

Field and Laboratory Protocol

WSU Extension Cereal Leaf Beetle Project

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IDENTIFYING CLB LEAF DAMAGE:

- CLB (adults and larvae) are very destructive to grain plants, including wheat, oats, and barley. They can, however, be found eating tall grass and such, especially if near wheat/oat/barley fields.
- Damage is identified by long strips of the chlorophyll layer missing from grain plant leaves (larval damage). Adult beetle damage can be only tiny holes missing from the plant leaves, or areas several inches long missing.
- Damage can occur on newly emerging shoots of crops, or on the leaves of mature plants.



CLB larvae feeding damage

CLB EGG COLLECTION:

Tools Needed In Field:

- *small, sharp dissecting scissors*
- *at least 2 plastic containers*
- *wax pencil*

CLB eggs are very small (about 1 mm in length) and are oval. Colors range between a very bright yellow-orange, to a dried-apricot color.

- The brighter the yellow-orange eggs, the “fresher” they are (newly laid)
- The darker the eggs get, the further along in maturity



- Eggs are located near the stalk of the plant, on the dorsal side of the leaf. This is the most common place to find eggs, but they can also be found along length of leaf, both dorsal and ventral sides. CLB eggs and adult feeding damage
- Using dissecting scissors, snip off the leaf that has the egg(s) on it. While it is fine to use your fingers to pull the leaf off, I have found it neater and less-likely to crush the eggs (especially near the stalk) if the scissors are used.
- Place the cut leaves in a plastic deli container. It's all right to fill the container with leaves.
- Eggs are usually laid wherever adults have been eating. If see CLB adult leaf damage, it is likely that eggs can be found on or near the same leaf. Collecting was much more successful if I scanned the plants very close to where I spotted the adult or where the CLB leaf damage was found.
- It appears that CLB adults have a preference for oats (rather than wheat). If having difficulty finding eggs in wheat, look to oats for further egg collection. (There are, however, always exceptions to the rule. The 2006 season has shown the CLB avoiding oats and feeding off of winter wheat.)

CLB EGG STORAGE:

- Since you will arrive back at the lab late on a collection day, CLB eggs can be stored in a cooler or refrigerator in the same container in which they were gathered, for several days.
- If condensation begins to develop while the containers are in the cooler, put a small, wadded up piece of paper towel in the cup with the eggs/leaves.
- My preference was to look at them when I got home from the field. The leaves didn't curl and more eggs remained attached to the leaves as opposed to falling off in the deli container.

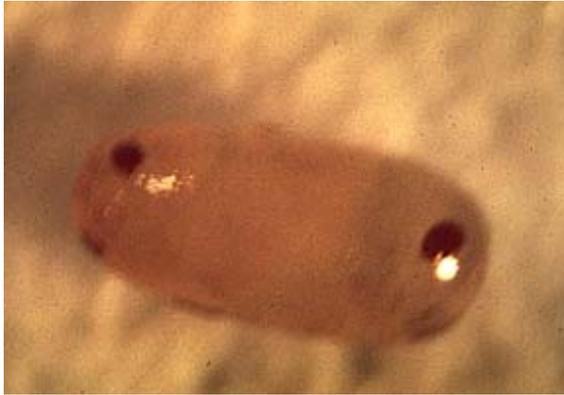
CLB EGG COUNTING:

Tools Needed In Lab:

- *Petri Plates*
- *small, sharp dissecting scissors*
- *wax pencil or dry erase pen*
- *dissecting microscope*
- *micro-forceps*
- *paper & pen*
- *garbage can*
- Removing each leaf from the container that they have been stored in with tweezers, trim excess leaf off so that there is only the CLB egg on a smaller section of leaf. The purpose of this is to reduce the amount of space that a full leaf will take up in the Petri dish. Also, dried leaves tend to curl up and around eggs that you are trying to view, and are very tedious to pull away to reveal the egg again. If possible, it is best to examine the eggs the

same day as collected. (note: the oat leaves do not curl up as much as the wheat leaves making egg count/observation a bit easier if the eggs are laid on oat plants).

- Examine egg under microscope. It is very difficult to tell if the eggs have been parasitized. At certain stages in development, red compound eyes will appear bilaterally at one end of the CLB egg. Since *Anaphes* commonly develop two per egg, it is possible
- to see two pairs of red eyes, one pair at each end of the egg. Unless the egg is ready to hatch and an *Anaphes* wasp is evident inside of the egg, the red eyes are the greatest determining factor in early identification of parasitized eggs.



