Project Title: Identifying sources of X disease in cherry orchards

Report Type: Final Project Report.

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Cooperators: Garrett Bishop, Scott Harper, Tianna DuPont

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 58,400
Total Project Request for Year 2 Funding: $ 55,849
Total Project Request for Year 3 Funding: $ 53,707

Other related/associated funding sources: Awarded
Funding Duration: 2020 - 2022
Amount: $249,360
Agency Name: WSDA/USDA Specialty Crop Block Grant
Notes: USDA SCBG funding to identify strains of phytoplasma in cherries and other stone fruit as well as weedy plants, and to conduct molecular gut content analysis on X-disease vectors. PI: Harper, co-PI's: Northfield, Cooper, DuPont

Other related/associated funding sources: Awarded
Funding Duration: 2021 - 2023
Amount: $244,750
Agency Name: WSDA/USDA Specialty Crop Block Grant
Notes: USDA SCBG funding to evaluate selective broadleaf herbicides as a management option for X-disease vectors. PI: Northfield, co-PI: Harper.

Other related/associated funding sources: Awarded
Funding Duration: 2022 - 2024
Amount: $295,376

Agency Name: USDA Crop Protection and Pest Management
Notes: USDA funding to develop phenology models for phytoplasma prevalence in plants and vectors to integrate into phenology models for leafhopper abundance (WTFRC project led by Nottingham). PI: Northfield, co-PI: Nottingham (WSU), Harper (WSU), Adams (OSU), Galimba (OSU).

Other related/associated funding sources: Awarded
Funding Duration: 2021 - 2023
Amount: $164,765
Agency Name: USDA AFRI
Notes: USDA postdoctoral fellowship awarded to Adrian Marshall (mentors: Northfield, Harper, and Cooper) to precisely estimate the time between acquisition to transmission for leafhoppers to better inform timing of control measures.

Other related/associated funding sources: Awarded
Funding Duration: Ongoing
Amount: $2 million per year indefinitely
Agency Name: USDA ARS congressional appropriations
Notes: Cooperative research project between USDA ARS and WSU to better understand little cherry disease (caused by X-disease phytoplasma and Little cherry virus). USDA PI: Cooper, WSU PI: Northfield.

WTFRC Collaborative Costs: $0

Budget 1
Primary PI: Tobin Northfield
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Item	2020	2021	2022
Salaries[1]	$39,629.00	$41,214.00	$42,863.00
Benefits[2]	$4,478.00	$4,657.00	$4,844.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[3]	$7,000.00	$4,000.00	$4,000.00
Travel[4]	$2,000.00	$2,000.00	$2,000.00
Plot Fees			
Miscellaneous			
Total	$53,107.00	$51,871.00	$53,707.00

Footnotes:

[1] new student position

[2] 11.3%

[3] Research consumables (e.g., cages, pots, soil), + molecular tests for disease presence

[4] In state travel

If project duration is only 1 year, delete Year 2 and Year 3 columns.

Budget 2
Co PI 2: W. Rodney Cooper
Organization Name: USDA-YARL
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Station Manager/Supervisor: Rodney Cooper
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Item	2020	2021	2022
Salaries			
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies[1]	$5,293.00	$3,978.00	
Travel			
Plot Fees			
Miscellaneous			
Total	$5,293.00	$3,978.00	$0.00

Footnotes:

[1] molecular supplies for gut content analysis

Objective Recap, Goals, and Anticipated Accomplishments:

Objectives

1. Conduct oviposition tests and life cycle analysis on leafhoppers on five host plants (cherry, clover, dandelion, peach, alfalfa).

We sequenced key species-barcoding genes of *Colladonus reductus* and *Colladonus montanus* and have determined that the two "species" are highly similar for the genes sequenced, supporting a 1957 USDA bulletin (Nielsen 1957) suggesting they may be the same species or very closely related (identified as subspecies in the bulletin). Importantly, Nielson (1957) noted that what was referred to in California life history studies as *C. montanus* was actually the *reductus* subspecies, allowing us to use the detailed life cycle description provided by Severin and Klostermeyer (1950) to inform *C. m. reductus* management (Table 1). Similarly, an unpublished Oregon State University MS thesis (Marsh 1965) with Nielson as an advisor (along with Knud Swenson) shows that *C. m. reductus* overwinters as eggs in the Pacific Northwest. We find that the life cycles conducted by researchers on *C. m. reductus* and *C. geminatus* in the 1940s in California are quite similar to those conducted in Oregon in the 1950s, providing confidence in the values.

Table 1: Life cycle analysis conducted by Mervin Nielson (Nielson 1968), or Severin and Klostermeyer (Severin & Klostermeyer 1950) for *C. geminatus* and *C. montanus reductus*.

