STANDARD OPERATING PROCEDURES FOR THE MASS-REARING OF *GALLERIA MELLONELLA*

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TABLE OF CONTENTS

page

LIST OF FIGURES

Figures page

1.1 Introduction

"Know your insect"

—Allen Carson Cohen

moth intro
This document will continue to be updated to include the newest research relevant to the DBCL's research goals, and should be considered a work-in-progress. This SOP includes some adaptions based on the resources available at our rearing facility at the DBCL, your facility may require significant adaptations in order to fulfill your rearing needs. With that being said, these operating procedures come with no *guarantee* of suitability for any particular purpose. Even so, we wish you the best of luck in your endeavors to mass-rear these beautiful invertebrates.

1.2 Moth Development Timeline

moths

- lifecycle

1.3 ETHICAL AND HUMANE TREATMENT OF ORGANISMS

We at the Dundee Biological Control Laboratory strongly encourage that each lab adopt a policy for the humane and ethical treatment of the organisms they work with.

1. Recognizing the diversity and complexity of life systems, we value all living things
2. In light of this value, we try to design our work to help us understand the nature of life and living systems with a minimum of harm to or discomfort of living organisms
3. We treat the subjects of our studies with respect, dignity, and with efforts to inflict a minimum of pain, trauma, or damage. This includes using, whenever possible, physical models instead of living systems to conduct our experiments.
4. We first consider ways that can inflict a minimum of potential harm to organisms and we ask the question: is the experiment necessary to give us the information we seek? Is the information of sufficient value to merit such sacrifices?
5. Furthermore, we subscribe to the tenet that the information derived from studies of organisms can be no better than the health, homeostasis, and minimization of stress of the organisms involved. Therefore, we strive to make our rearing systems and every other aspect of our handling of insects compatible with insect well-being.

Translation of these tenets into practice:

1. We use the concept of insect homeostasis of "comfort" in the sens that sensitive and reasonable thought about our insect handling can suggest guidelines about issues like food quality, space, management of waste, access to avoidance of adverse stimuli, sufficient gas exchange, protection from contaminants and pathogens, and all other aspects of insects' well-being as it would pertain to the populations of insects in the wild
2. We subscribe to the model that teaches that well-treated insects are closer to "normal" insects from the wild. Such insects are of higher quality than ones that have been reared under suboptimal conditions. Therefore, we believe that our science is better when all aspects of our inquires are conducted with the highest possible quality of components.
3. Therefore, our research into development and improvement of rearing systems keeps at its forefront the homeostasis of the insects subjects in our studies, and we further strive continuously to improve the base of knowledge for the scientific community to improve the conditions of rearing systems and the quality and well-being of the subjects.

2.1 Materials

- Datasheets (8.10.3.1)
- Insect cage

2.2 Methods

2.2.1 Insect Cages

Label each cage is with the following: I = date initially set, T= date when new adults are added, and # = current number of adults in the cage.

- Add new 'T' labels each time new adult weevils are added to a cage, and update the '#' as insect populations change.
- Write clearly and legibly, or print labels with a label maker to avoid misunderstandings.
- Record all information in the appropriate datasheets found in 8.10.3.1.

2.2.2 Selecting Healthy Adult Weevils

- beans

Figure 2-1. Cages for adult *Diaprepes abbreviatus*: One new cage is started each month with around 50 adult *D. abbreviatus* per cubic foot

3.1 Materials

- Diet ingredients, see below

3.1.1 Optimized Diet for *Galleria mellonella*

Table 3-1. From Hickin et al. (2021).

Ingredient	Amount	Unit	Percentage of diet
Oat bran	14	grams	4.9%
Wheat bran	34	grams	12.0%
Rice bran	20	grams	7.1%
Torula yeast	42	grams	14.8%
Beeswax	11	grams	3.9%
Honey	68	grams	24.0%
Glycerol	64	grams	22.6%
Deionized water	30	milliliters	10.6%

This is the preferred diet used for mass-rearing *Galleria mellonella.*

3.1.2 Historical Artificial Diet

Table 3-2. For reference only, not recommended for use in mass-rearing.