Anaphes development in CLB eggs: red eye stage (left) and larvae(right)

- As the *Anaphes* wasps develop more, the egg will begin to get very dark. Near the center of the egg, two (usually) bright yellow splotches are evident. These are indicative of a maturing wasp inside a CLB egg.
 - Due to the difficulty in identifying parasitized eggs, each egg is counted and tallied on a piece of scratch paper, then placed in a Petri dish. Generally, an egg count of 50 per Petri dish is sufficient.
 - Label the Petri dish with the following (as it will save a lot of hassle later when trying to look the information up!):
 - *CLB Eggs*
 - *# of eggs in the dish*
 - *Site of Egg Collection*
 - *Date of Collection*

Depending on instructions given by supervisor, the dishes are either stored in the cooler, or set out under grow lights at room temperature.

- By storing in the cooler, this greatly slows the development of the eggs
- By storing at room temperature, the eggs can be checked daily for growth and stages of development. The main purpose of doing this is to see if an *Anaphes* wasp hatches, or if development of the red compound eyes appears. If this does happen, congratulations! Be sure to let everyone know and see (as this isn't seen too often)!
- Some eggs might hatch into CLB larvae. Simply remove larvae (which are very small) with a soft bristled paintbrush (or on a leaf, if it doesn't have an egg on it), and take the larvae outside to a designated area. (Be sure to ask what to do with them first, though.)

Be careful in larval removal, because they are very fragile. If one is squashed, though, it is not the end of the world!

TALLYING EGG INFORMATION

- On a piece of paper, write all pertinent information down. This would include the collection site, date of collection, and date of counting. Next, in separate columns, use the headings “Eggs”, “Egg cases”, and “Larvae” (just in case some eggs have hatched in transport). Generally, it isn’t imperative that the egg cases be counted. However, it is good record keeping. The egg cases do not need to be saved, unless specified otherwise.
- In terms of counting eggs, it is easy to use hash marks in groups of 5. Make sure, though, that after putting a newly counted egg into the petri dish to make the tally immediately. Otherwise, it is very easy to lose count, and incredibly frustrating to recount everything again!
- Record the data on the Excel spreadsheet.

ANAPHES WASP RELEASE:

- The laboratory in Colorado will ship Anaphes-parasitized CLB eggs when requested.
- The best time for release occurs when CLB eggs become readily apparent in the insectaries. The purpose of waiting until this time is to insure that emerging Anaphes from Colorado will have a host to lay its eggs in (the CLB eggs).
- There are two different ways to perform the releases. These depend on the condition that the eggs arrive in from Colorado. In the past, Colorado has had trouble shipping eggs so that when they arrive in Washington they are either not desiccated or not being drowned due to too much condensation in the shipping containers.
- Before doing the release, make sure that you write down all of the information that is labeled on the outside of the carton. Also, when out in the fields, be sure to note which plot in the insectary the carton was placed (A or B). This is important when compiling data on the Excel spreadsheet.
- If the shipment from Colorado gets the approval from Terry Miller, the milk cartons that the wasps are shipped in can be taken immediately out into the fields. Once at the insectaries, take wooden stakes (one for each carton) and hammer them in the ground in random fashion around the field.
 - Tape marker flags at the top of each stake, so that they are easier to find once the fields start growing.
 - Next, tape one carton near the bottom part of the stake. Using duct tape, tape the carton at the level of the CLB eggs found in the field, so that the wasps have an easier time of finding their host.
 - When taping, the bottom part of the milk carton is placed against the stake so that the carton is on its side. Each carton should have holes of some sort on one side, usually in the way of wire mesh. Make sure that this side faces towards the ground. This is where the wasps will emerge, and we don’t want rainwater or moisture gathering in the container by facing the holes upward.
 - Finally, record the information with a Sharpie that was on the carton to the duct tape holding the carton into place. Later, when pulling the cartons from the field, this makes it easier to see which carton is which

- If the shipment from Colorado still appears too immature to withstand the elements out in the fields, each carton needs to be placed in its own plastic deli tub.
 - First, circles of paper towels cut to match the diameter of the deli tub are made. Next, write all of the information that is on the individual milk carton onto the plastic deli tub. This can be done either directly using a white board pen, or indirectly by writing the information on duct tape and placing that on the deli tub.
 - Carefully empty each carton into their assigned deli tub. Forceps will probably have to be used to pull all of the vegetation out. Using a paint brush, you can retrieve any eggs stuck to the walls of the carton or on the sponge that was shipped with the cartons.
 - After all of the cartons have been emptied into deli tubs, give them to Terry Miller, who will put them in the incubator in the quarantine part of the lab to speed up the development of the eggs.
 - The milk cartons can now be thrown in the garbage dumpster.
 - Terry will let you know when the eggs are ready to go into the fields.