Life stage	***C. geminatus*** (days) (Nielson 1968, peach host, OR)	***C. geminatus*** (days) (Severin & Klostermeyer 1950, Celery host, CA)	***C. m. reductus*** (days) (Severin & Klostermeyer 1950, Celery host, CA)
Egg	20	17.6	14.3
1st instar larva	4.0	7.1	5
2nd instar larva	5.0	4	4.6
3rd instar larva	8.0	4.3	4.3
4th instar larva	6.0	3.5	3.6
5th instar larva	9.0	7.4	5
6th instar larva	N/A (Only 5 instars)	N/A (Only 5 instars)	7.5
Total nymph stages	32.0	26.6	27.6
Total egg, nymph	52	44.6	42
Pre-oviposition stage	8	7-13 (only range given)	13.9
Total generation time	60 days	~55.6 days	55.9 days

Despite detailed life cycle numbers, prior to this study it was unclear what plants leafhoppers feed on or how other hosts affect their growth and reproduction. In particular, due to its low abundance at the time, *C. m. reductus* was not included in the extensive *C. geminatus* study by Mervin Nielsen study in Oregon (Nielson 1968) but is typically far more abundant than *C. geminatus* in Washington orchards. Here, we originally set out to build on this research by evaluating the generation time for *C. m. reductus* and *C. geminatus* on 5 plant species: cherry, white clover (*Trifolium repens*), dandelion (*Taraxacum officinale*), peach and alfalfa. Understanding host plant use will help inform management plans. In our surveys of cherry farms in the Wenatchee and Yakima regions in this project and in the project title,

Figure 1. Nymph deformation in colony. *Example of leafhopper deformation (left) compared to healthy (right) C. m. montanus in leafhopper colonies. It is not clear what is causing these abnormalities in colonies.*

"Field evaluation of leafhopper controls for X-disease management" we rarely observed *C. geminatus*, with *C. m. reductus* being >95% of *Colladonus* spp. individuals collected by sweep nets and sticky traps, and even fewer in 2021. In response to the abundance of *C. m. reductus* and lack of knowledge, we focused our trials on this species. Furthermore, when collecting leafhoppers, we noticed they were commonly found on mallow plants, so we included mallow in our trials. In two initial attempts to start a colony of *C. m. reductus* with a diverse offering of plants (attempt 1: pea plants, clover, alfalfa; attempt 2: alfalfa, clover, mallow) the leafhoppers died as older nymphs or newly emerged adults, often with deformations (Figure 1). The cause of this mortality appears to be related to high humidity, and we now have *C. m. reductus* colonies in Wenatchee (WSU, Northfield) and Wapato (USDA ARS, Cooper) using humidity-controlled growth chambers (WSU) and rooms (USDA). The Wenatchee colony is raised on a diverse range of broadleaf weeds, and the Wapato colony is raised on celery root. Given the presence of a diverse diet in wild caught leafhoppers (according to molecular gut content analysis led by Co-PI Cooper), we have focused trials on feeding behavior, and used an oviposition test to determine the number of generations per year for *C. m. reductus*, which is unknown (2 reported for *C. geminatus* in the 1950s) and was unclear from sticky trap data. The findings from this study have also led to broadleaf-weed specific herbicide trials funded by a specialty crop block grant.

2. Evaluate incubation time and acquisition probability for leafhoppers feeding on each, cherry and peach trees and transmission likelihood to cherry, clover, dandelion, peach, and alfalfa.
In our evaluation of acquisition and transmission studies we had planned to follow the methods of previous studies (Jensen 1971, Suslow and Purcell 1982), with the addition of molecular techniques to better evaluate acquisition and transmission success. While leafhoppers have long been known to acquire X-disease from infected cherry trees, a 1951 study was unable to get *C. geminatus* to acquire X-disease in 17 symptomatic peach trees, leading to the thought that it might be a dead-end host (Nielson and Jones 1954). However, research and discussions over the course of the current study suggests these peach trees may have actually been infected with peach yellow leaf roll phytoplasma (*Candidatus* phytoplasma pyri, a.k.a. pear decline, transmitted by pear psylla), rather than X-disease, given the common misidentification at the time. Indeed, we found a study in 1950 in Wenatchee conducted by Homer Wolfe that demonstrated that X-disease vectors can readily acquire X-disease phytoplasma from peach trees at similar rates to cherry (Wolfe et al. 1951). In light of this information, we have focused on acquisition from cherry trees, due to higher number of available trees and to take advantage of trees with high pathogen titers, and the lack of need to demonstrate that acquisition from peaches can occur. To evaluate acquisition in year 2 of the project we planned to identify cherry and peach trees exhibiting X-disease symptoms during harvest, and place *C. geminatus* and *C. m. reductus* leafhoppers in sleeve cages on the diseased trees. After 1 week of feeding (the maximum time needed according to previous research) we planned to cut the branch off the tree, keeping the sleeve cage intact and place the sleeve cage and branch immediately into a cooler with ice for transport back to the WSU TFREC without allowing leafhopper escape. The leafhoppers were to be collected from cherry trees will then be transferred to greenhouse cages containing one of five potential host plants: cherry, peach, alfalfa, dandelion, or white clover, and replicated 8 times (40 total cages). Each cage was to include 3 *C. geminatus* and 3 *C. m. reductus*

leafhoppers, to focus on the potential of the plant to host the disease and allow for either leafhopper species to transfer the disease. We have adapted these methods slightly to take advantage of highly infected cherry trees in a commercial orchard, and to focus on *C. m. reductus*, given how abundant it is relative to *C. geminatus*.