Ingredient	Amount	Unit
Cellulose	307	grams

3.2 Methods

3.2.1 Sanitation Before Making Artificial Diet

beans

3.2.2 Diet Preparation Instructions

4.1 Materials

- Wax or butcher paper

4.2 Methods

4.2.1 Preparing Storage Bags

Collect egg strips daily at the beginning of the day to avoid cross

5.1 Materials

- Büchner funnel
- Fast flow filter paper

5.2 Methods

5.2.1 Creating a Grub Shaker

Transferring newly hatched

5.2.2 Surface Sterilizing Neonate Larvae

Any fungal spores or bacteria on the skin of the larvae will be transferred directly to the diet cups, so it is necessary to sterilize the larvae before they are transferred to avoid contamination. Transferring larvae should be done in a clean room on a sanitized surface, while wearing surgical gloves, facemasks, and hairnets to reduce the risks of microbial contamination.

1. Sanitize any surfaces and utensils which will be used by spraying and wiping the work space with 75% isopropyl alcohol.

6.1 Materials

- Forceps
- Soft forceps

6.2 Methods

The original diet cups become overcrowded around 4 weeks (~28 days) as the larvae grow.

6.2.1 Sanitizing and Preparing Grubs for Transfer

1. Cups contaminated with bacteria or mold should be removed prior to separation of grubs and recorded as losses on the *Galleria* discard datasheet, 8.10.3.1.

Ship overnight on the sender's account number for institutions (Universities, USDA, researchers, etc.) and on our own account number for civilians. Include a return label as necessary. Remember to include a copy of our permit when shipping pests like *Diaprepes!*

7.1 Larvae and Pupae

Larvae and Pupae can be shipped in 50 ml centrifuge tubes with diet.

1. Place cups with diet and larvae in 50 ml centrifuge tube trays and place 2 trays in a large Ziploc®-style plastic freezer baggie.
2. Put bagged trays together in a larger bag or trash bag.
3. Put in appropriate size box, fill space with bubble wrap or air bags, etc.
4. Tape box well, reinforce the bottom of the box with tape.
5. Label with 'This side up' before shipping.

7.2 Adults

Teneral adults in their diet cups can be shipped as-is, in the same way we ship larvae.

1. Adults from cages can be shipped in an empty clean diet cup with a damp dental wick.
2. Ship with a ice pack wrapped a paper towel.
3. Do not use an ice pack for teneral adults.

7.3 Eggs

Eggs are more fragile, but can still be shipped if needed.

1. If shipping eggs, ship freshly laid eggs, eggs about to hatch do not survive well.
2. Double-bag eggs in small to medium ziploc baggies with the air gently squeezed out from the bag to prevent desiccation.
3. **Be careful not to squash eggs when squeezing out air!**
4. Ensure both zippers on the plastic baggie are sealed well.
5. Place the nested baggies into a large bag inflated with air, to help protect eggs from damage.
6. Pack the baggies in a small box with plenty of bubble wrap or air bags.

8.1 Materials

- beans

8.2 Methods

- beans

8.3 Daily Duties

- Sinks: Wash dishes using a mild detergent and rinse with 37.5 mls of bleach per gallon of water. Empty and rinse sinks in all rooms at end of day.
- Garbage: Remove garbage and place in freezer for 24 hour duration, after which the material is taken to the dumpster.
- Counters and Table Tops: Spray surfaces with 10% bleach at the beginning and end of work schedule.
- Floors: Sweep and Mop with bleach

8.4 Weekly Duties

- Floors: Sweep all rooms (Q.C. 1,2,and 3), and mop with detergent and water.

8.5 Monthly Duties

- Apply standard quaternary ammonia solution to ceilings, cupboards and drawers, lights, walls and doors using swivel head mop to the following surfaces in Q.C. 1, 2, and 3.
- AC vents: Remove from the ceiling and spray with water, followed by application of the standard solution of quaternary ammonia.

As the above sanitation duties are completed, it is recorded on the Sanitation Checklist Form, SFL 01-10 (see Appendix A-7).

8.6 Rearing Area Organization

Persistence of contamination in lab reared insect colonies has led to separation of colony stages.

- Q.C. 1, has been designated a “dirty area” and as such is reserved for the adult egg laying cages and contaminated cultures held for recovery.

- Q.C. 2, has been designated “semi clean”, and is reserved for cultures 30 days or older (neonate larvae which have been transferred to single cups, and later stages of the life cycle including pupae and teneral adults).

