

Prevention and Management of Insect Diseases in Laboratory Environments

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This article is being shared with butterfly farmers, through the Butterfly Boutique's Butterfly Farming Library and website - useful information on prevention and management of diseases of laboratory- and greenhouse-reared insects. We have used a question-and-answer format, first to general insect pathology and then to specific diseases of primarily lepidoptera, starting with the microsporidians, Nosema and Oe. In answering questions we will rely on our rearing knowledge and experiences plus the writings of primarily the following insect pathologists, Drs. Peter Sikorowski (deceased, Mississippi State University), Louela Castrillo (Cornell University), and George Soares (private business). I (Frank) have many years of experience in rearing lepidoptera and have worked on development of a multi-tactic approach to prevention and management of microbes causing diseases and diet contamination in our laboratory but I am not a trained insect pathologist. Amanda is a well-trained microbiologist with many years of experience in the field of insect pathology.

1. Question – What kind of diseases do insects have?

Answer – Insects are like all living organisms in their susceptibility to infection by a variety of microorganisms such as bacteria, viruses, protozoans, fungi, rickettsia, and nematodes. Some microbes are considered to be true primary disease-causing organisms, such as some baculoviruses which infect butterfly larvae causing them to die. These organisms are capable of causing disease under normal host conditions. Other microorganisms are known as facultative pathogens. They do not normally cause diseases under field conditions, but can under laboratory environmental conditions. These microbes can become associated with the insect through feeding on diet in the laboratory in which the microbes have contaminated. Examples of common facultative pathogens are some bacteria and fungi. Also, we must be aware that not all insect diseases are caused by microorganisms. These are referred to as amicrobial diseases. Such diseases can be caused by mechanical injuries (e.g., bruises caused by mishandling of insects); sub-optimal environmental conditions (e.g., high or low temperatures and/or moisture conditions); harmful chemicals (e.g., toxins, poisons, or insecticides); biological agents (e.g., parasitoids); genetics; and nutritional related.

2. Question – What are the modes of transmission for microbial disease organisms?

Answer – Disease microorganisms can be transmitted via contaminated food ingestion, contact with the insect's cuticle, trans-ovarially (within the egg of the female), trans-ovum (on the surface of the eggs), mating, and by vectors (e.g., ovipositor of parasitoids puncturing the cuticle). The most common mode of transmission is through feeding on microbial contaminated food (e.g., on leaves, artificial diet, and cannibalism of infected insect bodies). Another common source is by introducing disease infected wild insects into your rearing colony. These diseased insects can introduce the infectious microbes through mating (males as well as adult females can transmit some diseases organisms) and eggs (surface contaminated eggs or within the eggs), and by contaminating adult diet with the micro-organisms.

Prevention and Management Insect Diseases in Laboratory Environments

3. Question – How do disease microbes affect the insect?

Answer – Some diseases such as those caused by the baculoviruses cause acute reactions. Such a reaction results in death, usually in the larval stage but depends upon when infection occurs. Other disease organisms such as some protozoans (i.e., microsporidia) cause chronic effects within the insect colony: there can be a slight increase in developmental time and/or reduction in egg production and egg hatch. Some people have referred to this type of disease as being like the new fighter jet the “Stealth” which cannot be detected by radar. However, when chronically-infected individuals are stressed and weakened by say a sub-optimal environmental factor such as high temperature, the organisms increase rapidly and infect a high percentage of the adults. The results can be colony collapse and total destruction.

4. Question – What is the impact of microbial diseases on insect rearing programs?

Answer - Their impact can range from slight to major, depending upon whether the microbe involved causes acute or chronic effects. Acute diseases can cause major disruptions in production of quality insects by causing high mortality in immature stages. Examples of such diseases are those caused by the baculoviruses (i.e., the nuclear polyhedrosis [NPV] virus). Quantifying impact of chronic diseases is more difficult because they normally do not cause mortality. For example, their impact is often reflected in reducing the number of eggs produced per female, or lowering the percentage rate of egg hatch. However, as mentioned above, when the insects are stressed these disease organisms can create major problems for the colony. In fact, colonies can become so weak and vulnerable to these organisms that they must be discarded. In our opinion, microbial diseases are a major threat to rearing quality insects and must be dealt with in a knowledgeable and consistent manner. They can result personally in loss of revenue, research opportunities, and customer confidence in ones reliability as an insect rearer.

5. Question – How do you know that your lepidoptera colonies are suffering from a disease?

Answer – Rearing staff must be educated to recognize the signs and symptoms of disease and must be continuously looking for variations from normal healthy insects. Regular, careful monitoring of the various insect stages is imperative, to watch for signs and symptoms of diseased insects. These signs can be color change, abnormal body size, change in form and texture, odor, wounds (such as melanitic spots indicating point of entry of fungal pathogen), and presence of pathogen (definitive sign of infection in most cases). Symptoms are objective aberrations in function and behavior indicative of disease. Examples of symptoms are abnormal movement (movement to higher elevation, lack of coordination, twitching), abnormal response to stimuli (little or no response to stimuli like touching), reproductive disturbance (reduced matings and number of eggs produced, all male offspring), and variation in longevity (premature mortality, prolonged larval stage). The above information on signs and symptoms were obtained from notes prepared for our insect-rearing workshop by insect pathologist, Dr. Louela Castrillo.

Prevention and Management Insect Diseases in Laboratory Environments

6. **Question** - How do you know whether a microbe is causing the disease and if so, what microbe is involved?

Answer – You must first identify the factor causing the disease and if it is a microbe, which microbe is it. To answer these questions, my (Frank) choice is to consult with an insect pathologist or a microbiologist trained in insect pathology. There are pathology services available like the one that we offer here at Mississippi State University, run by Amanda.

If you want to identify the microbial organism to a major group of microbes (viruses, bacteria, protozoans) yourself, you must become educated in the microbiology procedures, such as:

- preparation of growth media for bacteria identification;
- preparation of specimens (slides) for identification;
- the use of equipment such as light microscopy for identifying microbial characters
- identifying the microbe by use of taxonomic keys.

You must acquire equipment and supplies for preparing the specimens and a suitable light microscope. Some diseases are caused by microbes that can only be identified by electron microscope techniques and equipment. In this case, expert help is required. To identify microbes to the species level does require professional assistance as molecular techniques often must be utilized.