LARVAL COLLECTIONS:

Tools Needed In Field:

- *Sweep Net (only if preferred – most field techs preferred clipping off the leaves that had CLB larvae on them)*
- *Larval Forceps or Dissecting Scissors*
- *Plastic containers (deli tubs) with air holes in the lids*

CLB larvae look nothing like the CLB adults. They are very plump, and have a bright yellow body with black head and legs. The yellow might be hard to see, as the larvae cover themselves in their own fecal matter. This is an ingenious form of camouflage, as well as protection from UV rays. Therefore, many larvae are a muddy-brown color. CLB larvae field collections can be performed by clipping the leaf where the larvae are feeding and placing the larvae and leaf in the plastic containers (deli tubs). One plastic container can easily accommodate a sample count of 50 larvae.

LARVAL DISSECTIONS:

- These are performed in the Pullman lab or in any location where a dissecting microscope is available for use. The purpose is to identify how many CLB larvae have been stung by T.julis wasps. Finding T.j. larvae inside the CLB larvae is what this project is striving to establish.
- Using a dissecting dish (preferably the candle lid dissecting dish or a dissecting Petri dish with silicon gel in the base), pour in alcohol solution provided (70%). Using larval forceps, pluck the CLB larvae out of the deli tub container and placed them in the alcohol solution.
- Wait until they drown to begin dissection.
- Perform the dissections underneath a dissecting microscope. This is the best way to identify T.j. larvae, as they can sometimes be impossible to see with the naked eye.
- To do the dissections, use whatever sharp instrument that you feel most comfortable handling. Scalpels or many different types of probes are at your disposal. The quickest

and most efficient way to dissect the CLB larvae is to pin it right behind the head with one hand (an insect pin works well for this purpose), and then chop the head off with the other. Then push the guts out of the larvae. If it was parasitized, the T.j. larvae will spill right out onto the dissecting dish.

- The T.j. larvae can range in size from very miniscule to almost the size of the CLB larvae itself. If they are that big, you can actually see them inside the CLB larvae under the dissecting scope, without cutting into them. The T.j. larvae look like bright orange-yellow bananas, surrounded by a clear capsule. You may find only one T.j. larvae, or as many as 20+ T.j. larvae. The number of T.j. larvae doesn't matter; only if the CLB larvae is parasitized or not matters.
- Indicate parasitization with a '+' sign in your notes, or a '-' sign to indicate non-parasitization. Record this data on the excel spreadsheet



Parasitized CLB larva in alcohol

***T. julis* WASP RELEASE:**

- Now that the T.julis wasp has a solid establishment in some of our insectaries, CLB larvae from the most heavily parasitized larvae are moved to other designated fields. The purpose of this is to settle the T.j. wasp into as many fields in Washington as possible, in order to prevent economic damage brought on by the CLB.
- Be sure to collect CLB larvae from the site to determine any level of parasitism **prior** to releasing parasitized larvae in that location.
- Parasitized CLB larvae are moved by collecting them in the fields. You can collect them in plastic containers (deli tubs). The deli tubs can be stored in coolers while you are performing the field collections and then transferred to a refrigerator until the releases can be performed.
- If moving them to a new state or region, hold the larvae in a quarantine facility (preferably) while you transfer them on to fresh, locally grown oat leaves so as not to spread any pathogens with them.
- Take the deli tubs of larvae out to the field in coolers.
- Stake the release areas with lath stakes and plastic flags. This is so any CLB larvae collections efforts in the same field/insectary do not occur within the release area. These stakes must be removed at the end of the field season so that harvest of the crop will not be hampered by the stakes/flagging.
- When performing the release, remove the leaf/leaves from the plastic container with the larvae attached and place the leaf/leaves in the canopy of the plants within the release area. Try to place the leaves in the canopy where the larvae have a good chance of moving on to the existing plant. In most cases, the leaves can be placed in the notch where the existing leaf attaches to the stem of the plant (leaf axil). This area acts as a good cradle for the clipped leaves and provides a path for the larvae to climb on to the new host plant. In some cases, larvae in the deli tub will not be on a clipped leaf. These

larvae can either be placed on a leaf for transfer to the plants or hand-placed on the new plants.

