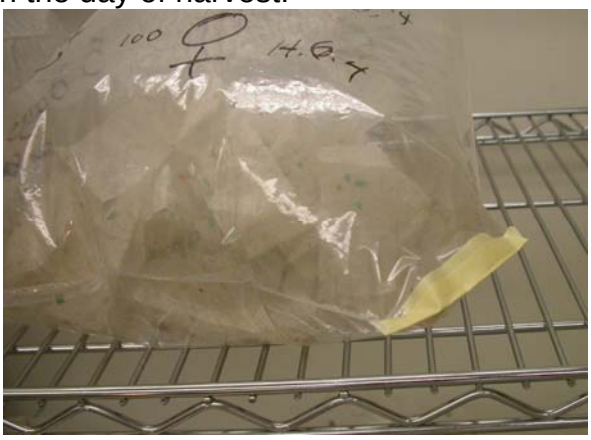
Preparing to collect adults for QC



Collecting adults for QC

2.8.5 Adults from each mating chamber shall be frozen until transfer to the QCU for subsequent analysis for pathogens.

2.8.6 Within a BSC, waxed paper strips with egg masses shall be placed back in their original mating chamber, sealed with a twist tie, corner taped shut, and moved to the wire shelf in the environmental chamber (23±3ºC, 55±10%RH and 16L:8D) for 24h to allow eggs to harden off before surface sterilization; eggs harvested on day 6 shall be surface sterilized on the day of harvest.



Hardening-off of eggs

2.8.7 The lower portion of the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3) shall be completed after the egg mass harvest (i.e., the date that adults are removed) from each mating chamber to indicate:
(a) adult discard date
(b) initials of the individual who harvested the eggs

2.8.8 The individual who subsequently surface sterilizes the eggs (as described in section **2.9**) shall initial the applicable portion of the *Co Tracking* form.

2.9 Egg Mass Surface Sterilization

2.9.1 Sufficient quantities of lab instruments (i.e., one set for each mating chamber to be dismantled; refer to photo below) shall be prepared ahead of time by wrapping in aluminum foil, sterilizing by autoclaving and maintaining on the shelf until required for use.

Lab instruments for surface sterilization of eggs

2.9.2 Wax paper strips with egg masses shall be removed from the mating chambers within a BSC or chemical fume hood (cleaned before and after opening each mating chamber); the staples shall be removed and the wax paper strips maintained separately by mating chamber in foil covered 2L beakers labeled with the ID code for the new generation (e.g., progeny of *Co*1-F_2 shall now be identified as *Co*1-F_3) and with the bag number.

Removing eggs from mating chamber

2.9.3 Egg masses that were laid on the inner surface of the bag shall be removed by bending the plastic or scraping with forceps; egg masses shall be added to the others from the same mating chamber.

2.9.4 Each beaker of egg masses shall be surface sterilized successively (not concurrently); mating chambers shall not be disassembled or egg

masses sterilized within the BSC while previous batches are hanging to dry in that unit.

2.9.5 Egg masses shall be surface sterilized within a BSC by adding 1 L of 1% Javex® (refer to 2.16.1) to the 2L beaker.

2.9.6 Using a gloved hand, wax paper strips shall be pushed beneath the surface to ensure complete contact between the egg masses and the Javex® solution.

Surface sterilizing eggs

2.9.7 Egg masses shall be allowed to soak for 10 minutes at room temperature; wax paper strips shall be pushed beneath the surface at least twice during the 10-minute interval to dislodge trapped air bubbles and to ensure complete contact with the Javex® solution.

2.9.8 The Javex® solution shall be decanted out of the beaker; to prevent losses, the solution shall be poured through a sterile sieve to capture dislodged egg masses; decanting and subsequent rinsing may be conducted on the lab bench due to lack of space and/or a drain within the BSC.

Collection of dislodged eggs

2.9.9 Egg masses shall be rinsed by the addition of 1 L autoclaved tap water to the beaker and re-submerging the wax paper strips for about 10 seconds.

