

FINAL REPORT

WTFRC Project #AE-03-334

WSU Project #13C-3643-6366

Project title: Laboratory and field-testing of protein markers to determine large-scale movement patterns of pests and their natural enemies

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Objectives:

1. Determine the importance of the different methods by which an insect may acquire our marking materials. This includes direct contact, contact with a treated surface, consumption of a treated surface, or contact with a marked individual.
2. Determine if parasitoids acquire the mark by feeding on marked hosts.
3. Determine the longevity under field conditions of the different markers.
4. Determine the cross-reactivity of the antibodies to other pesticides used in the system (e.g., other chloronicotynyls to Provado antibodies).
5. Perform a complete workup of the codling moth granulosis virus antibody to determine cross-reactivity, longevity and sensitivity.

Significant findings:

- OBLR or pear psylla walking across dried egg white residues less than 3 days old strongly acquired the mark.
- Egg whites were detected from apple leaves for >45 days when applied by airblast sprayer.
- Casein (non-fat milk) was detected from apple leaves for >18 days later during a field test in the fall.
- The casein, egg whites and codling moth granulosis virus (CpGV) assays are highly specific and do not cross-react.
- The markers cannot be applied with most of the non-ionic surfactants (Silwet, Regulaid, Reguard, Tween-20, Latron B-1956, Sylguard) or Nufilm-17. They are compatible with Orchex (650 ppm), Raynox (600 ppm) or Nufilm-P (650 ppm).
- The egg white marker can be detected at less than 100 ppb, the casein marker at less than 250 ppb, and the CpGV can be detected at 1 ppm of the formulated Virosoft™.
- The type of water used to mix up the markers (10-25% solution) has a significant effect on the sensitivity of the assays, and its effect varies by which assay is being tested. Tap water is best, followed by irrigation water, with type I laboratory (deionized, distilled and filtered) water completely suppressing activity.

Objective 1.

Methods:

To test the ability of an insect to acquire a mark by walking on a dried residue, we dipped apple leaves in either a 10 or 30% solution of the marker in type I lab water and allowed them to air dry. Leaves were then used to line the inside of a half-liter container. Adult pear psylla or OBLR moths were placed in the container for two days, then removed and tested for presence of the mark. Because neither pear psylla nor adult moths feed on the leaves, the only way to acquire the mark is by direct contact with the residue.

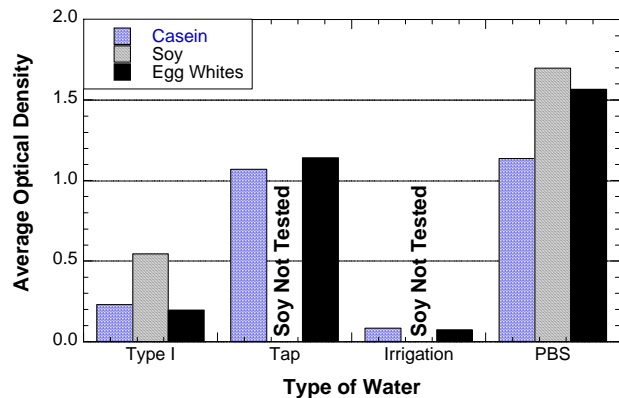
OBLR larvae were also reared on marked leaves, but because they actually ingest the marker we had to process the samples differently. In this case, we washed each larva in 1 ml of buffer, removed the buffer and stored it in a separate microcentrifuge tube to test for the external mark. A larva was washed an additional six times to remove as much of the external mark as possible, then ground up with disposable pestles, and the ELISA was run on the ground-up larva to assess it for an internal mark. We used third instar larvae and after two days removed them from the leaves and froze them. The fourth and fifth instars were allowed to continue feeding on the marked leaves and were removed only after they had attained the desired age. Thus, the older larvae had fed for a longer period on the marked leaves and should have acquired more of the mark internally.