- Q.C. 3, has been designated “clean”, and as such is reserved for new cultures only, and these cultures should be moved to Q.C. 2 at the time of transfer.

8.7 Order of Duties

Organization and order of duties are directly related.

- Clean work should be performed in the morning prior to exposure of equipment/ personnel to contaminants.
- Duties such as neonate transfers and sanitation of Q.C. 3 fall into this category.
- Semi-clean work should be performed next.
- This includes separation of neonate larvae, transfers to fresh diet, and sanitation of Q.C. 2.
- Dirty work should be performed last.
- This includes egg collection, cage maintenance, and sanitation of Q.C. 1.
- Moving from dirty areas to clean ones should be avoided.
- It is important to keep in mind where you have been, and what has been handled.

8.8 Supply Inventory

Supplies should be checked periodically to ensure that shortages do not occur.
A list of needed items should be given to the immediate supervisor or purchaser, based on lead time necessary to obtain these items. (See Appendix A-1 for suppliers.)

8.8.1 Disposal and Cleaning Used Materials

8.8.1.1 Disposal of flush removed from cage

1. Dispose of in trash bags and take to the steamer, or freeze prior to putting in dumpster.
2. Collect dirty vials, water cups, etc. and take to A105.
3. Wash vials and water cups and lids with dawn dish detergent and scrub with bottle brushes OR rinse and put in a dishpan with detergent to soak; then wash later.

8.8.1.2 Disposal of water cups

1. Prior to washing water cups, remove cotton wicks, do not get detergent on water wicks, can rinse with clean water, and gently brush with small bottle brush.
2. Can reuse until they become soiled to the point that the above cleaning methods are no longer effective.

8.9 Materials

- Parafilm®

8.9.1 Potato Dextrose Agar

Table 8-1. For culturing fungi. (FDA 2020, 2022)

Ingredient	Amount	Unit
Potato Infusion	200	grams
Dextrose	20	grams
Agar	20	grams
Sodium Chloride*	75	grams
Deionized water	1000	milliliters
Chlortetracycline-HCl (stock solution)**	4	milliliters

*Optional. Used to to make Potato Dextrose Salt Agar (PDS-A). Cultures fungi with spreading habits, such as *Mucor*, *Rhizopus*, and similar fungi (Mislivec and Stack 1991).
**Optional. See 8.10.2.3 and Mislivec and Stack (1991).

8.9.2 Nutrient Agar

Table 8-2. For culturing bacteria. (FDA 2020, 2022).

Ingredient	Amount	Unit
Beef extract	3	grams
Peptone	5	grams
Agar	15	grams
Deionized water	1000	milliliters

8.10 Methods

8.10.1 Testing for Environmental Contaminants

Follow the sanitation programs as described in 8 and 8.10.3.1 to prevent contamination of *Galleria* cultures. Monitor the levels of microbial contaminants weekly by 'air plating' agarose petri dishes to detect the relative quantities of mold, bacteria, and yeast present. Monitoring for microbial contaminants helps to ensure that the sanitation programs are effective and to make sure areas are adequately sterilized. The goal of sampling air quality is to quantify the number of particles of contaminant in the air, usually expressed in terms of the colony forming units (CFUs) per cubic meter of sampled air during a specific time period (CFU/m^3/time) (Romano 2015).
Our lab uses two types of enriched agarose media to culture and detect contaminants: Potato Dextrose Agar (PDA) and Nutrient Agar, for fungi and bacteria, respectively.

8.10.2 Preparing Agarose Media

8.10.2.1 Nutrient Agar

- Mix dry ingredients, then add to 1 L of DI water.
- Heat to boiling while stirring until ingredients are dissolved.
- Dispense into petri dishes.

- Once all the ingredients are completely dissolved, pour into containers that can be autoclaved and autoclave for 15 min at 121 °C.
- Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes.
- Adjust final pH as needed to be 6.8 ± 0.2.

8.10.2.2 Potato Dextrose Agar (PDA)

PDA medium powder is available commercially but you may need to add extra agar until there is a total of 20 g/liter of agar in the final product. For example, you would need to add 5 g of agar to BBL™ or Difco™ dehydrated medium to have a plate with the correct consistency. PDA medium should not be re-melted more than once.