Rinsing surface sterilized eggs

2.9.10 The rinse water shall be decanted off and replaced for a total of 3 rinses; to prevent losses, the water shall be poured through the sieve to capture dislodged egg masses.

Final collection of dislodged eggs

2.9.11 Within a BSC or laminar flow hood, the wax paper strips with egg masses shall be hung to dry for 1-2h; the ambient relative humidity of the work room shall be maintained below 50% RH for sufficient drying of the egg masses.

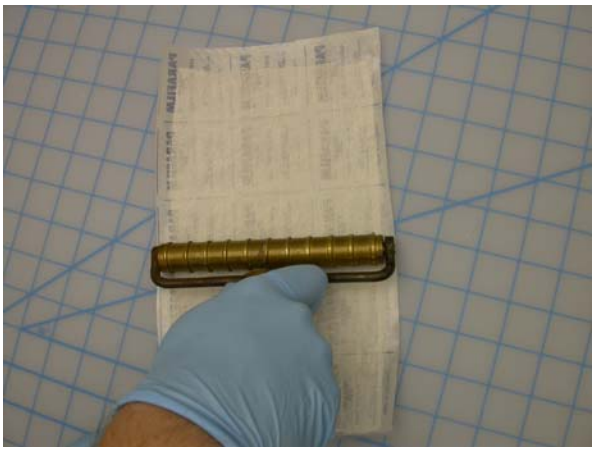
Hanging of wax paper strips for drying

2.9.12 Dislodged egg masses shall be placed on autoclaved paper towel (raised off the bench surface using a vented metal tray) within the BSC or laminar flow hood for drying; it is critical that these egg masses be separated prior to drying; care must be taken to keep egg masses separate by ID code and by mating chamber number by use of sufficient labeling.

Drying of eggs

2.10 Egg Hatch

2.10.1 Gauze/parafilm substrate (for subsequent spinning of hibernacula by newly hatched larvae) shall be prepared in advance of hatching pan setup; this shall be conducted with a BSC or laminar flow hood; both top and bottom sheets shall be prepared by attaching 5” x 9” pieces of autoclaved gauze to 16” x 20” (top sheet) and a 6” x 10” (bottom sheet) pieces of parafilm using a hand-held ribbed roller and a soft cutting mat; the gauze/parafilm substrate shall be stored in sealed plastic bags until use.



Rolling gauze onto parafilm while placed on cutting mat

2.10.2 Hatching pans (12” x 18”; sterilized by autoclaving) shall be prepared for each mating chamber within a BSC or laminar flow hood.

2.10.3 The bottom sheet of gauze/parafilm shall be securely adhered to the bottom of the pan (to prevent hatching larvae from getting underneath) by scoring the exposed parafilm edges with forceps and then pressing firmly with a gloved hand.

Placement of bottom gauze/parafilm in hatching pan

Attaching bottom gauze/parafilm to hatching pan

2.10.4 Within a BSC or laminar flow hood, wax paper strips from each mating chamber shall be folded lightly (avoid sharp creases; avoid crushing the eggs) and placed on edge on the bottom of the pans (but not touching the gauze or parafilm).

Adding strips of eggs to hatching pan

2.10.5 Dislodged egg masses shall be placed loosely in the pans (not touching the gauze or parafilm); all egg masses from a mating chamber shall be placed in one pan.

Adding loose eggs to hatching pan

2.10.6 Within the BSC or laminar flow hood, the top sheet of gauze/parafilm shall be placed loosely on the top of the pan.

Placing top sheet of gauze/parafilm on hatching pan

2.10.7 The pan may then be removed from the BSC or laminar flow hood and the top sheet shall be sealed by stretching the parafilm over the rim. Seal the four sides first, then the corners.