Results:

We found all the pear psylla and OBLR moths that were held on leaves treated with either 10 or 30% solution of egg whites to be strongly marked. The buffer used to wash the external mark off larvae reared for two days on egg-treated leaves tested positive during the first wash for all instars tested, but activity in the later washes declined dramatically. The marker was still detected after the third wash for the fourth and fifth instars, but not for the third instars. Larvae of OBLR reared on egg-treated leaves also tested positive for the internal mark in the third instar (62.5%) and during the fourth instar (37.5%), but not during the fifth instar. Frass collected from the treatment containers was marked at much higher levels than the larvae. These results suggest that the fresh residue contributes strongly to marking the insects not directly contacted by the spray, at least while the residue is fresh. The internal marking is not highly effective, probably because a large amount of the protein is not incorporated into the larvae (at least not in a form that is recognizable to the antibody).

None of the psylla reared on leaves treated with 10% soy milk acquired the mark, and only one psylla was weakly positive when reared on the leaves treated with 25% soy milk. Only two OBLR larvae reared on the soy-treated leaves acquired the mark, and those were at very low levels. The wash buffer was weakly positive in only the first wash for those larvae that tested positive. Frass collected and tested showed no trace of the mark.

Fig. 1. Effect of different types of water on the ELISA reactions for casein, soy, and egg whites.



The low activity of the soy milk assay was puzzling until we began to analyze some of the apple leaves treated in the field to determine field longevity (Objective 3). We had treated that orchard with a concentration of soy milk $>100,000 \times$ higher than the detection limit and were barely able to detect it from the leaves collected the next day. When we tested the solution applied in the field, it also had a low response (considering the level used). Further investigation revealed that the activity of the marker was strongly affected by the type of water used to dilute the marker. We used type I laboratory water, phosphate buffer saline (PBS), tap water or irrigation water to dilute the markers for the casein and egg whites marker and only type I water for the soy assay (Fig. 1).

Diluting the markers in type I lab water or irrigation water severely reduced the sensitivity of the tests. Tap water had the least effect on sensitivity, scoring similar to the PBS buffer with the casein test and in the egg test reducing the sensitivity about one-third compared to the PBS buffer.

Because the phosphate buffer is a packet of salts (KCl and NaCl) mixed up in type I lab water, we designed a test to check if the loss of activity was because of the removal of all the ions in the type I water. We tested type I water with a serial dilution of either NaCl or CaCl₂ at a diagnostic concentration of each marker. We found that activity was dose-independent; that is, even a small amount of the salts would restore activity (Figs. 2, 3 A, B). The soy assay was relatively unaffected by CaCl₂ but responded well to NaCl. However, the 8 g/L rate caused leaf burn, and we would need to test lower levels to be sure that activity was restored and leaf damage would be minimized. We settled on CaCl₂ for the egg white and casein assays, primarily because it is normally used to prevent bitter pit, and the effects on the leaves were more predictable.

Tests with the casein residues showed that 25% of the moths placed on a 3-day old field-aged residue of non-fat milk (in both the 100 and 25% milk treatments) could acquire the mark but at low levels. If moths were caged on a 6-day old residue for two days, 25% of the moths acquired the mark in the 100% milk treatment but none in the 25% milk treatment.

Tests to determine if the mark can be passed by direct physical contact (i.e., between mating pairs) will be run this winter.

Fig. 2. Effect of the addition of calcium chloride and sodium chloride to soy reaction when the soy was mixed in Type I water.