- Select release sites at random within the release area and avoid concentrating too many larvae in one spot.
- Record these releases on the Excel spreadsheet.

CLB ADULT COLLECTION:

Tools Needed In Field:

- *Sweep nets (if necessary)*
- *Aspirator*
- *Many small plastic vials with caps (if collecting hundreds to thousands of adults)*
- *Bug dorm*
- *Shipping tubes (if mailing adults to Colorado or Pullman)*

Three ways that collection can be done:

I Using sweep nets, the bug dorm, and the aspirators

- the fields can be swept and then all of the bugs in the net (including CLB) are dumped into the bug dorm
- then using the aspirator, reach inside the bug dorm and suck out the CLB, counting as you go

Pros= sweep nets gather large quantities of CLB adults

Cons= VERY time consuming to sweep, then aspirate CLB from bug dorm. Also, reaching inside the dorm filled with many other insects (including spiders, stink bugs, aphids, and flying things) can be kind of creepy.

II Using sweep nets and aspirators (no bug dorm)

- sweep the fields and, instead of emptying net into bug dorm, just aspirate CLB directly out of net, counting the number of adults and recording the number of adults after each sweep. The total collections for the day can simply be obtained by adding these individual sweep counts.

Pros= efficient use of time

- can count easily and quickly
- can see what is in the net if using the open weave variety
- many times nontarget insects fly out of the net and are out of the way
- CLB tend to drop to the bottom of the net making aspiration easy (some may take flight but usually they fall to the bottom and crawl up the sides).

Cons= becomes very frustrating if CLB adults are not present in large numbers

- some of the CLB can escape by flying out of the net – a bummer if numbers are scarce
- nets tend to drown most of the insects gathered in the net if fields are wet (for this reason the open weave nets are preferable)
- if CLB larvae are present in the fields, they become a big, slimy ball that prevents seeing if adults have been gathered
- later in the season as the wheat/oat plants mature, seed heads tend to collect in the nets. They are an impediment to finding/collecting CLB adults in the net

III Using only the aspirators

- walk through the field and aspirate beetles
- my personal preference, especially later in the season

- in some cases, collecting off the leaves early in the morning (while it is cool) makes this method easier. As the day heats up the beetles tend to become more active (drop off the leaves or fly away).

Pros= count as you go and CLB are immediately available to package for shipping

- it's much more entertaining

Cons= time-consuming

- it's easy to lose count if you are thinking of something else
- CLB adults tend to drop to the ground (defense mechanism) and are hard to find if there is lots of ground litter.

CLB ADULT COUNTING:

Tools Needed In Field:

- *Aspirator*
- *Plastic vials*
- Simply aspirate adults into vials, counting as you go along! In windy conditions this method may not be an option until the CLB adults were doing the 2 by 2. They were just too skittish – drop off the leaves with only the slightest movement.
- Generally, 100-150 CLB adults can fit in the vials. As more adults are aspirated into the vials and take up space, the harder it is to aspirate CLB adults
- Sometimes, especially if the vial is full, adults will sneak up and out the way that they were aspirated in. To prevent this, aspirate air randomly so that the adults are sucked back into the vial. I put a piece of oat greens or wheat curled in the bottom on the vial. It gave them something to crawl on, oxygen and seemed to help when transferring to the shipping tube – very few if any escapees.

SHIPPING CLB ADULTS:

Tools Needed:

- *Shipping tubes*
- *Shipping box with Styrofoam liner*
- *Ice packs (chilled, not iced)*
- *Small pieces of sponges*
- *Pen & Post-its*
- Before transferring the CLB to the shipping containers, place a slightly moist piece of sponge in the tube. Make sure, though, that no excess water can be squeezed out of the sponge before placing it in the tube. If it is too moist, it will cause the vegetation to mold quickly. When it got hot out I kept these moist sponges in the cooler to help reduce the stress on the adult CLB's.
- Place small amount of grass, etc. into tube, along with long strips of ripped paper. This is to give the CLB something to walk on.
- The transfer from whatever receptacle that the CLB were gathered in must be quick, because the CLB tend to fly out quickly if not careful.

- Tape the container lid, but only so that it will stay on during shipping. We don't want the CLB to escape, but we don't want them to suffocate, either. Also, poke some holes in the lid (if they are not already there).
- Tape a post-it to the container with the approximate number of CLB in each written clearly on there.
- Place ice packs in bottom of Styrofoam-lined box then newspaper, then the tubes, then another ice pack.
- Place CLB-containing containers gently in box. Try to make the tubes as stable as possible. Can prevent rolling/movement by stuffing box with newspaper or other (empty) shipping tubes.
- Place the Styrofoam lid on the box and seal the box with packing tape
- No need to worry about writing the addresses on the box → FedEx does that.