Sealing the parafilm from the top sheet to the hatching pan

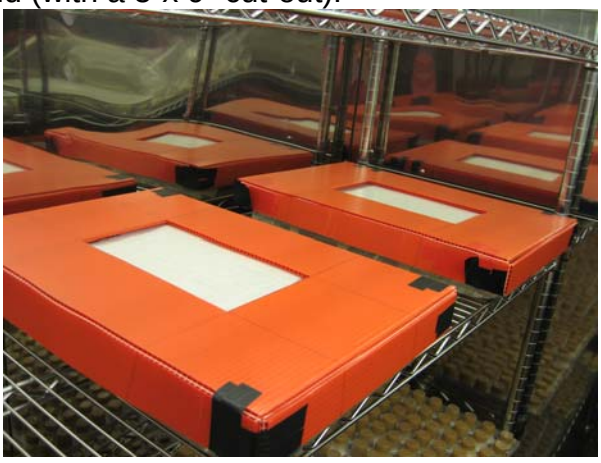
2.10.8 To ensure a tight seal, the edge of a pen/marker shall be run along the upper edge of the pan.

Ensuring a tight seal of the top gauze/parafilm to the hatching pan

2.10.9 Each pan shall be labeled with the applicable ID code, mating chamber number, date of pan set-up, and dates 3 and 13 days hence (i.e., dates when covers are to be installed and when L2s are to be counted). Hatching pans shall be maintained in an environmental chamber (23±3ºC, 55±10%RH and 16L:8D) for 13d to allow eggs to hatch and larvae to spin hibernacula (total 14d since egg harvest).

Completed hatching pan

2.10.10 Once the 3-day match is reached, each pan shall be covered with a coroplast lid (with a 5"x 9" cut-out).



Hatching pans with covers in place

2.10.11 The lower portion of the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3) shall be completed after the hatching pan set-up for each mating chamber to indicate the date of preparation and the initials of the individual who performed it.

2.10.12 Pans on upper shelves within the environmental chamber shall be exchanged with those on lower shelves on a daily basis, excluding statutory holidays or days of rest when personnel are not in the facility.

2.10.13 Upon completion of 13 days of hatching and spinning of hibernacula, larvae obtained from each pan shall be quantified as described in section ***2.11***; if there is insufficient time to conduct larval counts on the scheduled date, pans may be left for an additional 2-3 days and the length of pre-diapause reduced by the same amount.

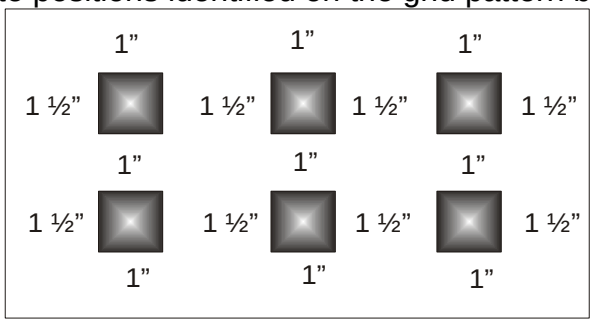
2.11 Quantification of Larvae

2.11.1 Prior to counting larvae, the top sheet of gauze shall be examined for the presence of fungus; this shall be conducted before breaking the parafilm seal; when 5 or more spots of fungal growth are observed, the L2s from the pan will not be counted (pans with fewer than 5 spots will be counted as specified in 2.11.2 through 2.11.5); the contaminated pan shall be set aside and disassembled in a BSC or chemical fume hood once the remaining pans for the counting session have been analyzed; L2 counts shall be estimated visually for both sheets of parafilm/gauze from the contaminated pan (as well as losses in the pan) and shall be maintained by packaging and labeling as specified in section ***2.12***, with the exception that the counts will be written in parentheses and will be identified with the level of fungus contamination as specified in ***2.11.6***.

2.11.2 Mean counts shall be determined using a fixed-focus magnifier from six 1"x 1" sample areas/fields on each top sheet of gauze.

Magnifier for counting L2 larvae

2.11.3 The location of the 6 sample areas shall be estimated visually at the approximate positions identified on the grid pattern below:



L2 sample locations

2.11.4 The count for each sample area shall be recorded on the *Co L2 Count* form (IPS Form Number 0128/002, Appendix 5), along with the ID code and mating bag number.

2.11.5 The following calculations shall be determined and documented on the form:
(a) sum of the 6 counts
(b) mean of the 6 counts
(c) estimate of the number of larvae on the 45 in^2 top sheet; determined by multiplying the mean by 45.