7. **Question** – How does one prevent and manage diseases from occurring in your lepidoptera colonies?

Answer – (Frank) I would suggest a multi-tactic approach to prevention and management of microbial organisms causing diseases. In my former USDA rearing laboratory we implemented a multi-tactic approach for prevention and management of diseases and microbial contamination of the insects' artificial diet. This approach has evolved over time and has resulted in successfully minimizing the problems associated with microbes. First, you and your staff must educate and train yourselves in disease recognition, transmission, and adverse effects of various microbes on the biological fitness (i.e., development, size and reproduction) of your insect(s) and microbes that commonly contaminate the insects' diet. Education in insect pathology is an ongoing process and should be taken very seriously. Secondly, you and your staff must be totally committed to solving your problems with microbes.

8. **Question** – Is prevention of disease organisms and management of these microbes the same?

Answer – No. In my opinion (Frank), prevention is taking the steps to avoid introducing the microbe into the colony in the first place. Management is minimizing the effects of the disease organism once the colony is infected. A good example of prevention is establishing your colony using only disease microbe-free individuals. A good example of a tactic for management of a colony where only a few of the individuals are infected with the disease organism is development of a rearing system that places a minimal stress on the insects.

Prevention and Management Insect Diseases in Laboratory Environments

9. Question – What tactics should one employ to prevent and manage microbes that cause diseases?

Answer – Here is a listing of tactics for prevention and management of disease causing microbes.

1. Disease microbe(s) free colony - The most critical prevention tactic is establishment of a laboratory colony free of disease causing microbes by obtaining a start (founder insects to establish colony with) using (1) insects obtained from other rearing facilities which practice strict sanitation Standard Operational Procedures (SOPs) to avoid disease problems and have a history of maintaining healthy robust colonies or (2) immatures or adults collected from the wild, screening them for disease causing microbes and using only progeny from the disease microbe free parents for colony establishment. When obtaining insects from other rearing facilities, we suggest that you ask the facility manager about the health of their colony(s) and if their insects have ever been screened for the common disease organisms known to infect their insect species. If the colony has not been screened, you should quarantine the new insects and screen them for infection before establishing it as your microbe disease-free colony. We also suggest that you communicate with the rearer in which you obtained the colony start the findings of your screening efforts. As colonies age often rearers want to increase the genetic diversity of the colony by introducing genes from wild individuals. When doing this, one should quarantine the field-collected insects from the established colony, screen for microbial disease infected individuals using appropriate techniques, and use only microbe free insects for crossing into the existing colony. Later in this series when the disease caused by the microsporidian, Nosema, is discussed, a technique for screening for this microbe will be described that does not require waiting to the end of the insect's reproductive life to determine if they are infected.
2. Egg sterilization - Most rearers surface sterilize their eggs (only those to be used to replenish colony) to destroy transovum- (egg surface) transmitted microbes. This procedure involves washing the eggs in chemical solutions such as bleach containing sodium hypochlorite or formaldehyde. Some rearers use an additional tactic in an effort to kill microbes that are inside the eggs (transovarially transmitted). This tactic involves heat treating the eggs in a regulated water bath to destroy the microbes. When attempting to use these tactics research/experimentation must be done prior to implementation to insure effectiveness of the treatment in microbe elimination and to insure that the treatment does not adversely affect the normal development of the eggs.
3. Sanitation/Sterilization- This tactic involves the development of effective SOPs to insure strict personal hygiene as humans are chief carriers of a variety of problem microbes and to sanitize facility floors, walls, work surfaces and equipment used in preparing the diet and to rear the insects in (i.e., larval rearing containers and adult cages). These SOPs must be practiced daily and monitored often to insure that all employees are following them to the letter. We have used janitorial supply businesses that furnish hospitals with anti-microbial supplies and associated equipment as a source of our supplies, equipment, and advanced technology. This tactic should also involve sterilizing the insects' food before feeding. If you are using plant grown tissue such as leaves one should consider sterilizing their surfaces with a solution of 10% Clorox bleach (containing sodium hypochlorite an active sterilizing agent for a variety of microbes) for 5 to 10 minutes and then washing the leaves thoroughly in running tap water to remove the bleach prior to feeding.

Prevention and Management Insect Diseases in Laboratory Environments

For some rearers who use our pathology service for controlling bacteria affecting their butterfly larvae, we have suggested that they dip their leaves in a solution containing a selected antibiotic before feeding. The antibiotic of choice is first selected by screening potential antibiotics for their effectiveness against the bacteria affecting their larvae and then recommending the one that exhibited the most effectiveness against the bacteria. These rearers have reported some success using this tactic.

4. Artificial diets - For those that are using an artificial diet to rear their insects, the diet can be sterilized/sanitized by heating in the preparation (cooking) process and by adding mold and bacteria inhibitors and antibiotics to the diet. Through experimentation you can select a rate for each of these microbial inhibitors that are effective against an array of microbes (i.e. bacteria and fungi) and at a "safe" level which does not adversely affect the biological fitness of the insect that you are rearing.
5. Air Filtration - Air filtration is another critical tactic for preventing microbial caused diseases and diet contamination. Filtration and removal of up to 95% of the particles from the air can be accomplished by forcing contaminated air through a combination of special low and high efficacy filters. Such filters can be inserted directly into the air ducts or by using equipment separate from the heating and cooling system. In addition to overall air purification in your rearing facilities, we suggest the use of clean-air hoods which use high efficiency HEPA filters when handling the food for the larvae and when infesting the food with larvae. Remember, microbes can often piggyback on dust particles or on shed moth/butterfly scales. When these airborne microbial contaminated particles land on the insects' food and are consumed by the larvae or adults, disease can result.
6. Laboratory Design - When designing a rearing laboratory attention needs to be made for separation of clean and dirty rooms. Examples of clean rooms are those where you want to minimize occurrence of microbes such as those for preparing and handling the diet, infesting the diet with first instar larvae, and holding the insects during their development to pupae/chrysalis. Dirty rooms are those in which microbes generated during the rearing process are often released into the air. Commonly these are rooms where you harvest the chrysalis, clean up the rearing containers, remove the refuse generated during the immature developmental period, and maintain the adults.
7. Personnel and traffic control – To maintain an aseptic environment for rearing, we suggest limiting personnel especially into the clean rooms to only authorized individuals. Also, we suggest that rearing personnel restrict their movement from clean to dirty areas and not from dirty back into clean rooms for fear of contaminating these sensitive areas.
8. Stress Minimization – Rearing insects in the laboratory inherently creates stress on the insects which makes them more vulnerable to microbes which cause diseases. Efforts should be made to minimize the stress by not overcrowding larvae or adults, by insuring stable optimum environmental conditions (temperature and moisture) and offering the larvae and adults high quality food. This is a principle tactic used to manage chronic diseases.
9. Monitoring for disease incidence – The insects being reared must be monitored for disease symptoms and signs on a regular basis. Staff must be trained in recognition of symptoms and signs and communicate immediately to supervisor when disease

