

**Suggested Procedures:
USING SENTINEL EGG MASSES (COLONY-PRODUCED) & WILD EGG MASSES (LAID IN FIELD) TO
MONITOR BMSB PARASITISM & PREDATION**

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USING SENTINEL EGG MASSES

Removing egg masses from colony substrates (for those with their own colony):

- Inspect BMSB colonies each morning for freshly-laid egg masses (EM).
- EM laid on leaves often fall off when leaves dry out in the field. Therefore, to ensure colony EM that have been deposited on leaves remain in place, they should be attached to card stock with double sided sticky tape for placement in the field.
- If the leaf type has a smooth surface it is OK to cut a small piece of the leaf containing the EM and attach this to the tape; but EM is likely to come loose from setose leaf types so it's best to remove them entirely from the leaf and place directly onto tape (**Fig. 1**).
- Areas of tape that remain exposed (i.e., not covered by leaf/eggs) should be coated with a thin layer of fine vermiculite (sawdust, sand or other fine soil would also work) so that searching parasitoids won't become stuck in the glue (**Fig. 2**).
- Take care to attach the EM to the tape with the operculum (=lid) side up.
- EM that have been laid on the cage surfaces - top, sides, and bottom – can be moistened with water to soften the oviposition adhesive and ease their removal (a small squirt bottle or dropper can be used to add a few drops to moisten the edges of the EM). After about one minute the EM can be removed intact and whole from the surface by sliding a stiff, thin flat surface under the EM (plastic sheet, razor blade, etc. used in the manner of a paint scraper), placed on filter paper and allowed to dry. The dry filter paper is then attached to card stock with double sided sticky tape as described above.

NOTE: BMSB eggs are very cold-sensitive. Prolonged storage at 10-12 degrees C will prevent further BMSB development.

Field placement and collection of egg masses:

To assess parasitism of sentinel egg masses

- The number of eggs per EM should be recorded on each card before field placement.
- EM should ideally be fresh (24 hours or less of age) and definitely not more than 48 hours of age when placed in field. If EM will be left in place for a minimum of 48 hours, egg masses should be no more than 24 hours of age.

- Placement of EM will depend on the host plant: in trees and shrubs we place at 4-6 ft above ground level; on herbaceous plants including crops, we suggest placement within the upper 3rd of the canopy unless experimental needs dictate other placements.
- To mimic natural EM placement, we recommend sentinel EM placement on the underside (abaxial surface) of leaves.
- Cards with EM should be securely attached to leaves. We suggest two colored 1" plastic "clothespins" to attach the cards, one along each edge of the card (**Fig. 3**). The colored pins are helpful in locating the EM when it's time to collect them. Cards may also be attached by threading two insect pins into the leaf, one along each margin of the card (**Fig. 4**).
- EM should be left in the field for at least 2 days but not more than 4 days to minimize losses due to predation or weathering. Fresh egg masses will not be acceptable to parasitoids after 2-3 days of age, so longer exposure is detrimental.
- For best results, egg masses should be placed on plants in which BMSBs are active. Stink bug parasitoids are known to use chemical cues left by stink bugs in locating egg masses.
- We are testing cold-treated egg masses to facilitate shipping and handling; but even if these prove suitable for parasitoid development and give an extended period of acceptability, frozen EMs should not be left in the field longer than fresh egg masses would be exposed or the rates of parasitism will not be accurate or comparable. If the objective is simply to capture and identify native egg parasitoids, this may not be an important consideration.
- When retrieving sentinel EM, be prepared to collect adult parasitoids that are often found on or near the EM for later identification, and/or any predators encountered. If parasitoids are found on the egg mass, it is possible that they are simply guarding EM already parasitized, or that parasitism has not occurred and will be interrupted by collection [if parasitoids are parasitizing or examining egg masses for host suitability, they may be found backed up to the eggs with their wings at 90 degrees from their body or antennating and circling the eggs, respectively. Guarding females are generally motionless on/near the EM.]. If it is important to rule out interrupted parasitism, mark the location of the egg mass and allow it to remain in the field for another 24 hour, then collect the egg mass and parasitoid if still present.
- Do not make the assumption that parasitoids found on EMs are the same species that parasitized the EMs. Separate and verify the identities of parasitoids that emerge from egg masses and "guarding" parasitoids.

To assess predation of sentinel egg masses

- Attach EM to plants as described for parasitism.
- EM should be no more than 48 hours of age when placed in field.
- Once placed on the plant, EM may be examined in place after 24 h to detect predation, and if possible, to observe and record identity and behavior of natural enemies found associated with EM.
- To provide a more accurate assessment of predation rate, EMs should remain in the field until they hatch or all are preyed upon. This will allow predators ample time to locate the eggs. Egg masses should then be collected from test plants and examined in the laboratory for predation of individual eggs.
- If a card is recovered, but the entire egg mass is absent with no visible remains, the egg mass should be classified as missing (M)

- If partial remains of one or more eggs are found an EM may be categorized as attacked by a predator(s).
- Whenever possible, eggs attacked by chewing predators (CP) should be distinguished from those attacked by sucking predators (SP).
- The total number of eggs within each category should be recorded for each egg mass.
- Undamaged eggs, if possible, should be held for emergence of adult parasitoids and if there is no evidence of parasitism and parasitoids do not emerge; these eggs may be classified as unknown or unascertained (U) mortality.