2.11.6 Within the BSC, the covering sheet of parafilm/gauze shall be flipped back for 20 minutes to allow excess moisture to evaporate; the contents of the pan shall be examined for the presence of fungal contamination and classified as one of the following:

Level 0: No visible sign of fungus found on any substrate (egg masses, wax paper or gauze sheets).

Level 1: Any of the following:
-Fewer than 10% of egg masses are contaminated
-Trace amount on wax paper
-Up to 2 spots of fungus on gauze (both sheets combined).

Level 2: Any of the following:
-10-50% of egg masses are contaminated
-Significant amount on wax paper
-5 or fewer spots of fungus on gauze (both sheets combined).

Level 3: Any of the following:
-More than 50% of the egg masses are contaminated
-Large amount on wax paper
-More than 5 spots of fungus on gauze (both sheets combined).

Note: Batches that are borderline between Level 2 and 3 will be designated as 3; when fungus levels are different for each sheet within a pan, they will both be designated with the higher level.

2.11.7 L2s in the bottom sheet shall be counted (as specified in 2.10.7) only when the fungus level for the pan is 2 or lower; when there is level 3 fungus contamination, the pan shall be closed up and set aside within the BSC for disassembly once the remaining pans for the counting session have been analyzed; both sheets of parafilm/gauze from the contaminated pan shall be maintained by packaging and labeling as specified in section ***2.12***, with the exception that they will not be labeled with precise counts (unless the top has already been quantified) but will be identified with a visual estimate written in parentheses and with the level of fungus contamination.

2.11.8 Counts of L2s from the bottom sheet shall be determined and documented using the same procedures specified in 2.11.2 through 2.11.5; to prevent crushing of larvae during counting, the bottom sheet shall be placed over another empty pan to which parafilm has been fitted; the sum of larvae from both sheets shall be recorded on the Co *L2 Count* form.

2.11.9 When fungus is found in a pan, the level of contamination shall be recorded on the:
(a) *Co Tracking* form (IPS Form Number 0120/002; Appendix 3),
(b) packaging for the gauze sheet,
(c) *Co Bag Label* (IPS Form Number 0129/002; Appendix 2), and
(d) *Co L2 Count* form (IPS Form Number 0021/00; Appendix 5).

2.11.10 Spots of fungus shall be cut out from contaminated sheets of gauze when there are 5 or fewer.

2.11.11 A visual estimate of the number of larvae not entering the gauze sheets (and subsequently discarded) shall be determined for each pan and shall be recorded on *Co L2 Count* form; the completed form shall be maintained with IPU records.

2.11.12 The date counted, the number of larvae not entering diapause, the number of larvae within each sheet, and the fungus level shall be recorded on the *Co Tracking* form, along with the initials of the individual(s) performing the count.

2.12 Pre-diapause and Diapause

2.12.1 Top and bottom sheets from each pan shall be placed separately in individual clear plastic bags (after cutting off extraneous parafilm), sealed with twist ties and labeled with the corresponding ID code, mating chamber number, quantity of larvae, level of fungus contamination (only when present) and either top or bottom sheet identifier.

2.12.2 All plastic bags with larvae from the first 5 mating chambers shall be placed into one paper bag and those from the last 5 mating chambers shall be placed into a separate paper bag; each bag shall be closed with a paper clip and shall have a completed label attached (refer to IPS Form Number 0129/002, *Co Bag Label;* Appendix 2); bags of larvae shall be maintained in an environmental chamber (23±3ºC, 55±10%RH and 16L:8D) for 2 weeks of pre-diapause; the date that diapause is to commence shall be written on the label as well as on the bottom portion of the *Co Tracking* form (IPS Form Number 0120/002; Appendix 3); diapause for all progeny from the cohort shall commence 27 days after the pan set-up date for the last mating chamber (i.e., 13d egg hatch + 14d pre-diapause).

2.12.3 The IPU shall provide a copy of the *Co Tracking* form to the QCU at this time; the original form shall be maintained with IPU records.