Prevention and Management Insect Diseases in Laboratory Environments

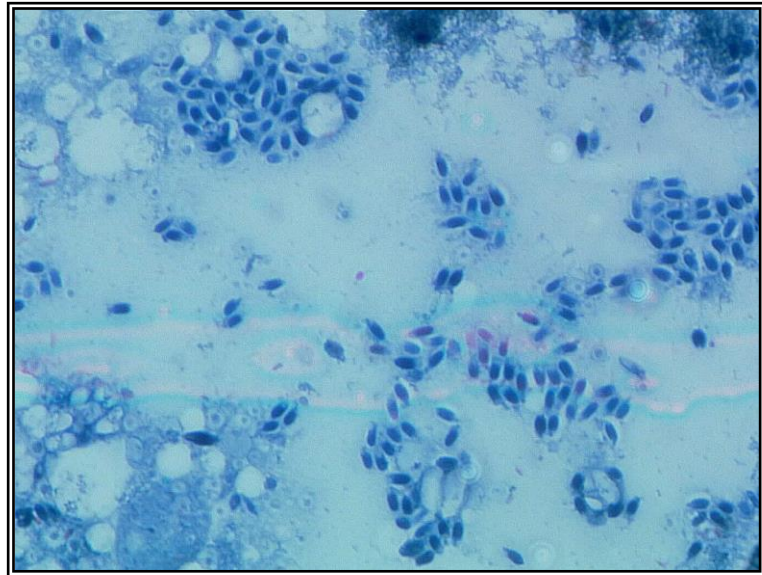
symptoms and signs are observed. Also, we suggest periodic microscopic screening of randomly-selected insects from the colony(s) for disease causing microbes.

10. Insect Pathologist Assistance – It is essential to develop a working relationship with an insect pathologist or microbiologist trained in insect pathology to assist you in prevention and management of microbes causing diseases.

The next part of our information on prevention and management of microbial diseases of insects reared under laboratory conditions deals with individual disease organisms.

Microsporidia

Nosema – The microbe *Nosema* (slide photo by Amanda Lawrence, right) is a protozoan and belongs to a group referred to as Microsporidia. Microsporidia are considered to be among the most important and widespread group of pathogens in insectaries. Microsporidia have unicellular spores containing sporoplasm and extrusion apparatus with a polar filament and polar cap. Most insect orders are susceptible to microsporidian infections, but infection is more common in the orders Lepidoptera and Diptera.



Transmission can occur by ingestion, transovarial, transovum or cuticular with ingestion of spores being the most common route. The spore stage is the only stage capable of existing outside of the host and is resistant to most environmental conditions.

Microsporidian infection normally occurs in the cytoplasm, rarely in the nucleus. Disease produced by these organisms may be sub-acute and chronic to acute. Infection can be limited to a single tissue or organ (e.g., epithelium, muscles, or fat body). Tissue specificity varies with host infected. The same microsporidian species may be limited to certain tissues in one species, but produce systemic infection in another. Chronic infections may be very prolonged, lasting months to a year, while systemic infections may lead to death after a short period of time. Infections limited only to the fat bodies produce chronic infection and infected larvae survive to adulthood, which leads to transovum transmission. The usual signs and symptoms of infection are as follows:

- altered color, size and form depending on tissues infected;
- translucent larvae may turn opaque or milky white;
- growth is retarded; or
- larvae appear sluggish.

Prevention and Management Insect Diseases in Laboratory Environments

An example of adverse effects of the microsporidian, *Nosema heliothidis*, on the bio-fitness of the corn ear worm (*Helioverpa zea*) are reduced egg production per female, longevity and mating success, deformities in pupae, and retarded growth in larval stage.

The above statements concerning disease caused by Microsporida were taken from notes provided for our insect rearing workshop by Dr. Louela Castrillo.

10. Question – Since microsporidian diseases caused by species of the genus *Nosema* are so prevalent in Lepidoptera being reared under laboratory/insectary how does one prevent and manage their occurrence?

Answer – The best way to prevent the microbe from entering the rearing system is to establish a laboratory colony from progeny of only *Nosema*-free adults.

11. Question – How would one test adults for *Nosema* infection?

Answer - One would need to prepare slides with smears of tissues taken from dissected adults or from meconia (a whitish fluid voided from the adult emerging from the chrysalis) for observation using a compound light microscope. The use of meconia for detection of *Nosema* infection is a nondestructive technique that has shown utility in rearing certain moths such as the corn earworm. This technique allows one to select disease-free adults prior to mating and egg production.

12. Question – What equipment and supplies are necessary to screen adults for infection with *Nosema*?

Answer – Amanda suggests the following equipment and supplies with estimated costs. The prices were obtained from Fisher Scientific (fishersci.com).

- a compound light microscope with magnification of 4X, 10X, 40XR, and 100XR (Achromats) – (\$375 to \$563)
- a large slide warmer which can handle about 30 slides at a time – (\$650) or a small hot plate which can handle about 6 slides – (\$230)
- glass slides (\$45/gross)
- cover clips (\$50/~ 100 or so)
- acetic acid (\$51/500ml)
- methanol (\$30/500ml)
- Naphthol Blue Black (\$50/100g)
- immersion oil (\$10/10oz bottle)
- miscellaneous supplies include toothpicks, plastic gloves, and clear plastic rearing cups.

Prevention and Management Insect Diseases in Laboratory Environments

13. Question – What are the steps or procedure for preparing slides for detection of Nosema spores?

Answer – According to Amanda, when preparing a slide using meconia, the following steps should be followed.

Preparing a Slide Using Meconia

Step 1 – Transfer a chrysalid to a suitable clean cup with lid.

Step 2 – Within 24 hours of adult emergence, transfer the butterfly to a new container (number the cup containing the meconia and the cup you placed the adult in using the same number).