1. Create potato infusion by boiling 200 g of sliced, unpeeled potatoes in 1 L DI water for 30 mins.
2. Filter solution through cheesecloth and retain the liquid, which is potato infusion. Alternatively, purchase dehydrated potato infusion and prepare according to the manufacturer's instructions.
3. Mix in 20 g Dextrose and 20 g Agar with the 1 L of potato infusion, then stir while bringing to a boil to dissolve the agar.
4. Once all the ingredients are completely dissolved, pour into containers that can be autoclaved and autoclave for 15 min at 121 °C.
5. Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes.
6. Adjust final pH as needed to be 5.6 ± 0.2 pH.

8.10.2.3 Preventing bacterial growth in PDA media

We want to focus on the fungi present in the PDA media, so if bacterial contamination becomes a problem, we can add an antibiotic to the media to avoid this issue. Mislivec and Stack (1991) mentions their preference for Chlortetracycline-HCl, but suggests that other antibiotics such as chloramphenicol or streptomycin can be used in addition to chlortetracycline-HCl. If adding an additional antibiotic, use the same concentration as the chlortetracycline-HCl (40 ppm).

1. Prepare a stock solution of Chlortetracycline-HCl by dissolving 19 g of antibiotic in 100 ml of sterilized DI water
2. Filter stock solution through a 0.45 µm membrane (Nalge Sybron Corp., Rochester, NY). and pour into an aluminum-wrapped amber bottle.
3. Store stock solutions in the dark at 4-8 °C. A properly stored solution should last for at least a month.
4. Allow stock solutions to reach room temperature immediately before use.
5. PPM calculation: add 1 ml of 100 ml stock solution for every 250 ml of media to obtain a concentration of 40 ppm.
6. Add Chlortetracycline-HCl to PDA media at a rate of 40 ppm (final concentration) after agar has been autoclaved and cooled to 47-50 °C.
7. Mix thoroughly and dispense 20 ml portions into 15 × 100 mm petri dishes.
8. Dispense 4 ml of stock filter-sterilized chlortetracycline HCl (1 g/100 ml) per liter of agarose medium.

8.10.3 Sedimentation Plates

An inexpensive and simple method of sampling for aerial microbes is with sedimentation plates, aka. settling plates. Sedimentation plates rely on gravity to cause airborne particles to land on the exposed surface of petri dishes filled with agarose media. These plates are then incubated for 48 hours and the number of CFUs are counted. Viable particles will grow into CFUs, which then can be counted to quantify the level of contamination in the area. In practice sedimentation plates have a bias towards larger particles, and tend to underestimate the number of smaller particles (Bourdillon et al. 1941, Schneider 2009). Due to these shortcomings, the use of sedimentation plates should be used as a preliminary assay only.

8.10.3.1 Sedimentation plate assays

1. Leave a petri dish of PDA and a dish of NA uncovered for 1 hour in each area where insects or diet are handled. More plates can be added for larger areas, or for specific point samples.
2. Replace lid of petri dish, and wrap the edges of the dish with Parafilm®
3. Incubate plates in lab oven at 30 °C
4. Incubate for 2 days (48 hours) for NA media, and 4 days (96 hours) for PDA media.
5. Record the number and type of microorganisms on the Environmental Testing Datasheet (@ref()) .

REFERENCES

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(Principles and procedures for rearing high quality insects) . 2009. Principles and procedures for rearing high quality insects. Mississippi State University, Mississippi State, MS.

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APPENDIX

SOURCE DIRECTORY FOR *GALLERIA* MATERIALS

1oz Cups 1oz Cup Lids 1. Heritage Paper 822 N.W. 107 terr. Gainesville, FL 32606 (352-332-1417) 10.75" x 13" Plastic food container lids 1. Merton Restaurant Supplies. 207 W. Gore St. Orlando, FL 32806 (407-425-4557) Citrus Root Weevil Premix 30 Cup Plastic Trays 1. Bioserve. P.O. Box 450 Frenchtown, NJ 08825 (908-996-2155) Benzoic Acid Methyl Paraben 3.5", 5" Petri Dishes 1. Fisher Scientific. 3970 Johns Creek ct. ste. 500 Suwannee, GA 30024 (1-800-766-7000) Agar 1. Coll-Chem Corp. P.O. Box 721 Ridgewood, NJ 07451 (201-445-6662) Zipper Top Bags (all sizes) Mineral Oil Organic Carrots Dawn Dish Soap Wax Paper 1. Publix. 125 S.W. 34th st. Gainesville, FL 32607 Bleach 1.Home Depot P.O. Box 9903 Macon, GA 31297-9903 Dental Wicks 1. Richmond Dental. P.O. Box 34276 Charlotte, NC 28234 (1-800-277-0377)