2.12.4 Upon completion of pre-diapause, bags of larvae shall be transferred to the diapause cold room to complete diapause.

2.12.5 The length of diapause required to facilitate *colony maintenance* will be determined as specified in section ***2.15***.

2.13 Destiny of Larvae

2.13.1 During the diapause period, the QCU will provide written notification to the IPU of results of QC screening of parental adults from each mating chamber; the IPU will be instructed as to the destiny of the larval progeny from each mating chamber [e.g., retain for *colony maintenance*, *distribution*, or *discard* (or distribute to willing clients)].

2.13.2 Upon notification by the QCU, the IPU shall sub-divide the larval progeny (by destiny) into separate paper bags labeled with the ID code and identification as either *colony*, *distribution* or *discard*.

2.13.3 Larvae destined for *distribution* shall be subdivided into those that are to be shipped to external clients as diapausing L2s and those that may be set up in the IPU facility for distribution prior to thinning; progeny that have been identified by the QCU as having *trace* or *low* levels of pathogens shall be distributed in the diapause stage and shall not be reared in the insect production facility (IPU shall inform clients of potential pathogens).

2.13.4 Distribution and/or rearing of larvae that have been identified as being contaminated with fungus shall be as follows:

(a) Level 1: larvae may be distributed (after cutting out spots) but not reared in the insectary.

(b) Level 2: larvae may be distributed (after cutting out spots) only if client demand is extremely high and our supply is low; these will not be reared in the insectary.

(c) Level 3: larvae will be discarded.

2.13.5 In consultation with the QCU, some larvae destined for *distribution* may be retained by the IPU if additional colony scale-up is required, or to replace diseased bi-weekly batches.

2.14 Distribution of Insects

2.14.1 IPU personnel shall minimize labour by distributing larvae to external clients during diapause, or to internal clients prior to thinning; metal trays shall not be used for distribution.

2.14.2 No larvae shall be distributed to clients until QC screening for the cohort has been completed by the QCU.

2.14.3 Distribution of insects (at any stage of development) to clients shall be documented on IPS Form Number 0127/002, *Co Distribution* form (Appendix 1) as specified in section ***2.2***; the form shall be maintained with IPU records.

2.14.4 All client complaints received by the IPU regarding insect or diet quality shall be forwarded to the QCU.

2.15 Rearing Schedule

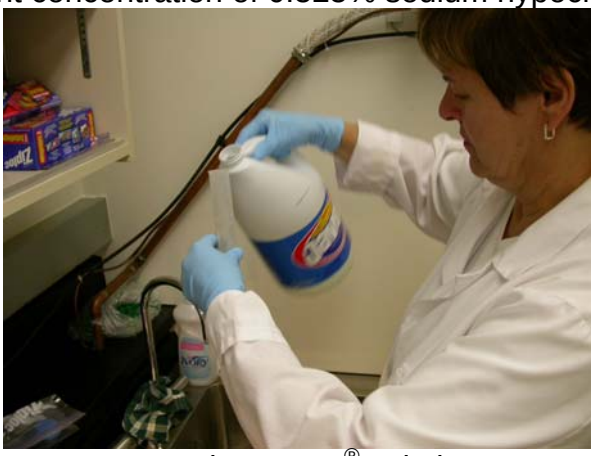
2.15.1 The *Co Rearing Schedule* (IPS Form Number 0131/002; Appendix 6) shall be maintained electronically on the Insect Production drive and shall be predetermined by discussion between the IPU supervisor and the technician having responsibility for the colony. The schedule shall facilitate 20-37 weeks diapause for each family, although 24 weeks is

optimum. Allowances will be made to facilitate breaks (e.g., Christmas, Easter, summer vacations) and splitting of families to replace diseased ones. Typically, L2s shall be taken out of diapause every 2 weeks (typically on a Monday) to meet demand and to sustain 24 families for each generation.

2.16 Calculations

2.16.1 1% Javex® shall be prepared daily by adding 10 ml Javex® (i.e., 5.25% sodium hypochlorite) to 990 ml autoclaved tap water to yield an active ingredient concentration of 0.0525% sodium hypochlorite.