Step 3 – Add a couple of drops of sterile water on top of the meconia.

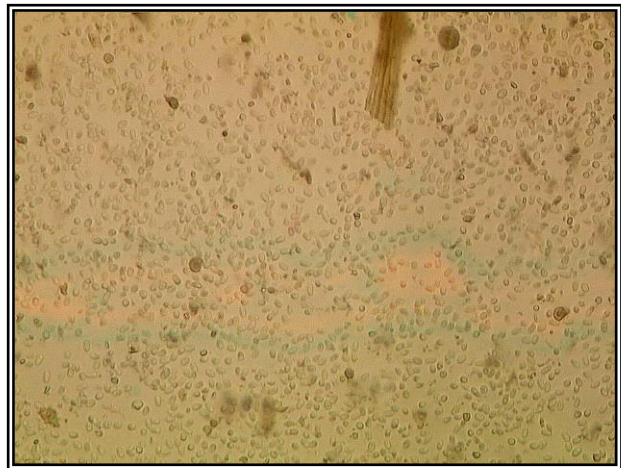
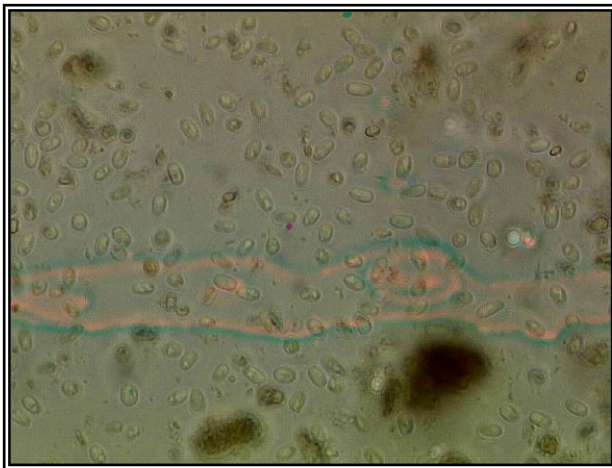
Step 4 – Mix/stir the meconia in the water drops using a sterile toothpick.

Step 5 – Using the toothpick make a fairly heavy stripe on a glass slide (see photo below by Amanda Lawrence).

Step 6 – Allow to air dry.

Step 7 – Stain the slide using the procedure described below. Step 8 – Place a drop of immersion oil on the stained area on the slide and examine the specimen under 100X magnification optical. 100X provides a much better magnification than 40X to see the spores clearly.

Below are photos of wet-mount Nosema spores (Amanda Lawrence). Photo at left is at 100X magnification and the photo on right is at 40X.



It is easy to see how much better the 100X magnification is, and important to note that in a wet mount, unless the organism is severely infected, at lower magnification it might be difficult to distinguish the pathogen from the host material.

Prevention and Management Insect Diseases in Laboratory Environments

Preparing a Slide Using Tissue From an Insect (Immature or Adult Stage)

Step 1 – Transfer the insect to a clean cup or container.

Step 2 – Drop a small amount of sterile water into the cup.

Step 3 – Using a sterile toothpick grind or macerate the insect.

Step 4 – For wet mounts – place a drop or two of the homogenate on glass slide and then place cover slip on top of homogenate. Make sure volume of water is sufficient not to have air bubbles or dry spots, but not so much the cover slip is floating. For staining, use a toothpick to streak a fairly heavy stripe of tissue homogenate on a glass slide (see photo), then allow the slide to air dry before staining using the procedure described below.

Step 5 – Examine the slide under the microscope at 100X magnification as describes above.

Technique for Preparing Buffalo Black Stain

Step 1 – Measure out and mix together the following ingredients

- 0.15 grams of Naphthol Blue Black
- 45 milliliters of methanol
- 15 milliliters of distilled water
- 30 milliliters of acetic acid)

Step 2 - Pour the mixture through a filter (Whatman's filter paper or simply a coffee filter)

Step 3 - Store the mixture in refrigerator.

Staining the Slide

The staining technique is as follows

Step 1 - Place air-dried slides on warmer at 40° C

Step 2 - Immediately flood slide(s) with Buffalo Black stain and let slide(s) stay on warmer for 5 minutes (do not let the stain dry on the slide)

Step 3 - Rinse the slide(s) by gently swishing in a beaker of clean water, and

Step 4 - Allow the slide(s) to air dry before examining under the microscope.

14. Question – How many pairs of disease microbe free adults would you need to start a new colony to insure enough diversity to avoid genetic problems?

Answer - Dr. Alan Bartlett, a well-known insect geneticist, recommends a minimum of 250 pairs to start a colony.

Prevention and Management Insect Diseases in Laboratory Environments

15. Question – What other tactics should one use to prevent and manage Nosema?

Answer – There are several key tactics that we would suggest. They are as follows. Basic to prevention and management of diseases caused by microbes is sanitation and sterilization techniques. Standard Operational Procedures (SOPs) should be developed, followed and strictly enforced concerning personal hygiene and facility sanitation/sterilization which includes floors, work benches/spaces, equipment, re-useable larval rearing containers, and adult oviposition cages. As mentioned previously, we purchase our sanitation supplies from a janitorial supply company that sells to hospitals. For sanitizing/sterilizing floors, work spaces and equipment, we are presently using an ammonium chloride compound by Johnson Wax called “virex 128”. It is a one-step disinfectant, cleaner and deodorant for prevention and management of bacteria, viruses, and fungi. We use on occasion, old faithful, 10% Clorox bleach for some of the same purposes.

Also, basic to maintaining the brood colony, we surface sterilize the eggs with a 3% solution of Clorox. Through experimentation one can establish an effective and safe SOP for surface sterilization of the eggs. The SOP should result in adequate elimination of the Nosema spores on the egg surfaces and should not adversely affect the normal development of the eggs.

To eliminate Nosema within the egg, one might try heating the eggs in a controlled water bath. Experimentation will be required to determine the minimum temperature and time required to kill the microbes. (Please see the Healthy Butterfly section on the website for a paper by Frankenhuysen and co-workers concerning prevention and management of a microsporidian in the eastern spruce budworm). This publication covers prevention and management by water bath treatment of eggs, addition of the fungicide fumagillin to the larval artificial diet, and using only disease free offspring to maintain brood colony.