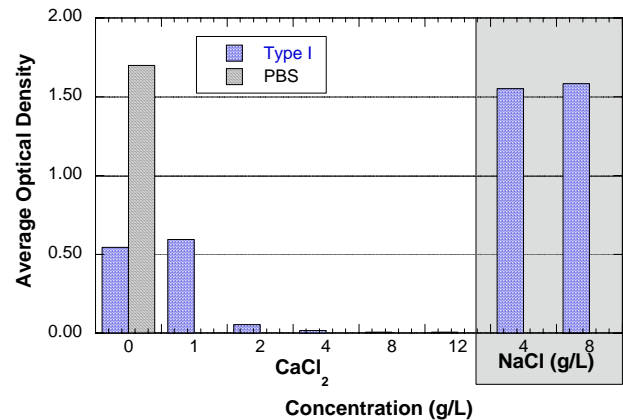
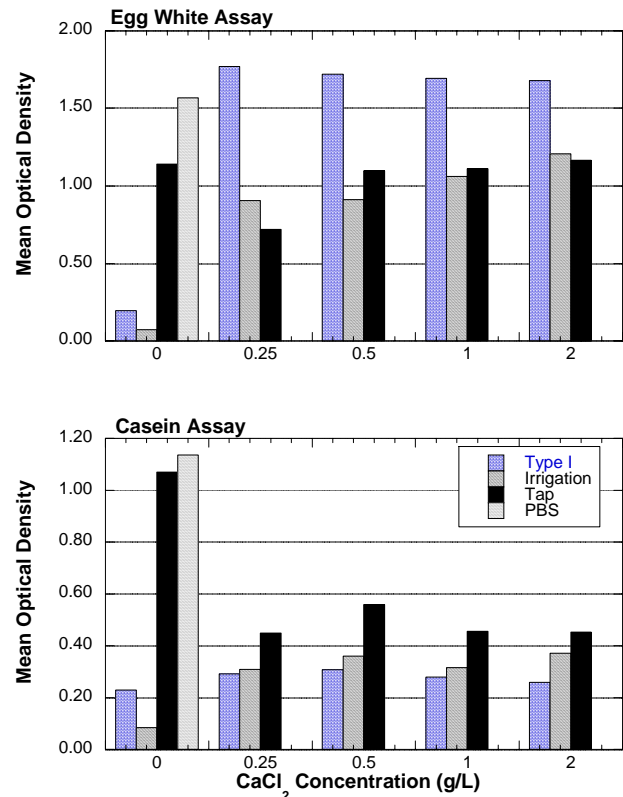


Fig. 3. Effect of adding calcium chloride to different types of water spiked with the appropriate marker.



Objective 2. These tests will be completed by early spring.

Objective 3.

Methods:

Our field trials consisted of two large-scale tests and several smaller trials. The large-scale plots were one acre each in two separate blocks at the WSU Columbia View orchard. The first trial was applied on 16 June (10% egg to one block and 10% soy milk to the other), and the second was applied on 11 Sept (10% egg to one and 15% casein to the other). For each trial, a Proptec sprayer was used to apply 30 gal/acre of the different markers. In the first trial both markers were diluted in irrigation water, and in the second trial irrigation water was used for the egg marker but 1.0 g/L CaCl₂ was added to the water for the casein marker. We collected 10 ml of the treatment solution before the solution was applied; this served as a test that the marker actually applied to the trees was active. Yellow sticky traps (to collect a broad range of insects) and CM and PLR pheromone traps were placed in the field near the interior of the plots. We also collected leaves separately from outside and inside the canopy to help determine longevity of the mark in the environment without having to worry about movement between areas or emergence curves that would be complicating factors with the insect samples.

Samples were processed by collecting insects directly from the traps, placing them in buffer and then using the standard ELISA protocol to determine if the mark was present. Leaves were processed by using a cork borer to remove a 7-mm diameter disc from each leaf that was placed in buffer and tested as above. The cork borer was thoroughly cleaned before and after processing each leaf to prevent cross-contamination.

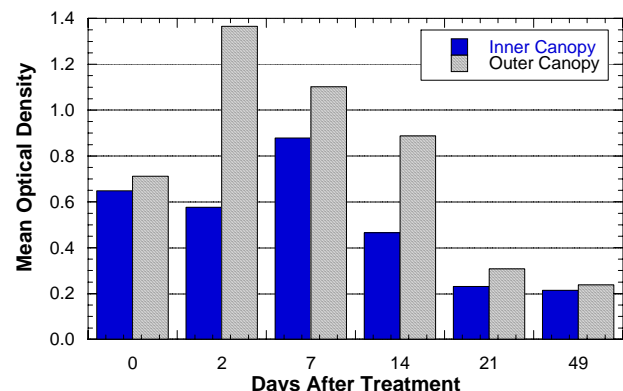
Results:

The egg marker was detectable on all the leaves tested for the entire duration of the first test (21 days). The activity of the marked leaves showed an interesting pattern where the level was good initially but increased before declining slowly over the period of the test (Fig. 4). For the outer canopy leaves, the increase in activity occurred by the 2-day sample, and the inner canopy leaves showed the pattern by the 7-day sample. This may be the result of redistribution of the marker by dew over time. Before the second test was run, we collected a pre-treatment sample to be sure that none of the marker from the previous test was still present. This sample was taken 49 days after the initial treatment, and 74% of the inner canopy and 58% of the outer canopy leaves still tested positive.