2.16.2 10% Javex® shall be prepared daily by adding 100 ml Javex® (i.e., 5.25% sodium hypochlorite) to 900 ml autoclaved tap water to yield an active ingredient concentration of 0.525% sodium hypochlorite.



Preparing Javex® solution

2.17 Documentation and Reporting

2.17.1 Compliance to this SOP shall include the completion and maintenance of the following forms:
- (a) IPS Form Number 0127/002, *Co Distribution* (Appendix 1)
- (b) IPS Form Number 0129/002, *Co Bag Label* (Appendix 2)
- (c) IPS Form Number 0120/002, *Co Tracking* (Appendix 3)
- (d) IPS Form Number 0130/002, *Co Discards* (Appendix 4)
- (e) IPS Form Number 0128/002, *Co L2 Count* (Appendix 5)
- (f) IPS Form Number 0131/002, *Co Rearing Schedule* (Appendix 6)

2.17.2 Any other pertinent information (e.g., malfunction of environmental chamber, justification for delaying procedures identified in this SOP, observation of unusual occurrences, etc.) shall be documented on the applicable tracking forms.

2.17.3 The *Co Tracking* form shall be copied to the QCU once larval progeny enter pre-diapause.

2.17.4 The IPU shall make all records available to the QCU.

3.0 DISTRIBUTION AND ARCHIVING

3.1 Distribution

This SOP shall be distributed by the IPS manager to all IPU personnel.

3.2 Archiving

3.2.1 The IPS manager shall maintain a historical copy of this SOP when it is replaced by a new version.

3.2.2 The IPU supervisor shall ensure that files of all documentation identified in 2.16 are maintained for expedient retrieval.

3.3 Destruction of Outdated SOPs

When new versions of this SOP are available for distribution, all persons in possession of a controlled copy shall ensure the retired version is returned to the IPS manager upon request.

4.0 ASSURING SOP VALIDATION AND COMPLIANCE

4.1 Responsible Individual

4.1.1 The IPU supervisor is responsible for assuring that this SOP is valid.

4.1.2 The IPU supervisor is responsible for assuring that this SOP is followed by IPU personnel and that these persons have been appropriately trained in its use.

4.1.3 IPU personnel are responsible for complying with procedures specified on a *Controlled Copy* of this SOP and shall never use non-controlled copies which could be outdated.

5.0 REVISION OF THE SOP

5.1 Responsible Individual

The IPU supervisor is responsible for assuring that this SOP is current. If necessary, the IPU supervisor shall initiate the revision process.

5.2 Revision Schedule

This SOP shall be revised when its provisions no longer agree with current practices or GLFC policies, and shall be approved by the IPS manager.

6.0 CONTINGENCIES

When IPU personnel find circumstances that do not permit compliance with this SOP, the IPU supervisor shall be consulted.

7.0 CONFIDENTIALITY

IPS SOPs are not considered to be confidential documents and may be distributed to outside parties. *Controlled Copies* shall not be reproduced.

8.0 REFERENCES

(a) SOP Number IPS/009, *IPU Personnel Responsibilities*

9.0 APPENDICES

Appendix 1: IPS Form Number 0127/002, *Co Distribution*
Appendix 2: IPS Form Number 0129/002, *Co Bag Label*
Appendix 3: IPS Form Number 0120/002, *Co Tracking*
Appendix 4: IPS Form Number 0130/002, *Co Discards*
Appendix 5: IPS Form Number 0128/002, *Co L2 Count*
Appendix 6: IPS Form Number 0131/002, *Co Rearing Schedule*

Appendix 1

***Co* Distribution**

ID Code:

(from *Co* tracking form) ❶ Total # larvae in diapause:
(from QC report) ❷ # kept for colony maintenance:
(from QC report) ❸ # discarded for QC reasons:
❶ - (❷ + ❸) ❹ # available for distribution:

Lab Supply

Date Out of Diapause (DD/MM/YY)	# Larvae	# Cups	Diet ID	Distribution			# Cups Discarded
				Pick-up Date (DD/MM/YY)	# Cups	Recipient	
	∑= ❺	∑= ❻			∑= ❼		∑= ❽