Handling egg masses after retrieval:

- After collecting the sentinel and wild EM, return them to the laboratory to rear parasitoids and record data. Record the placement (for sentinels) & recovery dates for each EM and place them in a sealed container. After 3 days, check EM daily and remove any BMSB nymphs that have hatched. Young 1st instars do not feed, but eventually they may feed on remaining parasitized eggs.
- Record the number of eggs intact, damaged and missing. Damaged eggs can be further categorized as due to sucking or chewing predators. Egg masses with un-hatched eggs can be placed in a smaller container. We use tight-seal 21 mm Falcon petri dishes to hold EM until parasitoid emergence. If moisture is a problem, consider using containers with ventilated lids to prevent mold development (strips of lab tissue placed with one end inside the container and the other end outside also make effective wicks). However, the use of wicks may not be appropriate in all situations as it may contribute to mold development. Each EM should be isolated in a separate container.
- EM with 100% hatch of BMSB nymphs may now be discarded; the rest are held for development and emergence of parasitoids.
- Emerged parasitoids are counted and tentative ID recorded and/or placed in gel caps or alcohol vials for later identification (for future taxonomic study, storage in 95% ETOH is preferred when possible). For future molecular work, store them in - 20 C (ideally - 80 C if a low temp freezer is available) and out of direct light. They should be placed in these conditions soon after emergence to prevent their DNA from degrading. For, many basic molecular assays, storage in an ordinary freezer may be adequate. Include labels with relevant information or codes.
- After all parasitoids have emerged (allow several weeks minimum), EM remains may be inspected more closely for additional evidence of parasitism and predation. Save remnants in a dry container.

MONITORING NATURALLY LAID (WILD) EGGS FOR PARASITISM

- Similar methods as described above can be used for monitoring and collecting naturally laid BMSB egg masses.
- Wild EM collected should be labeled and their data recorded separately from sentinel EM since the conditions of their exposure in the field are not known and cannot be directly compared with lab-reared sentinel EM.

PROTOCOLS FOR MEASURING WILD (NATURALLY DEPOSITED) EGG MORTALITY IN FIELD

Protocol I:

Recording parasitoid species composition (without measuring parasitism rate):

- Fields/host plants should be examined weekly for the presence of stink bug egg masses during the growing season.
- During search outings, visually inspect plants within the field or search area for egg masses. Pure white eggs may have just been laid and may not have had time to be parasitized.
- If surveying a cropping system, the field perimeters (especially sides facing woodlots and other host plants) should be searched first, but searches should not be limited to field borders if stink bugs are in the fields' interior area.
- Collect EM by removing a section of leaf containing the EM and placing it in a small dish.
- Label the dish and/or associated data sheet with date, site, plant species, location within field, surrounding habitat, etc and store out of direct sun.
- Once taken back to the lab, egg masses can be handled as described above.

Protocol II: Recording species composition and parasitism rate:

- Fields/host plants should be examined weekly for the presence of stink bug egg masses during the growing season.
- During each search outing, once egg masses are found, mark their location on the leaf by placing a small permanent mark next to the egg mass and tying a ribbon on the leaf's petiole or stem.
- Information can be written on ribbons such as dates egg masses were found and other relevant information described in Protocol I.
- Whenever possible, behavior of any adult stink bug egg parasitoids found should be observed and recorded (e.g., searching leaf, examining EM, ovipositing, guarding).
- Retrieve marked egg masses 48 hours later by removing the section of leaf containing EM and placing it in a small dish. This will ensure that EMs are exposed long enough to be discovered while still acceptable to parasitoids. However, there is risk that EM will be discovered by predators before removal.
- If dark gray to black EMs are found during searches, such as in **Fig. 6**, eggs are already parasitized and they may be collected immediately (not left for 48 hours).

Protocol III: Measuring total mortality due to combined parasitism and predation:

- If the purpose is to determine total mortality in EM whether due to parasitism, predation or unknown causes, EM locations should be marked as described in Protocol II.
- When possible, the number of eggs in each EM should be counted and recorded.
- Eggs should then be checked every 48 hours for a week to allow time for nymphs to hatch. During each inspection, identify predators, parasitoids, or herbivores that are found on or near EM and record their behavior.
- Conditions of eggs and their numbers within EM (e.g., hatched, grayish, partially consumed, etc.) should be recorded during each inspection.
- EM status should be classified as hatched (**H**), if BMSB nymphs emerged; missing (**M**), when eggs disappear entirely from the surface of the leaf; mortality unknown or unascertained (**U**), when eggs do not hatch yet show no obvious signs of predation or parasitism; and parasitized (**P**), when parasitic wasps emerge from the eggs (or are clearly visible within even if they fail to successfully emerge). Eggs attacked by chewing predators (**CP**) should be distinguished if possible from those attacked by sucking predators (**SP**);

and when this cannot be determined and eggs are clearly damaged, record as general predation (**GP**).

- Some EMs will contain multiple classifications, with some eggs parasitized, hatched, eaten by predators and unknown.
- If EMs are dark gray to black eggs during field inspections they may already be parasitized and may be provisionally classified as **P**, although they will have to be monitored further to determine if parasitoids emerge (evidenced by emergence holes).
- If it is important to minimize disruption of parasitoid activity in the experimental plots, only a representative proportion of parasitized BMSB egg masses should be collected and returned to the laboratory for emergence of adult parasitoids for species identification. In collecting a representative sample, it may be best to collect entire egg masses instead of dividing masses, as the egg mass may be parasitized by more than one species. Note, if parasitism is low, this will not result in many specimens and may cause some species to be overlooked.
- After one week, or when 1st instar nymphs have hatched and moved away from the egg mass, eggs or their remains should be collected, taken back to the lab, and number of eggs per EM verified and further examined under a microscope if needed.



Fig. 1. EM taped to card stock



Fig. 2. EM on tape with vermiculite



Fig. 3. Sentinel EM clipped to leaf



Fig. 4. Sentinel EM sewn to leaf



Fig. 5. Sentinel EM held for initial BMSB hatch



Fig. 6. Parasitized stink bug eggs (probably *Euschistus servus*)