Since ingestion is a common way in which insects become infected with microbes such as Nosema, the larval food (i.e., leaves) should be surface sterilized before feeding in a solution of 10% Clorox bleach as previously described.

Another key tactic is development of a rearing system that places minimum stress on the insect. Do not overcrowd the insects in a rearing container. In fact, rearing them singly in containers is a good way of avoiding disease spread and helps in managing the disease. Make sure that the larvae and adults have adequate quality food to develop and reproduce normally. Rear the insects in an environment where temperature and humidity is optimum and stable.

Do not forget to rely on the assistance of an insect pathologist or trained microbiologist!

Prevention and Management Insect Diseases in Laboratory Environments

Ophryocystis elektroscirrha



Ophryocystis elektroscirrha (Oe) is an obligate, neogregarine protozoan that infects monarch butterflies (*Danaus plexippus*). As of now, there are no other known hosts of Oe. From our study of the following websites concerning Oe and Monarch butterflies, we gather that this disease organism causes classic chronic effects on the butterfly. Photos show Oe spores on Monarch scales (Monarch Watch).

(http://en.wikipedia.org/wiki/Ophryocystis_elektroscirrha
<http://www.monarchparasites.org>
<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd>), and
<http://www.monarchwatch.org/biology/ophry.htm>)

At high dosages of Oe, the adverse effects on the Monarch was a decrease in

- larval survival
- adult size, and
- shorter life spans

(Altizer, S.M. and K.S. Oberhauser. 1999. Effects of the protozoan parasite ophryocystis elektroscirrha on the fitness of monarch butterflies (*Danaus plexippus*). *Journal Invertebrate Pathology*: 74(1):76-88.)

The chief mode of transmission is by spore-contaminated food and eggs. This contamination occurs on the milkweed leaves during oviposition as infected females shed scales covered with Oe spores. Other modes of transmission are during copulation (mating) of the adult butterflies and by touching each other during overwintering.

16. Question – Would one use the same technique as described for *Nosema* to identify OE as the microbe infecting the monarch?

Answer – Yes, with slight addition the other technique works as is and does not need modification. Spores of Oe can be sampled and identified by applying a piece of clear scotch tape to the adult's abdomen to remove a thin layer of scales. Then the tape is applied to a glass slide and examined under a light microscope at 100X for identification and counting of Oe spores. See photos above.

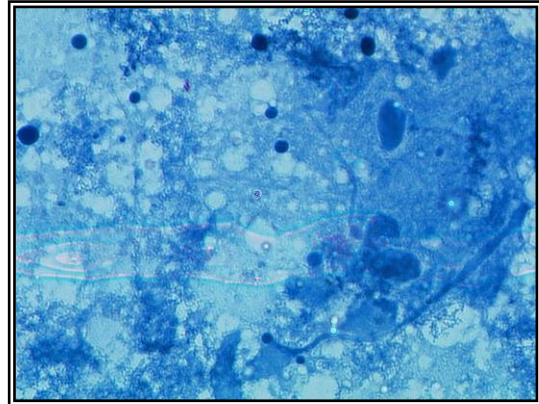
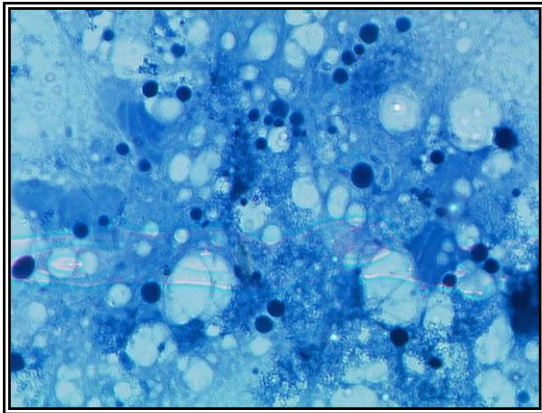
Prevention and Management Insect Diseases in Laboratory Environments

17. **Question** – Would the same prevention and management tactics be used for Oe as described for Nosema?

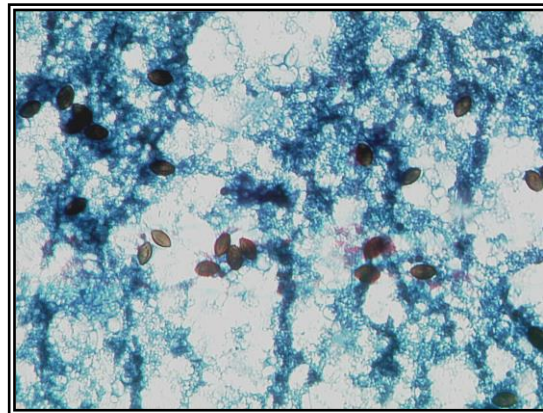
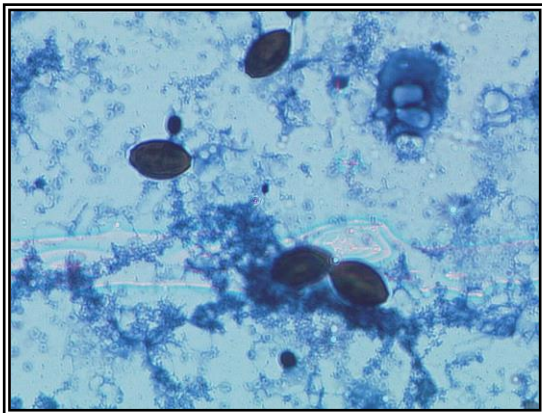
Answer – Yes. The best tactic for both Oe and Nosema is prevention by starting your colonies from progeny produced from disease microbe free adults.

Oe Slide Photographs

Amanda captured these two photographs of Buffalo Black stained homogenate smears from a healthy Monarch adult. All of the material visible on the slides is normal host tissue.



Below are photographs from Amanda, of Buffalo Black stained homogenate of Oe spores. The slide on the left is at 100 X magnification and the slide on the right is at 40 X magnification.



Prevention and Management Insect Diseases in Laboratory Environments

VIRUSES – Viruses are submicroscopic, intracellular, obligate pathogens that affect their hosts by causing host cells to replicate virus genome. They consist of protein and DNA or RNA nucleic acid depending on the type of virus and require the living host cell to reproduce. Viruses are often more resistant to certain environmental factors than some other pathogenic groups. For example, they can survive freezing and desiccation and have been known to remain viable in the soil for many years.