Distribution during diapause (i.e., L2s in gauze)

Date distributed (DD/MM/YY)	# L2		Recipient	
	Requested	Distributed	GLFC	Other
		❾∑=		

❻ = ❼ + ❽

discarded after 37 weeks diapause: ❿
Discard Date (DD/MM/YY):

❹ = ❺ + ❾ + ❿

IPS form Number 0127/002

Appendix 2

Co Bag Label

ID Code:		Start Diapause: DD/MM/YY		
Mating Bag #	Top gauze	Bottom gauze	Fungus level	Mating Bag Total
1				
2				
3				
4				
5				
			Total L2 this bag:	

ID Code:		Start Diapause: DD/MM/YY		
Mating Bag #	Top gauze	Bottom gauze	Fungus level	Mating Bag Total
6				
7				
8				
9				
10				
			Total L2 this bag:	

IPS Form Number 0129/002

Appendix 3

Co Tracking (Colony Maintenance)

ID Code_____ Created from: □ Previous generation □ Other _____

Date out of diapause (DD/MM/YY)	# wks*d	# out	Patch Set-up # cups	Patch Set-up Diet ID	Initials	Thinning Date (DD/MM/YY)	Thinning # cups	Thinning #cups with dead	Thinning Diet ID	Initials

Dead larvae frozen for QC: Yes No
Patches frozen for QC: Yes

Pupa Harvest

	1st Date: (DD/MM/YY) ♂	1st Date: (DD/MM/YY) ♀	2nd Date: (DD/MM/YY) ♂	2nd Date: (DD/MM/YY) ♀
# Kept				
# Discard				
# cups with Dead				
cups frozen for QC	Yes	No	Yes	No
Initials				

Total ♂	Total ♀	Total Pooled
ξ=	ξ=	ξ=
% discard	% discard	% discard

75%
♂= ♀=

Mating Chambers

Bag #	Date initiated (DD/MM/YY)	Initials	Date completed (DD/MM/YY)	Initials	Adult discard date (DD/MM/YY)	Initials	Egg wash Initials	Pan set-up date (DD/MM/YY)	Initials	Number of L2 Top	Number of L2 Bottom	Number of L2 Lost	Fungus Level	Date Counted (DD/MM/YY)	Initials
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
										ξ= x= SD=		ξ= %=		Start diapause: (DD/MM/YY)	

Pupa cases saved for QC: Yes

IPS Form Number 0120/002

Appendix 4

Co Discards

ID	Date into Diapause	Date out of Diapause	Total L2	Kept for Colony	QC Discard	Available	Distribution During Diapause	37 Week Discard Date	Actual Discard Date	Total used	Discarded	% Disc.

IPS Form Number 0130/002

Appendix 5

***Co* L2 Count**

ID Code: _____ Mating Bag # _____

L2s lost to top edge of pan:
L2's lost on pan bottom/sides: _____
Total L2s lost in pan: Fungus Level: _____

Top Gauze Count	Field 1				
	Field 2				
	Field 3				
	Field 4				
	Field 5				
	Field 6				
	Total	÷ 6 fields = _____	X 45 in^2 gauze area =	_____	#L2s in top gauze
Bottom Gauze Count	Field 1				
	Field 2				
	Field 3				
	Field 4				
	Field 5				
	Field 6				
	Total	÷ 6 fields = _____	X 45 in^2 gauze area =	_____	#L2s in bottom gauze
				_____	Total L2s in gauze

IPS Form Number 0128/002

Appendix 6

***Co* Rearing Schedule**

ID Code	Date into Diapause (DD/MM/YY)	Date out of Diapause (DD/MM/YY)	#weeks $^{+d}$ Diapause	37 week Discard Date (DD/MM/YY)
Co 1-F_				
Co 2-F_				
Co 3-F_				
Co 4-F_				
Co 5-F_				
Co 6-F_				
Co 7-F_				
Co 8-F_				
Co 9-F_				
Co 10-F_				
Co 11-F_				

IPS Form Number 0131/002

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