DIAPREPES REARING SUPPLIES AND SOURCES:

Product Product # Source Link Dental Wick 200404 Richmond Dental Wrapped Rolls | Richmond Dental
24"x24"x24" Lumite Cage 1450DS BioQuip Search (bioquip.com) 1 oz (30 ml) clear portion containers (cups) Dart/Conex 100pc Webstaurant Store
https://www.webstaurantstore.com/dart-conex-complements-100pc-1-oz-translucent-plastic-souffle-portion-cup-case/301100PC.html clear plastic lids for 100 PC cups PL100N Webstaurant Store
https://www.webstaurantstore.com/dart-solo-pl100n-small-clear-plastic-souffle-cup-lid-case/301PL100N.html
Pre-mix for Diaprepes Root Weevil F9855 Frontier Scientific Services
sales@insectrearing.com +1 302-533-3540 *Special order by request Agar 7060 Frontier Scientific Services https://insectrearing.com/product/product-7060-agar/ Cup Trays – 30 well 9040 Frontier Scientific Services Cup Tray-30 Wells (9040) - Frontier Scientific Services Agriculture (insectrearing.com)

DIAPREPES EGGS AND NEONATE PRODUCTION DATASHEET

Cage ID	EC Week (Gen)	No. Eggs	No. Clusters	Infest Date	No. Larvae	No. Cups

COMMENTS:

rev. 09.02.2022

DIAPREPES GRUB, PUPAE AND ADULT PRODUCTION DATASHEET

Cage ID	EC Week (Gen)	Transfer Date	No. Grubs	Pupation Date	No. Pupae	Emerge Date	No. Adults	No. to Cages

COMMENTS:

rev. 09.02.2022

DIAPREPES DISCARD DATASHEET

Month:

Generation	Date	No. Cups	L	G	P	A	Mold	Mites	Bacteria	Dead	Slow	Elytra	Other

COMMENTS:

rev. 08.26.2022

AUTOCLAVE LOGSHEET

All loads containing biohazardous waste must be autoclaved at 121 °C for a minimum of 30 minutes

Autoclave make/model					Location					
Facility name					Supervisor name					
Lab manager					Phone number					
Date	Contents	Cycle No.	Cycle Type	Time (min)	Pressure (bar)	Temp	Tape Result	CI?	BI?	Operator

COMMENTS:

rev. 08.30.2022

DIAPREPES SHIPPING DATASHEET

Month:

Generation	Ship Date	E	L	G	P	A	Recipient Name	Use Category	Notes

COMMENTS:

rev. 08.26.2022

SANITATION CHECKLIST

AREA: Grub Rooms and Clean Room

DATE: FROM: TO:

	Cleaner	SUN	MON	TUES	WED	THURS	FRI	SAT
CLEAN DAILY								
Dustmop/sweep								
Empty/Clean Sinks	bleach							
Clean Countertops	bleach							
Clean Utensils	autoclave							
Spot mop	bleach							
CLEAN WEEKLY								
Wet mop floors	detergent							
Recycling/garbage								
Clean horizontal surfaces	quat (NR_4^+)							
CLEAN MONTHLY								
AC vents	quat (NR_4^+)							
Ceilings	quat (NR_4^+)							
Humidifiers	quat (NR_4^+)							
Cupboards	quat (NR_4^+)							
Lights	quat (NR_4^+)							
Walls/Doors	quat (NR_4^+)							

COMMENTS:

rev. 08.26.2022

ARTIFICIAL DIET LOG

Initials	Date	Notes	Initials	Date	Notes

COMMENTS:

rev. 08.26.2022

TODO: CAGE FEEDING AND MAINTENANCE CHECKLIST

- Fresh flush bouquets in cage
- Fresh water cups in cage
- New egg strips in cage
- No standing water in bottom of cage
- Check around cage and under shelf for any escapees and place back in cage if found
- Check that tape and marker have been returned to storage box and the lid is closed
- Lid is on trash can
- At end of week take trash bag of trash out
- Cage and room door are closed securely