18. Question – How are viruses classified taxonomically?

Answer – Insect viruses are classified according to the nucleic acid they contain (DNA or RNA). They are further separated according to those in which the virions are embedded in a protein matrix or inclusion body (known as occluded viruses) and those in which the virions occur free in the infected cells (known as non-occluded viruses).

19. Question – What are some of the major taxonomic families of viruses that attack insects?

Answer - The major virus families are:

1. Baculoviridae (DNA, occluded, nuclear polyhedrosis viruses (commonly referred to as NPV) and granulosis viruses);
2. Reoviridae (RNA, occluded, cytoplasmic polyhedrosis viruses (commonly referred to as CPV));
3. Picornaviridae (RNA, non-occluded, Enterovirus);
4. Poxviridae (DNA, occluded, Entomopoxvirus);
5. Parvoviridae (DNA, non-occluded, Densovirus); Iridoviridae (DNA, non-occluded, Iridovirus) and Picoraviridae (RNA, non-occluded).

20. Question – How does one know that their insects have a disease and specifically a virus?

Answer – By monitoring the various stages of an insect on a regular basis, one can note individuals that do not appear to be normal in appearance. One must be able to recognize abnormal signs (e.g., color change, abnormal body size, change in form and texture, and odor) and symptoms (objective aberrations in function and behavior, such as, abnormal movement, abnormal response to stimuli, reproductive disturbances, and variation in longevity). Secondly, one must determine if the abnormality is caused by a microbe or another factor. Insect pathologists have developed keys to determine what entomopathogen group is causing a disease based on signs, symptoms and microbe's morphology. One can determine if occluded viruses, such as, NPV and CPV are the causative disease organism by light microscopy. The procedure requires making smears of the dying or dead insects on slides and staining the smears using similar procedures described above for preparing slides for Nosema. Non-occluded viruses cannot be seen by light microscopy techniques. Transmission Electron Microscopy (TEM) is required to identify the presence of non-occluded viruses. For most rearers assistance by an insect pathologist or insect pathology service is required to determine

Prevention and Management Insect Diseases in Laboratory Environments

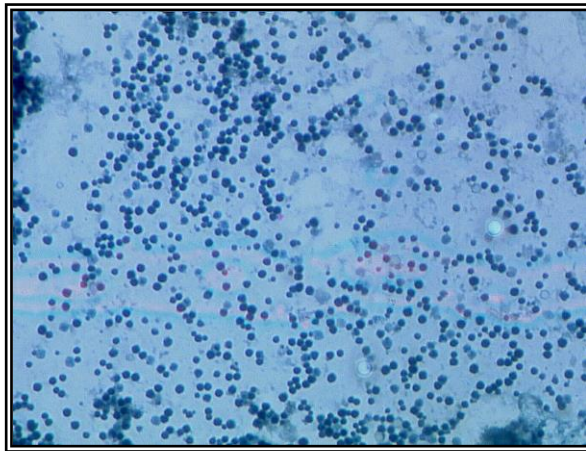
if a virus or some other microbe is responsible for the insects' abnormality. If a microbe cannot be found, the rearer should consider non-biotic, diet, and genetic problems as possible causative factors.

21. Question – Which of the above viruses are the most important to insect rearing?

Answer - The majority of insect viruses of importance to insect rearing are the baculoviruses. The baculoviruses (NPV) are of major concern to Lepidoptera rearing.

22. Question – How would one describe NPVs?

Answer - NPVs produce inclusion bodies (IB) within the cell nucleus that are large enough to be seen using light microscopes (see NPV Inclusion Bodies photo below). Their virions are enclosed by an outer membrane and maybe embedded singly in the inclusion bodies (referred to as a single embedded virus, SEV) or embedded in packets of varying numbers of virions (referred to as a multiple embedded virus, MEV).



NPV Inclusion Bodies

23. Question – How do lepidopterous larvae become infected by a NPV?

Answer – The most common way is by ingestion of the virus. Also, the virus can be transmitted by the female on or within their eggs and an infected adult male/female can transmit the virus during mating.

24. Question – What are some of the signs and symptoms of larvae infected with a virus?

Answer - Affected larval tissues may change color (i.e., to a milky white for some granulosis viruses or bluish for iridoviruses). Infected larvae show a slowed growth and often appear sluggish before cessation of feeding. Larvae infected with NPV often climb to the tops of plants and quit feeding illustrating an abnormal behavioral response. NPV infected dead larval tissue appears disintegrated, liquefied and black in color.

Prevention and Management Insect Diseases in Laboratory Environments

25. Question – Do some viruses cause acute effects while others are more chronic in their effects?

Answer – Yes. The NPVs are acute in their actions and have been used widely as bio-control agents of especially lepidopterous pests. An example of a chronic virus is CPV. CPV infected larvae develop slower than uninfected larvae. Infected female adults oviposit fewer eggs than uninfected females. Please remember chronic diseases can become acute when the insects are reared under stressful conditions.

26. Question – Are viruses specific (infect and cause disease in only one insect species) or non-specific (infect and cause disease in multiple insect species)?

Answer – Generally, viruses are considered to be species or genus specific. However, there are viruses that can infect and cause disease across insect species. An example is the CPV. Some strains of CPV have a broad host range. These strains can cause serious problems where rearers are raising multi-species of Lepidoptera in the same facility.

27. Question – How does one bring in viruses from the field?

Answer – Viruses can be brought into the rearing facilities by viral infected soil and on plant parts used as food for the insects. Also, insect stages collected from the wild can be infected with the virus and can introduce it to the laboratory insects through their eggs, mating and feeding (contaminating the adult food).

28. Question – Once a virus has been introduced into an insectary how can it be transmitted to uninfected insects?

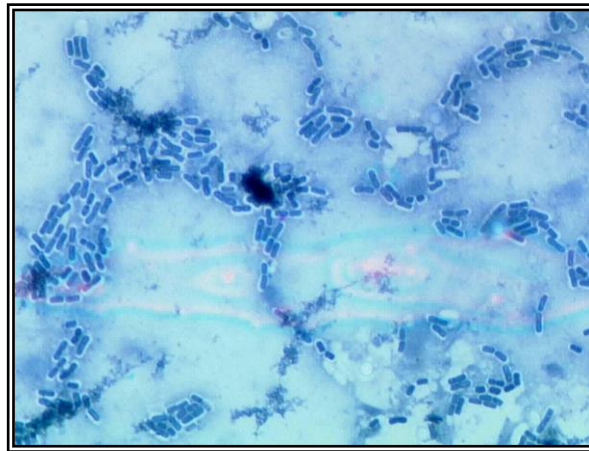
Answer – Viruses can piggy back on dust particles and adult scales and setae being circulated by air currents through out the insectary. Virus particles can land on the insects' food where it can be ingested thereby causing increased infections or on a workspace where food can be contaminated with the virus. Also, infected adults can mate with uninfected adults.