3. Use molecular analysis on leafhoppers raised on different host plants to evaluate the reliability of gut content analysis to identify previous hosts of leafhoppers collected in orchards.

Research conducted by co-PI Rodney Cooper and colleagues on purple top disease in potatoes (Horton et al. 2018, Cooper et al. 2019), caused by a phytoplasma vectored by beet leafhoppers has included the development of molecular methods to identify previous plant hosts of leafhoppers collected from crops. While the methods have been focused on beet leafhoppers, rather than the *Colladonus* spp. that vector X-disease, we expected the methods to be directly applicable to identifying non-cherry plants as sources of leafhoppers. Here, we used leafhoppers arising from experiments described in objective 1 as a cost-effective evaluation of such methods for cherry-X-disease research. These data can then be used as pilot research justifying federal funding identifying alternative leafhopper hosts and their potential importance for disease transmission in cherry orchards. Thus, at the end of the life cycle analysis in year 1 we planned to send leafhoppers from the field trials to the USDA lab in Wapato for molecular analysis to identify the host plant within the insect's gut. Assuming identification success in year 1, in year 2 we planned to collect adult leafhoppers from the end of experiments and place them on cherry seedlings, raised separately for each host plant. We will then collect 5 leafhoppers from each seedling at 0, 1, 2, and 3 weeks to identify the timeframe in which the previous host plant can be detected.

Objectives timeline

Objective	Y1	Y2	Y3
1 Life history tests	x	x	
2 Transmission tests		x	x
3 Gut content analysis	x	x	

Significant Findings from this project:

- We have conducted oviposition studies on second and third generation leafhopper adults, and they readily laid eggs on broadleaf weeds and grasses. However, in each of two trials, all adults feeding on grass-only died (n = 6 cages), while adults feeding on broadleaf weeds alone or with grasses survived (n = 12 cages).
- Leafhopper eggs developing in grasses and broadleaf weeds did not survive when cut from the plant. Further studies are needed to see if this suggests mowing after oviposition reduces egg survivorship.
- We have developed and published methods for molecular gut content analysis for leafhoppers, including *C. m. reductus* and *C. geminatus* (Cooper et al. 2022).
- Field-collected *C. m. reductus* successfully transmitted X-disease phytoplasma to each, mallow (*Malva neglecta*) and alfalfa (*Medicago sativa*). These have not previously been reported as hosts. The other broadleaf plants (dandelion and white clover) did not test positive, but were less preferred feeding hosts and have been reported as phytoplasma hosts elsewhere (https://www2.ipm.ucanr.edu/agriculture/cherry/X-disease-cherry-buckskin/)
- Potted alfalfa plants infected in fall feeding trials were left outside for the winter, and tested positive for X-disease phytoplasma the following spring, suggesting they can host the phytoplasma from year to year.

- *C. m. reductus* and *C. montanus montanus* are genetically similar and may be the same species. Furthermore, literature at the time suggests that *C. montanus* in California in the 1950s was most likely *C. montanus reductus*, allowing us to use early *C. montanus* research on life histories and incubation period to inform management of *C. m. reductus*
- Of the plants included in the trials (cherry, peach, mallow, alfalfa, white clover, and dandelion), *C. m. reductus* have a strong affinity for mallow and alfalfa. Given how common these plants are in orchard groundcover, these hosts should be considered in management strategies. *C. m. reductus* may also benefit from a diverse diet, that includes tree feeding.
- Leafhopper feeding rates on cherry trees ranged from 14% to 51% of the observed feeding observations, depending the available herbaceous plants, with highest feeding when mallow was not present. Cherry feeding rates were highest when mallow was not present.
- Leafhopper feeding rates on peach trees ranged from 22% to 41% of the observed feeding, depending on the available herbaceous plants. Peach feeding was highest when mallow was not present.
- We conducted molecular gut content analysis on 5 *C. m. reductus* and 5 *C. geminatus* leafhoppers from a commercial orchard in Wapato and found all *C. m. reductus* had fed primarily on dandelion, with little else in their guts. Four of the five *C. geminatus* had fed on dandelion as well, demonstrating the importance of ground cover broadleaf weeds as leafhopper feeding hosts. Dandelion was the dominant weed at the location and time sampled. Subsequent analysis of leafhoppers over a much wider geographic range supports dandelion and mallow as an important host, amongst other common broadleaf weeds.
- In the second generation (August) leafhoppers in growth rooms deposited eggs on the underside of the leaves of cherry, mallow, and clover.
- *C. m. reductus* leafhoppers collected during the final generation (late September) laid eggs on grassy weeds in potted plants, but not broadleaf weed or trees, suggesting they may overwinter as eggs on tall grasses.