Marked insects from the first egg trial have been tested for two sample dates. The day after application, 24% of the insects tested were marked. At 14 days, 14% were still marked.

In the second Proptec-applied egg trial, we partially completed the testing for those sampled up to eight days. In this trial, there were enough white apple leafhopper (WALH) and noctuid moths to test them separately. We were able to detect the egg marker on 22% of the WALH the day after treatment, on 39.5% by four days, and on 35% by eight days. With the noctuids, 38.4% were marked one day after application of the marker, and by six days 23.8% were marked.

Fig. 4. Longevity of egg white marker applied by proptec sprayer on inner and outer canopy apple leaves.



The insects used in the first Proptec experiment with soy have not been tested, primarily because none of the leaf samples showed positive. As mentioned above, this is probably related to the use of irrigation water and its severe depression on detectability of the soy ELISA assay. The egg assay worked better than expected from the lab assay (Objective 1), probably because we were using such a high rate compared to the sensitivity of the test (egg assay sensitivity down to 100 ppb, we used 100,000 ppm).

Tests of the casein marker were run in both a large-plot (CV19 in September) and in several small trials using a backpack mist blower in one case and leaf dips in two others. The leaf dips were used primarily to determine the effects of the addition of salts on longevity so no data on insects were taken (only branches were dipped). To date, we have only examined data from leaves collected up to eight days after application from the large-plot WALH trial. At eight days, 95% of the inner canopy leaves tested positive and 100% of the outer canopy leaves tested positive. Unfortunately, none of the WALH adults tested positive. However, the studies with the backpack mist blower, which were run on single-tree replicates that were surrounded by trees with high populations of (unmarked) WALH, allowed us to mark up to 40% of the population four days after treatment using the same concentration of marker (15%) but mixed in tap water.

Objective 4.

Results:

We decided to drop the imidacloprid assay because of problems with being able to determine that insects had not contacted the pesticide. The type of ELISA used for imidacloprid (an indirect competitive ELISA) works differently from the types of ELISA that we use on all the other marker candidates (an indirect ELISA). In the indirect ELISA, the greater the concentration of the marker in the initial sample, the darker the reaction at the final stage; conversely, if there is no marker present in the sample the well is clear. With the indirect competitive ELISA, the lower the concentration of the marker in the sample, the darker the sample at the final stage. Unfortunately, although we could easily detect the presence of the imidacloprid, the negative controls were highly variable, which made it extremely difficult to tell when it was absent.

We did test if imidacloprid was cross-reactive with the soy, egg white or casein antibodies (it was not) but did not test the other chloronicotinyls since we would not be using the imidacloprid assay. Tests for cross-reaction between the soy, egg white and casein antibodies showed that each was specific and did not respond to any of the other markers (e.g., egg white did not react to soy milk, casein or imidacloprid).

We also tested the effect on the assays of mixing the markers with low concentrations of a variety of agricultural adjuvants. In general, non-ionic surfactants (Tween-20™, Silwet™, Sylguard™, Regulaid™, Regard™, Latron B-1956™) almost completely inhibit detection by the indirect ELISA. Nufilm-17 also was extremely detrimental to sensitivity, but Nufilm P™ and Orhex™ horticultural oil could be used up to 650 and 1300 ppm, respectively. Raynox™ had no detrimental effect up to 600 ppm but by 1200 ppm reduced the sensitivity of the tests by ≈50%.

Although we did not field test the surfactants, we did mix up different concentrations, apply them to moths and observe whether the droplets would wet the moth. At virtually any concentration where wetting was observed, our studies showed that the ELISA reaction was inhibited. We also performed lab tests by spraying in a fume hood moths with markers mixed at rates that had low effects on the ELISA reaction, and we found that there was no benefit compared to just applying the markers in buffer or tap water.

Objective 5.

Results:

The antibodies for the codling moth granulosis virus were obtained from Canada. These antibodies were developed by Dr. Ken Eastwell, who is now a faculty member at WSU-Prosser, to detect CpGV-infected codling moth. We tested three formulations of the codling moth granulosis virus as markers, Virosoft™, Cyd-X™, and Carpovirusine™. Our studies showed that Carpovirusine™ was a poor antigen, probably because the UV stabilizers and the formulation inhibit the ELISA reaction. However, either Virosoft™ or Cyd-X™ could be used, and each had approximately the same activity. We were able to detect Virosoft™ mixed in buffer down to 1.0 ppm of formulated material. In the field, we only used the Virosoft™ material, primarily because the manufacturer recommends that Cyd-X™ be applied with Nufilm-17, which greatly reduced the sensitivity of the test.