FedEx-ing CLB ADULTS:

Have to get the package in the mail by the following times, for next-morning delivery:

- Pullman: 3:00 pm
- Spokane: 5:00 pm
- Pasco: 4:00 pm

NOTE! Pullman does NOT ship living creatures!! Also note that some FedEx outlets (eg Kinkos) will not ship live creatures. If possible, go to a FedEx office for shipping. It is okay to ship empty containers to CO from Pullman, but if you have adults that need to be shipped it must be done from Spokane.

Bill To:

3rd Party

FedEx# (whatever number is being used for the season)

Send package "Overnight Express" for arrival in the morning (not afternoon)

Mailing Address:

Colleen Jandreau
 Colorado Department of Agriculture
 Division of Plant Industry
 750 37.8 Road
 Palisade, CO 81526
 Office Phone: (970) 464-7916

Sender Address:

Diana Roberts
 WSU Cooperative Extension
 222 N. Havana Street
 Spokane, WA 99202
 Office Phone: (509) 477-2167

When they ask if items in box cost less than \$100, just say 'yes'.

FIELD JOURNALS:

The purpose of these is to record what you are seeing in each of the insectaries. The following are examples of what to keep track of in the journal:

- Date, time, weather, location
- Crop condition
- Presence of CLB eggs or larvae or adults
- Observations or thoughts that might be of later interest
- Conversations had with farmers
- Approximate amounts of eggs/larvae/adults gathered in the field
- Anything else you might find important
- Record these into a computer for review by your supervisors.

GAS CARDS FOR PROJECT VEHICLES:

The WSU motor pool will give instructions on how to work each card at the pumps

Voyager Credit Card

WSU Gas Pumps

Be sure to save receipts for Voyager credit card- just in case (WSU Gas Pumps don't give receipts)

CONTACTS LIST:

- Field Technicians should add any new contacts (farmers and field agronomists) to the Contact List in Excel
- Company Field Representatives may not provide the phone and address of clients but the Field Tech can note in the contact list the name and number of the field rep who can be used to contact the specific farmer if needed.

2% STERILE SOLUTION

Purpose is to sterilize egg and leaf that it is on. This will help to ensure that mold does not grow while the eggs are being reared.

- Mix 2 ml of bleach with 100 ml of water
- Using forceps, dip the leaves in the solution
- Place the leaves on a paper towel, which is resting in a tray of some sort
- Cover the leaves with another paper towel
- Let the leaf and egg dry completely before placing them in plates or vials for rearing out.

MAKING MESH LIDS FOR PLASTIC DELI CONTAINERS

- Using a leather hole punch, make holes in the lids of the plastic containers
- Use silicon glue to attach the mesh netting across the holes
- Silicon glue can be found at the hardware store

SWEEP NETS:

- Used for collecting CLB adults – the best type are the openweave mesh. Canvas ones get waterlogged easily and drown the insects.

- Can be used for collecting CLB larva, but this is not preferred because it gets very messy. See *Larval Collections* for more information and the preferred way of gathering larvae.
- Most useful when wheat/oats are under 2 feet in height; after that, aspirator is most useful in adult collection (especially if adults numbers are high)
- While it is up to the person sweeping the fields, I prefer doing ~25 by 180°sweeps to equal one total sweep. To clarify, doing 5 total sweeps means that I have swept the field 125 times. After each total sweep, collect CLB adults with an aspirator, counting each adult gathered. Then, note the amount of adults collected after each total sweep. (I varied the number of sweeps by the density of CLB adults and how moist the field was. A wet field meant you would drown the adult CLB if you did too many. So I always did a few sweeps, analyzed the contents and proceeded according to my findings. We were told to discontinue tracking the number of sweeps very early on in the season. I found this very acceptable – gave me more liberty to observe field/weather conditions while I was collecting.)
- For example, 1 total sweep of 25 is done. Turn net inside out, except for bottom portion where CLB adults should be gathered, then aspirate each adult. If 5 adults are collected, note this number and the number of sweeps done. It should look something like this: *1 total sweep of 25=5 CLB adults*. The total number adults collected can be calculated as the summation of the individual sweeps
- All of the above is merely for the sake of thoroughness.

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