Key findings from federal funding leveraged by this project:

- Molecular gut content analysis led by co-PI Cooper continues to reveal the importance of broadleaf weeds in orchard ground cover as important X-disease vector hosts.
- Surveys led by cooperator Harper for X-disease phytoplasma hosts continue to highlight the importance of broadleaf weeds in orchard ground cover as hosts for phytoplasma
- Surveys led by PI Northfield suggest that X-disease phytoplasma transmitted in the field in October can overwinter in broadleaf weeds and be detectable in the plant the following spring.
- Ongoing broadleaf-specific herbicide trials to control leafhopper vectors in groundcover suggest promise for reducing leafhopper numbers.
- Ongoing electropenetragraph research suggest X-disease vector leafhoppers readily feed on each, phloem and xylem and it may be possible to use this technique to quantify feeding on each in a variety of plant types to determine when and where phytoplasma can be acquired.

Methods:

Feeding trials

We initiated feeding trials in 24in × 24in × 56in (w × w × h) cages with a combination of white clover, alfalfa, dandelion, mallow, Early Red Haven peach trees, and/or Bing cherry trees, with each plant in a separate pot (Figure 1). Each trial lasted 5 days and each cage contained 10-15 leafhoppers, depending on mortality after collection. In the first trial, we conducted observations every two hours from 8am to 11pm. However, leafhoppers rarely moved in the span of the two-hour intervals and did not appear active in observations made at 9pm and 11pm, which were in the dark and made with red headlamps to avoid disturbing insects. Therefore, in subsequent trials, observations were made at 8AM, 1PM, and 6PM, doing 3-minute time searches in each cage. Trials were conducted in environmentally controlled growth rooms set at 75F, with a 16:8 L:D daylength. During each observation, we counted how many leafhoppers were on each plant, what plants they were on and if actively feeding or not by visually observing stylets piercing the plant. We present data only on actively feeding leafhoppers summarized across the insects within a cage.

Figure 1 Feeding trial cages in the growth room.

The trials included the following treatments:

- 2 trials of cherry, alfalfa, clover, dandelion; each with 2 cages
  - Initiated June 11 and August 3, 2020
- 2 trials of peach, alfalfa, clover, dandelion; each with 2 cages
  - Initiated June 11 and August 3, 2020
- 1 trial of cherry, clover, mallow, dandelion; each with 2 cages
  - Initiated September 22, 2020
- 1 trial of peach, clover, mallow, dandelion; each with 2 cages
  - Initiated September 22, 2020
- 1 trial of peach, alfalfa, mallow, dandelion; each with 3 cages
  - Initiated August 22, 2020
- 1 trial of cherry, alfalfa, mallow, dandelion; each with 3 cages
  - Initiated October 6, 2020

Transmission tests

After the completion of the feeding trials, Scott Harper's team tested the plants for X-disease phytoplasma.

Field oviposition test

Based on yellow sticky card data, in the Pacific Northwest *Colladonus* species leafhoppers typically have three periods of abundance: May, late July/early August, and October. However, it is difficult to determine the number of generations per year from yellow sticky card data. This is because the October generation may be the same generation as the August generation, just moving into orchards after loss of alternative host plants. Because leafhoppers overwinter as dormant eggs, we evaluated the potential for eggs laid in field conditions in August to hatch into nymphs. Development of these eggs would then suggest that the August adults represent a distinct generation that gives rise to the adults collected in October. Therefore, during the first week of August 2020 we collected *C. m. reductus* and placed them in cages 24in × 24in × 24in mesh cages with combinations of herbaceous plants next to the

Brunner building at the WSU Tree Fruit Research and Extension Center. The cages were monitored periodically to identify the emergence of nymphs and/or adults.

Second generation oviposition test

2021 field season. Second generation oviposition trials took place in 2021 within two growth rooms under two different temperature regimes: 60°F, 30% relative humidity (RH) and 80°F, 30%RH with a 16:8 L:D daylength. Due to a growth room malfunction in the first replication, the first room fluctuated around 75-80°F with about 70% RH in the beginning. High humidity was corrected in subsequent trials by placing a dehumidifier in the rooms and set to 30%RH, but the temperature could not be corrected. This took place from July 22nd to July 29th 2021. For both temperatures, four rearing observation cages (24x24x56"; BioQuip) were set up individually with two cages with Bing cherry, two cages with Early Red Haven peach, and each with Dutch white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), dandelion (*Taraxacum sp.*), and common mallow (*Malva sp.)*. Two additional cages of only clover, alfalfa, dandelion, and mallow were set up to test preference without the presence of fruit trees. Two rearing observation cages (24x24x56"; BioQuip) were placed in field conditions outside of the lab with clover, alfalfa, dandelion, and mallow as well to serve as a control. To each cage we introduced 5 male and 5 female field-collected *C. m. reductus*. Sex determination was conducted by anesthetizing them with CO2 using a modified sparkling water maker (SodaStream Inc.), and a microscope for identification. Two days were given before the start, and timed checks happened twice a day at 8-9AM and 5-6PM for 3 minutes. Leafhoppers were counted and we recorded what plants they were on, and if they were actively feeding. We made oviposition observations using the Simplified Leafhopper Egg Detection by Autofluorescence method, also known as the Blue Light Detection Method, to detect eggs within the plants (Hermann and Boll 2003; Yao et al. 2020). Using a blue LED flashlight with a 455-460nm wavelength (LEDwholesalers; Amazon) and wearing blue light blocking computer glasses (UVEX; Amazon), we scanned each plant for eggs. Plants that had eggs were recorded as well as where on the plant they were laid.