29. Question – How do you prevent and manage viruses within a rearing facility?

Answer – The multi-tactic approach should be used to effectively prevent and manage viruses. Strict sanitation of personnel, facilities, work benches, equipment, rearing containers, adult cages, and floors, egg surface sterilization, air filtration, and quarantining field collected stock from laboratory stock (until you are sure that field collected stock does not have viruses), are extremely important tactics for preventing and managing diseases caused by viruses.

Prevention and Management Insect Diseases in Laboratory Environments

BACTERIA – Bacteria are minute unicellular microbes that reproduce asexually by binary fission or sexually by conjunction. The bacteria found in insects vary from symbiotes (good bacteria), to entomopathogens (bad bacteria), to saprophytes (bacteria which consume dead plant/animal tissue for food). They are both spore formers and non-spore formers. Bacteria including the entomopathogenic species vary in shapes [Rod-Shaped Bacterium (see image below), cocci (circular), spiral and pleomorphic (polymorphic)] and may occur singly or in chains. They may occur in aerobic or anaerobic environments. Another characteristic commonly used to distinguish them apart is whether their cell wall composition stains using the Gram stain. Bacteria in which the cell walls stain are referred to as Gram – positive. Those in which the cell walls do not stain are referred to as Gram – negative. See image of a rod shaped bacillus bacteria.



Rod-Shaped Bacterium

30. Question – How would you (Amanda) go about determining whether the insect being reared was caused by a bacterium?

Answer - The only way to really know if a bacterium is responsible for causing a disease is by following Koch's postulates:

1. Isolate bacterium from preferably larvae that are showing symptoms and signs of the disease or from dead larvae (when isolating from a dead larva you have to be careful because secondary decomposing microbes, especially, bacteria can mask the true pathogen organism);
2. Inoculate the insects' diet with the bacterium and feed to healthy larvae;
3. Observe treatment larvae for signs and symptoms of disease; and
4. Re-isolate the bacterium from larvae showing symptoms/signs.

The exception to the above would be if one isolated a known bacteria or pathogen in which someone had already demonstrated using Koch's postulates.

Prevention and Management Insect Diseases in Laboratory Environments

31. Question - What are some common bacteria which cause insect diseases?

Answer – Examples of entomopathogens are *Bacillus thuringiensis* (commonly referred to as BT), *B.sphaericus* and *B. popillae*. BT is a gram-positive bacterium of worldwide distribution, commonly found in soil, and abundant in insects, especially Lepidoptera. Other bacteria that can cause disease are two Gram-negative bacteria *Serratia marcescens* and *Pseudomonas aeruginosa*. These are not entomopathogens or true pathogens but are considered to be non-invasive potential facultative pathogens. This simply means that these two microbes can cause diseases when the insects are reared under stressful environmental conditions or when the insect is injured causing breaks in the gut wall or integument.

32. Question – How can, for example, the BT micro-organism find its way into a rearing facility?

Answer – BT can be brought into a rearing facility by BT contaminated soil and field grown plant material used to feed the larvae and from field workers entering the facility with contaminated soil on their shoes and clothes and on their unwashed contaminated hands. Bt can also be brought into a facility by infected wild insects.

33. Question – How are bacteria transmitted to insects?

Answer – Primarily by ingestion of bacterial cells on contaminated food. Bacteria in the laboratory can be moved around by air currents on dust particles and by dirty hands and clothes.

34. Question – What are some of the signs and symptoms associated, for example, with BT diseased Lepidoptera?

Answer – Larval symptoms and signs are generally as follows:

1. reduced feeding at early stages of infection,
2. sluggishness,
3. cessation of feeding,
4. paralysis,
5. larvae become darker in color as internal tissues are broken down, and
6. body becomes flaccid.

35. Question – Are bacteria a problem for rearers that utilize excised plant parts (e.g., leaves) to feed their larvae?

Answer – Yes

36. Question – Can you eliminate the bacteria prior to feeding leaves or other plant parts to the larvae?

Answer - Yes. You can wash your leaves in a bleach solution containing sodium hypochlorite as described previously. Also, you can spray or dip the leaves using solutions of an antibiotic capable of destroying the bacteria that you are having trouble with. The above statements refer only to bacteria on the surface of the plant tissue.

Prevention and Management Insect Diseases in Laboratory Environments

37. Question – Is bacteria a common microbial problem in rearing facilities that use an artificial diet to feed their lepidopterous larvae?

Answer - Yes, bacteria are a common problem associated with rearing larvae on an artificial diet. Most of these bacteria are facultative pathogens in that they utilize the diet as a food and are ingested by the larvae. A good example would be *Serratia marcescens* (see image) as mentioned above.



Moths infected with the Bacterium *Serratia marcescens* - Note Red Pigment

38. Question - Where do the facultative bacteria pathogens come from to contaminate the diet?

Answer – They can come from primarily bacteria contaminated food/diet ingredients, dirty air and floors, contaminated workbench surfaces, and unclean hands and clothing.

39. Question – How would you prevent/manage bacteria that contaminate the insects' artificial diet?

Answer – By routine monitoring rearing containers for signs of bacterial contamination and a foul odor, by removing contaminated containers without opening, strict sanitation; air filtration; using heat generated by hot plates or steam-jacketed kettles to prepare (cook) the artificial diet which kills most non-spore forming bacteria but not spore formers and using autoclaves and flash sterilizers to kill both spore formers and non-spore formers by intense heat; addition of antibiotics and anti-microbial agents (e.g., sorbic acid and methyl parasept) to the diet; and adjusting the ph of the diet to maximize the action of the anti-microbial agents.

40. Question – How do you know what antibiotic to use and how much of it should be added to the diet to control the bacteria so as to not adversely affect the biology of the reared insect?