The field tests of the CpGV antibody were run in conjunction with an efficacy trial with single-tree replicates. We followed only the Virosoft™ treatment that was applied at 3.24 fl oz/100 gal (253 ppm of formulated material) and only after the second application had been applied. The treatments were repeated at ≈10-day intervals so our tests could not follow the insects longer than that period without an additional spray being applied.

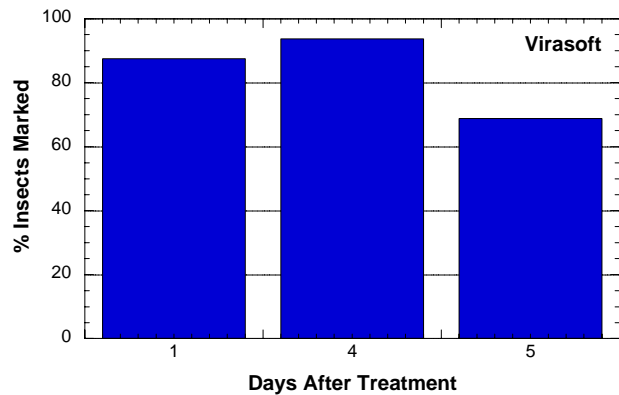
We were unable to detect the Virosoft™ on the leaves, even on leaves collected the day after treatment. However, insects collected from this trial were very strongly marked, with more than 60% being positive five days after treatment (Fig. 5). Tests are underway of whether the insects can pick up the enough Virosoft to become marked by coming into contact with recently dried residue.

Overall comments:

The way each insect acquires the mark is probably a unique combination determined by its mode of feeding (leaf chewers vs. phloem feeders vs. predators), surface of its body (scales vs. bare, the wax layers present, etc.) and its behavior (activity level, location on tree). These differences and others mean that what works for one insect with one particular marker may not work for another insect or marker and that the only way to know is actually to test those insects of interest.

We expect that much of the difference in ability to acquire the mark that is unrelated to the surface properties of the insect will come from two sources: 1) the quality of the water and 2) the method used to apply the marker to the field. The quality of the water can be overcome by using tap water (preferably) or with proper testing beforehand and the use of irrigation water with calcium chloride. The method of application is an area we need to investigate further. Our studies show that leaves are easily marked using the 30 gal/acre rate that we used with the Proptec sprayer. However, the insects collected in those tests were marked at lower rates than we expected. Conversely, even using the backpack mist blower resulted in higher numbers of marked WALH. Finally, the CpGV application (Objective 5) showed a much higher proportion of marked individuals, despite the fact that the egg and casein assays can detect much lower levels of their respective markers. It is even more important in light of the fact that the dose applied to the CpGV was only 253-fold higher than the detection level compared to the egg and casein markers that were applied at >100,000 times their respective

Fig. 5. Percentage insects on sticky cards testing positive for CpGV in single tree plots. Formulated material (Virosoft) was applied by handgun at 3.24 fl. oz./acre.



detection levels. This suggests that the handgun application, or at least an increase in the amount per acre when using the Proptec sprayer, would be beneficial and we will be testing this as soon as trees leaf out in the spring.

Budget:

Project title: Laboratory and field-testing of protein markers to determine large-scale movement patterns of pests and their natural enemies

PI: Vince Jones

Project duration: one year (2003)

Project total: \$24,497

Category	Year 1 (2003)
Salaries (Associate in Research) ¹	\$12,200
Benefits (30%)	3,660
Wages	1,230
Benefits (16%)	197
Equipment ²	3,000
Materials and supplies ³	3,810
Within-state travel ⁴	400
Total	\$24,497

¹ Associated with this project alone.

² Equipment required was a high quality still to produce distilled water that was used in all the assays.

³ Supplies included ELISA supplies, telecom charges, traps, miscellaneous lab and field supplies.

⁴ Included rental of a vehicle for this project.

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