2021 Overwintering oviposition test

To see if eggs laid by the third generation that overwinter as eggs are laid in a different location, we set up an oviposition test that was similar to the "second generation" oviposition test. These trials took place from September 20th to September 24th, 2021, and then repeated again from September 28th to October 1st, 2021. The two growth rooms were maintained under two different temperatures: 70°F, 30% RH and 80dF, 30%RH with a 16:8 L:D daylength. For both temperatures in both replicates, two rearing observation cages (24 × 24 × 56"; BioQuip) were set up with two cages of Bing cherry, each with Dutch white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), dandelion (*Taraxacum sp.*), and common mallow (*Malva sp.)*. Two additional cages of only clover, alfalfa, dandelion, and mallow were set up to test preference without the presence of fruit trees. Two rearing observation cages (24 × 24 × 56"; BioQuip) were placed in field conditions outside of the lab with clover, alfalfa, dandelion, and mallow as well to serve as a control. To each cage we introduced 10 females and at least 5 field-collected *C. m. reductus*. The leafhoppers that were placed in the second replication were put into the same cages as the first. Additionally, half of the cages (one with weedy hosts in the growth rooms and the field, and one with cherry in the growth rooms) were used to test a method of inducing oviposition in leafhoppers (Tipping *et al*. 2005). To do this, we placed the 10 females and around 5 males in a plastic tube with mesh secured on both ends to allow airflow and ran a hairdryer through both ends on cool for 2 minutes, flipping the side halfway through. Sex determination for both replicates was done by anesthetizing them with CO2 using a modified sparkling water maker (SodaStream Inc.), and a microscope for identification. Two days were given before the start of the first replicate, and checks happened once a day over a 5-day period for however long was needed for a thorough search of the plants (around 5-10 minutes). For the second replicate we allowed 24 hours for leafhopper acclimation before observations were initiated,

which included one check for the same amount of time, and halfway through, barley was added for additional observations. Egg detection was conducted using the Blue Light Detection (Simplified Leafhopper Egg Detection by Autofluorescence) method by using a blue LED flashlight (LEDwholesalers; Amazon) and wearing blue light blocking computer glasses (UVEX; Amazon) (Herrmann and Boll 2003; Yao et al. 2020). Plants were scanned for eggs using this method and plants with eggs were recorded.

2022 field season

We conducted the same experiment from August 9-12th, from September 1-9, and from October 28 – November 7 2022. For each experiment, we set up a total of 9 cages to evaluate oviposition in 3 treatments: broadleaf plants only (2 pots of alfalfa, 2 pots of clover), broadleaf plants and grasses (1 pot with alfalfa or clover, 1 pot containing perennial ryegrass or creeping red fescue), or grass only (2 pots containing perennial rye grass and two creeping red fescue), with 3 replicates per treatment. The key difference between the August and September experiments is that in the August experiment we realized there was too much plant material to search for eggs, making them difficult to find. Therefore, in the September and October experiments, we switched to seedling trays to reduce the amount of plant material in cages and improve egg identification. Cages were kept in a growth room set to 16:8h Light:Dark, 70°F (21°C), and 30%RH (controlled with a dehumidifier). To each cage, we added 5 females, field-collected *C. m. reductus* leafhoppers and began observations same day. We searched for eggs daily over 7 days, scanning each plant using a blue light and blue light filtering classes to find eggs. On each day, we removed plant material containing eggs when found and place in small deli cup on top of soil labeled by cage number and what plant it was found on. Because the blue light detection method can also confuse thrips feeding with eggs, we confirmed eggs in plant material under microscope. For each egg, we recorded the number of eggs, when they were found, and what plant they were on. Deli cups with eggs were kept in the same growth room that cages were in previously (16:8 L:D, 70°F (21°C), 30%RH) to monitor for nymph emergence. We also monitored each plant in separate cages and checked for nymph emergence after observations were finished.

Gut content analysis

C. reductus and *C. geminatus* were collected from orchards *C. geminatus* and *C. reductus* were collected in a commercial cherry orchard near Wapato, WA on 22 May 2020 from herbaceous ground cover between orchard rows using a sweep net and stored in -80C freezer for processing. Methods for molecular gut content analysis were similar to those previously described for psyllids (Cooper et al. 2019). Briefly, DNA was extracted from leafhoppers using a commercial kit, and plant DNA was amplified by polymerase chain reaction. The plant DNA amplicons were then sequenced and used to identify the dietary history of leafhoppers.