Answer – There are insect pathology services like ours at Mississippi State University that will culture the bacteria and screen a variety of antibiotics to determine which ones show the best

Prevention and Management Insect Diseases in Laboratory Environments

potential suppression of the bacteria. I (Davis) would suggest sending diseased insects to a pathology service or an insect pathologist. In doing so, I would suggest providing the insect pathology service with some insects that demonstrate signs and symptoms of disease along with dead ones. After a suitable antibiotic has been identified, the rearer must run experiments using insects reared on diet with the antibiotic versus those reared on diet without the antibiotic to determine if the antibiotic is adversely affecting the insects. From the literature insect pathologists have shown that antibiotic concentrations of 0.05 to 0.1 % of the diet have been sufficient to be effective against the bacteria and do not generally affect the reared insects adversely at these concentrations. However, concentration depends on antibiotic used and the species of insect being reared.

41. Question - How would one determine exactly what bacteria species are causing rearing problems?

Answer – To identify your problem bacteria to the species level, you will need to send them to an insect pathologist with taxonomic identification capabilities.

FUNGI – Fungi are widely distributed in nature and commonly cause problems in insectaries. Some fungi species are true entomopathogens while others are contaminants of the insects' diet and can become a facultative pathogen.

42. Question – What are some examples of true fungal entomopathogens?

Answer – *Beauveria bassiana*, *Nomuraea rileyi* (see images) and *Metarhizium anisopliae* are deuteromycetous fungi that can infect and kill a wide range of insect species.



Beauveria bassiana Infected Larva



Nomuraea rileyi Infected Larva

43. Question – What is a common way in which a true fungal entomopathogen can enter an insectary?

Answer – The most common way is by bringing in fungal infected wild insects from the field.

Prevention and Management Insect Diseases in Laboratory Environments

44. Question – What are some of the signs and symptoms of fungal infection?

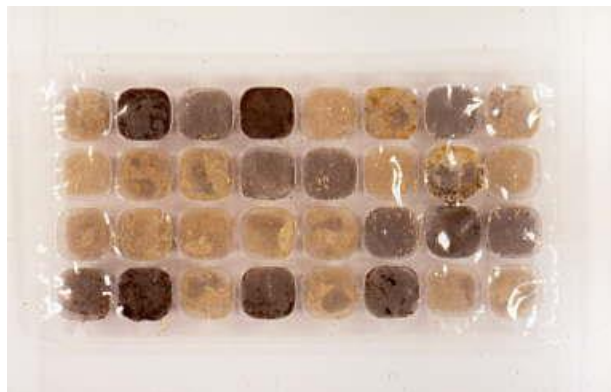
Answer – Early infected insects become sluggish and have a decreased rate of growth. Generally, there will be a color change with the infected insect. There will also be yeast-like cells or hyphal bodies showing up in the insects' blood (hemolymph). Mycelium (appear like small strings) will appear as the infection progresses. At this point the consistency of infected larvae will become cheese-like and later will change to a leathery consistency and eventually will become hard and mummy like. Finally, the fungal mycelium will grow out of the cadaver and sporulation will occur on the surface of the insects' integument.

45. Question - How does one prevent these true fungal entomopathogens from becoming a problem within a rearing facility?

Answer – When you bring wild insects into a facility, isolate or quarantine them from your laboratory colony? Place the larvae in individual rearing cups. Monitor the larvae as they develop to see if any diseases are occurring. Cups containing diseased larvae should be thrown out without removing the lid. If you open the lids of cups containing larvae that have been infected by these fungi in designated clean rooms within your facility, spores from the fungi will be released into the air and will be dispersed through out the facility by air currents.

46. Question – What are some of the more common contaminants and facultative fungi?

Answer - Here are some fungi species that belong to the groups (= Orders): Deuteromycotina, Zygomycotina, and Ascomycotina which are common contaminants and facultative entomopathogens. Within Deuteromycotina you have *Aspergillus* spp. and *Penicillium* spp. In our experience *A. niger* (especially the bread mold *A. niger* – see image) have caused us the biggest problem. Within Zygomycotina you have *Rhizopus* spp. and *Mucor* spp. Yeasts are fungi (found in the Ascomycotina group) can also be a diet contaminate.



Artificial Diet In Rearing Cells Infected With The Fungus *Aspergillus niger*

Prevention and Management Insect Diseases in Laboratory Environments

47. Question – How much problem do contaminants and fungal facultative pathogens cause in a rearing facility?

Answer – These fungi can create major problems as they are common contaminants of the insects' food (natural or artificial diets). They can become facultative entomopathogens under conditions that stress the insects. These fungi prosper under high rearing temperatures and humidity conditions.

48. Question – How do you prevent these fungi from causing problems when using natural foods such as leaves?

Answer – We suggest that you sanitize the surface of the food using bleach as mentioned previously. Also, do not place the sterilized food on paper toweling that has not been autoclaved or place moistened non-sterilized toweling within rearing containers. Non-sterilized paper toweling is a major source of bacterial and fungal microbes. Also, since fungal spores are carried by air currents, the rearing facility should have a system for purifying the air as described previously.

49. Question – How do you prevent these microbes from contaminating artificial diets?

Answer – The rearing facility should have an air filtration system using a combination of low and high efficiency filters and clean-air hoods which are used to keep fungi and other microbes from contaminating the prepared diet prior to infesting with the larvae. The facility should follow strict sanitation SOPs. Mold inhibitors and even some fungicides can be added to the artificial diet to control fungi. Remember some mold inhibitors work more efficiently at preferred Ph values. Also, cooking the diet helps sanitize the diet. To kill all microbes in a diet requires autoclave temperatures. Contaminated rearing containers should be thrown away without opening and harvesting of chrysalis should be harvested away from the areas in which the diet is prepared and the larvae develop.

This concludes our question and answer section. In summary, we want to stress again the importance of preventing and managing microbes that cause disease and those that contaminate the insects' diet.

Microbes can and do seriously disrupt production of insects and reduce the quality of those reared. Development of a multi – tactic approach to successfully prevent and manage microbes is highly suggested. Staff members must be trained to monitor the colonies of insects for diseases and diet contamination, to strictly follow sanitation SOPs and motivated to help develop and execute the multi-tactic approach for prevention and management of harmful microbes. Educational opportunities that increase your knowledge of the microbes and how to deal with them should be taken advantage of. Seeking the assistance of an insect pathologist and insect pathology service for help in dealing with harmful microbes is strongly encouraged.