Results & Discussion:

Feeding trials. We observed active feeding on all plants offered during the feeding trials (Figure 3). In the feeding trials that included cherry trees, the order of *C. m. reductus* preference appeared to be: mallow, alfalfa, cherry, white clover, and dandelion. Indeed, when offered mallow, alfalfa and a cherry tree we did not observe feeding on dandelion. In the feeding trials that included peach trees, the order of preference

appeared to be: mallow, alfalfa, peach, white clover, and dandelion. However, interestingly, when offered mallow, alfalfa and peach together they fed more on peach than alfalfa. The fact that leafhoppers always fed on cherry or peach trees, regardless of what herbaceous plants were there begs the question of whether there is something important about feeding on trees that provide important nutrients to leafhoppers. However, future research is needed to determine whether this is the case.

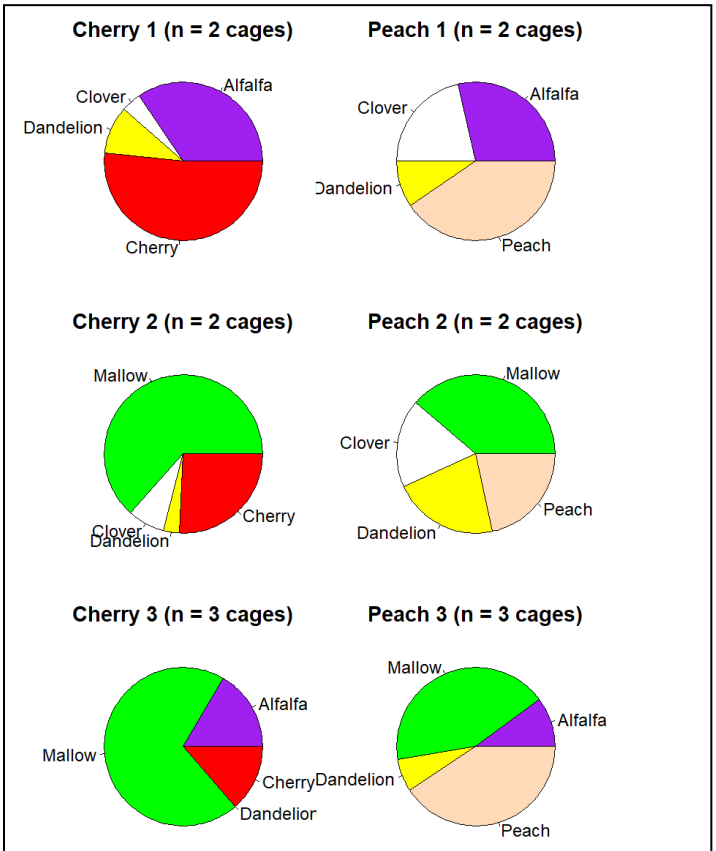

Figure 3 *Proportion of feeding observations made on each of the different plant species in cages: cherry (red), white clover (white), dandelion (yellow), alfalfa (purple), peach (peach), or mallow (green) during feeding observations.*

Field oviposition tests: Adult *C. m. reductus* leafhoppers collected in the first week of August and introduced to outside cages with mallow and clover readily laid eggs that hatched into nymphs and began reaching the adult stage in October, suggesting that the August generation is a separate generation from the first generation that emerges in May from overwintering eggs and from the October generation that lays eggs that remain dormant for the winter. Given that these two later generations typically occur after cherry harvest, leafhopper control after harvest is likely critically important.

Transmission test

Of the plants from the feeding trials, alfalfa and mallow tested positive for X-disease phytoplasma following the experiment. One of the two alfalfa plants tested positive with a Ct score of 36.82, and two of the three mallow plants tested positive with Ct scores of 38.71 and 38.29. In addition, one alfalfa, one mallow, and one dandelion plant was kept outside all winter and tested again the following April to see if the phytoplasma could survive the winter in the roots. Of those, the alfalfa tested positive with a 39.31 Ct score. Therefore, we found that alfalfa and mallow can host X-disease, and that it can survive the winter in broadleaf roots.

Second generation oviposition test

2021 experiment. During this experiment, most of the leafhoppers died within the acclimation period so there were fewer feeding results. From the data collected, there were no records of feeding on dandelion, cherry, or peach, but they did feed on (in order of preference) clover, mallow, and alfalfa. In the cages without a fruit tree, there were more observations on clover than there were on mallow, alfalfa, and dandelion. Overall, the feeding proportion for clover was 53%, for mallow was 37%, for alfalfa was 11%, with no feeding observations on either dandelion and cherry/peach. During this experiment we were able to find some eggs deposited in the first growth room (75-80°F conditions) despite the lack of feeding data. In the cages that had fruit trees, we found eggs deposited on the underside of the leaves of mallow, and clover, and did not observe any eggs on alfalfa and dandelion. Although no eggs were found in the cages without fruit trees, we were able to observe young instar nymphs on clover, as well as other nymphs on cherry, mallow, and clover within the fruit tree cages. Due to lack of leafhoppers in the field by the end of this experiment (being at the end of the second generation), we were not able to replicate this experiment before the final generation began.

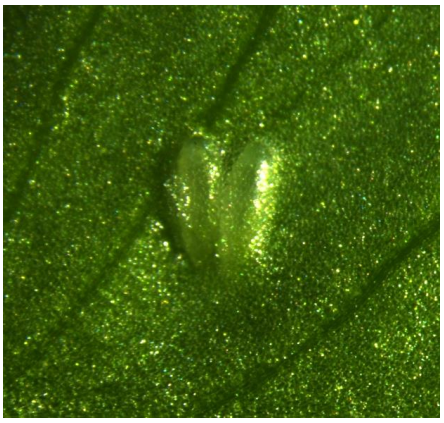

Figure 4. Leafhopper eggs in alfalfa (approximately 1mm long)

2021 Final generation oviposition test. No eggs were laid in any of the broadleaf plants in any of the cages. However, one of the pots with the dandelion plants had grass growing from the pots and we found 100 leafhopper eggs deposited in the grass. While it was a single cage that had eggs deposited, it suggests that leafhoppers may lay eggs in grass in the final generation to overwinter. This would make logical sense, given that they may be more likely to persist throughout the winter than tree leaves, or even leaves on perennial broadleaf weeds. With this observation on grass, barley was added halfway through the experiment, finding that they would feed on the barley, but no eggs were laid on it in any of the cages.

2022 experiments. In the August experiment, no eggs were found using the blue light detection method. At the end of the observation period, all plants were taken out of the cages and searched for eggs. The first set of plants were sorted through for about 1 hour and found 4 total eggs (cage B1: 1 clover, 2 alfalfa; cage BG2: 1 clover). Due to the large amount of plant material to sort through, egg searching was reduced to 15 minutes per plant, and no other eggs were found. Plants were then separated into individual cages and checked for nymph emergence, but no nymphs were found. This could be because the nymphs were too small to observe, the plants died before finding nymphs, or no/very few eggs were actually laid.

In the September experiment, plants were planted in seedling trays together for each cage to reduce amount of plant material needed to go through. Eggs were first found on 9/6, mostly with plain sight. On the last day, all trays were removed, and plants were sorted through to find eggs with no time restrictions. Overall, 48 eggs were found on barley, 28 on perennial ryegrass, 20 on creeping red fescue, 5 on clover, and 2 on alfalfa. A majority of the eggs were found in the cages that had only grass in them, and the eggs found in the broadleaves were found in the mixed broadleaf/grass cage. At the end of the trial, it was noticed that the leafhoppers in the grass cages had all died, and the leafhoppers in the cages with broadleaves had mostly survived. In our October/November trial we were more careful to document adult survivorship over the course of the experiment. When summed across the treatments, we observed 61 eggs laid on clover, 89 eggs laid on alfalfa, 69 eggs on perennial rye grass, 36 laid on red fescue, and 60 eggs laid on barley. However, all of the adults in the three grass-only cages died by the end of the 9-day experiment. In contrast, all cages in the treatments that included broadleaf weeds had live adults at the end of the experiment, with an average of 3.3 adults in the broadleaf only cages, and an average of 2.3 adults in the broadleaf-grass treatment cages. This consistent mortality in grass-only treatments suggests

that *C. m. reductus* relies on broad-leafed weeds as feeding hosts and only uses grass as reproductive hosts. In the September trial all of the eggs removed by cutting leaves from plants, but none of them emerged, and either developed partially (exhibiting eye spots in developing nymphs) before dying or had eye spots beginning to form.

Gut content analysis:

We conducted gut content analysis on 5 *C. m. reductus* and 5 *C. geminatus* collected on May 22, 2020 from our control blocks in the WTFRC project "Field evaluation of leafhopper controls for X-disease management." The block had many dandelions in the ground cover, and dandelion comprised the vast majority of plant DNA in all five *C. m. reductus*. Dandelion species included both common, and red-seeded dandelion. We did not detect cherry in the guts, but did identify small amounts of clover, alfalfa, and chickweed. Dandelion also dominated the plant species within guts of *C. geminatus* but they also had a more diverse group of plants in their guts, including mallow, chickweed, an *Oxybasis* species, and cherry. These findings support the feeding trial data that ground cover is an important part of the diet of these key X-disease vectors. These results will be built on by a WSDA/USDA Specialty Crop Block Grant to Scott Harper (PI), Tobin Northfield (co-PI), Rodney Cooper (co-PI), and Tianna DuPont (co-PI) that includes gut content analysis for known vectors. Furthermore, these results are documented in a recently published scientific publication describing methods for leafhopper gut content analysis (Cooper et al. 2022).

References

Cooper, W.R., D.R. Horton, M.R. Wildung, A.S. Jensen, J. Thinakaran, D. Rendon, L.B. Nottingham, E.H. Beers, C.H. Wohleb, D.G. Hall, and L.L. Stelinski. 2019. Host and non-host 'whistle stops' for psyllids: molecular gut content analysis by high-throughput sequencing reveals landscape-level movements of Psylloidea (Hemiptera). Environmental Entomology 48:554-566.

Cooper, WR, AT Marshall, J Foutz, MR Wildung, TD Northfield, DW Crowder, H Leach, TC Leskey, SE Halbert, JB Snyder. 2022. Directed sequencing of plant specific DNA identifies the dietary history of four species of Auchenorrhyncha (Hemiptera). *Annals of the Entomological Society of America*, 115: 275-284.

Herrmann, J. V., & S. Böll. 2003. A simplified method for monitoring eggs of the grape leafhopper (*Empoasca vitis*) in grapevine leaves. *Journal of Plant Diseases and Protection*, *111*(2), 193–196.

Horton, D.R., W.R. Cooper, K. Swisher Grimm, D.W. Crowder, Z. Fu, T.D. Waters, C.H. Wohleb, K. Frost, A.S. Jensen, and M. Blua. 2018. The beet leafhopper odyssey in North America: a brief overview.

Jensen, D.D. 1971. Herbaceous host plants of western X-disease agent. Phytopathology **61**:1465-1470.

Marsh, T.G. 1965. Induction of diapause in Colladonus monatanus reductus (Van Duzee). Oregon State University Masters Thesis.

Nielson, M.W. 1957. A revision of the genus Colladonus (Homoptera Cicadellidae). USDA Technical Bulletin No. 1156.

Nielson, M.W. 1968. Biology of the geminate leafopper, *Colladonas geminatus*, in Oregon. Annals of the Entomologial Society of America **61**:598-610.

Nielson, M.W. & L.S. Jones. 1954. Insect transmission of Western-X-little-cherry virus. Phytoplathology, 44: 218-219.

Severin, H.H.P. & E.C. Klostermeyer. 1950. *Colladonus geminatus* and *C. montanus* life histories on virus-infected and on healthy plants. Hilgardia, 19: 553-560

Suslow, K. G., and A. H. Purcell. 1982. Seasonal transmission of X-disease agent from cherry by leafhopper *Colladonus montanus*. Plant Disease **66**:28-30.

Tipping, C., R.F. Mizell III, B.V. Brodbeck, P.C. Anderson, R. Lopez-Gutierrez. 2005. A novel method to induce oviposition of the glassy-winged sharpshooter, *Homalodisca coagulata* (Hemiptera: Auchenorrhyncha: Cicadellidae). J. Entomological Science, 40: 246-249.

Wolfe, H.R., E.W. Anthon, G.H. Kaloostian, & L.S. Jones. 1951. Leafhopper transmission of Western X-disease. Journal of Economic Entomology, 44: 616-619.

Yao, Q., H. Zhang, L. Jiao, X. Cai, M. Wang, & Z. Chen. 2020. Identifying the biological characteristics associated with oviposition behavior of tea leafhopper empoasca onukii matsuda using the blue light detection method. *Insects*, *11*(10), 1–14. https://doi.org/10.3390/insects11100707

EXECUTIVE SUMMARY

Title: Identifying sources of X disease in cherry orchards

Keywords: X-disease, *Colladonus montanus reductus*, leafhoppers

Abstract:
At the start of this project it was unclear where the X-disease vectors reproduced, or for *Colladonus montanus reductus* basic life history information or the number of generations of this key X-disease vector. Over the course of the study, we identified broadleaf weeds as a key reproductive host for *C. m. reductus*, identifying management of X-disease vectors within orchard ground covers as a key part of management. In feeding trials, *C. m. reductus* fed extensively on mallow and alfalfa, as well as potted peach and cherries trees. In oviposition trials, *C. m. reductus* readily laid eggs on broadleaf weeds and grass, but only survived when there was access to broadleaf weeds. Preliminary molecular gut content analysis results support the importance of broadleaf weeds as feeding hosts for X-disease vectors. Furthermore, we found that X-disease phytoplasma can survive the winter within broadleaf weeds, and that there are 3 generations of the key X-disease vector in the Pacific Northwest, *C. m. reductus*. Molecular analysis and further literature review provided detailed life cycle data on this species, conducted previously in California, and identification of eggs as the overwintering stage. This project provided preliminary data for a wide range of federally funded projects that 1) identified broadleaf weeds in orchard ground cover as commonly hosting X-disease phytoplasma, 2) used molecular gut content to demonstrate broadleaf weeds as common leafhopper hosts, 3) used molecular tests to identify multiple X-disease phytoplasma genotypes, 4) evaluated broadleaf-specific herbicide as a management strategy, 5) evaluated entomopathogenic fungi sprayed in groundcover to kill nymphs feeding on broadleaf weeds, 6) seasonal sampling of weeds, leafhoppers, and trees to better understand phenology of phytoplasma within host plants to determine the time points in which it can be acquired, and 7) use of electrical penetration graphs, which thus far has provided detailed information on leafhopper feeding and suggests that X-disease vectors may feed on each, xylem and phloem. This last project is important, as better distinguishing xylem versus phloem feeding using this technique may distinguish plants on which they phloem feed and potentially transmit X-disease phytoplasma from xylem feeding where water is accessed, but transmission cannot occur.