AN ABSTRACT OF THE DISSERTATION OF

Danielle Lightle for the degree of Doctor of Philosophy in Entomology presented on May 9, 2013.

Title: Interactions of *Amphorophora agathonica* Hottes with Raspberry Viruses and Resistant Red and Black Raspberry

Abstract approved: _____
Jana C. Lee

In recent years, red raspberry production in Washington and British Columbia has been severely limited by a newly emerged virus complex that causes symptoms of crumbly fruit. The complex is comprised of three viruses: *Raspberry bushy dwarf virus* (RBDV), *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (RpLV). Both RLMV and RpLV are transmitted by the aphid *Amphorophora agathonica* Hottes. The objectives of this work were to monitor seasonal phenology of *A. agathonica* and study the aphids' behavior on infected plants as well as resistant red and black raspberry cultivars. The lower developmental threshold of *A. agathonica* was calculated to be 2.7°C and field populations in northern Washington began increasing rapidly at approximately 800 growing degree days and peaked at approximately 1000 degree days. Evaluations of aphid performance on infected plants revealed that single infection plants (RLMV or RpLV) and co-infected plants (RLMV+RpLV) significantly increased aphid longevity over the healthy controls, while the co-infected plants also significantly increased aphid fecundity. Electrical penetration graph (EPG) studies of *A. agathonica* feeding behavior showed no differences in feeding between healthy and infected plants. However, EPG studies revealed two distinct mechanisms of resistance against *A. agathonica* in red and black raspberry. The red raspberry resistance gene Ag_1 results in extended periods of salivation in the phloem sieve elements with little subsequent phloem ingestion, a behavior commonly associated with plant defense mechanisms related to a phloem

recognition factor. Three novel aphid-resistant black raspberry selections were also studied. Aphids feeding on the resistant black raspberries were unlikely to salivate in the phloem sieve elements, which may point to a mechanism that causes aphids to be unable to recognize when they have punctured the sieve elements. Overall, this research discovered new information about *A. agathonica* biology, feeding behavior, and interactions with viruses that can be incorporated into future management strategies.

Interactions of *Amphorophora agathonica* Hottes with Raspberry Viruses and Resistant Red and Black Raspberry

by
Danielle Lightle

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APPROVED:

Major Professor, representing Entomology

Head of the Department of Horticulture

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Danielle Lightle, Author

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CONTRIBUTION OF AUTHORS

Dr. Jana Lee oversaw the planning and design of this project. Dr. Michael Dossett and Dr. Chad Finn assisted with the background of red and black raspberry resistance and behavioral assay design (Chapters 4 and 5). Dr. Diego Quito and Dr. Bob Martin provided expertise and assistance on virus testing protocols (Chapter 2). Dr. Elaine Backus and Dr. Tim Ebert trained me on proper EPG experimental design and wrote the programs used for statistical analysis (Chapters 4 and 5, respectively). All coauthors helped to edit and approve the final versions of the chapters with which they are associated.

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DEDICATION

To my Mom and Dad. I love you.

Chapter 1:

Introduction

Danielle Lightle

Oregon and Washington (Pacific Northwest, PNW) are the country's largest producer of red and black raspberries (*Rubus idaeus* L. and *R. occidentalis* L.). In 2012, 11,000 acres of red raspberries were harvested in Oregon and Washington with a production value of 44 million dollars (USDA NASS 2013). Black raspberry is a much smaller industry primarily in Oregon. 900 acres were harvested in 2012 for a production value of 5.6 million dollars (USDA NASS 2013). Production in both industries in the Pacific Northwest is limited in part by multiple virus disease complexes, including black raspberry decline in black raspberry and raspberry crumbly fruit disease in red raspberries.

Black raspberry decline is characterized by leaf mosaic symptoms and a general field decline of black raspberry plants that results in short replanting intervals, often after the 4th growing season (Halgren *et al.* 2007). The major virus associated with black raspberry decline is *Black raspberry necrosis virus* (BRNV), a member of the family *Secoviridae*, genus unassigned, that is non-persistently transmitted by the large raspberry aphid, *Amphorophora agathonica* Hottes in North America and *Amphorophora idaei* Börner in Europe. BRNV spreads very quickly, and fields in Europe may reach 100% infection after only a single growing season (Stace-Smith 1987). BRNV does not cause symptoms in red raspberry. Raspberry crumbly fruit disease symptoms include decreased fruit weight and drupelet count that result in the fruit crumbling upon harvest. *Raspberry bushy dwarf virus* (RBDV), a pollen and seed transmitted *Idaeovirus*, was long considered the causal agent of crumbly fruit (Converse 1973). However, recent research shows that in the Pacific Northwest, the disease is caused by a complex of RBDV with two additional viruses: *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (RpLV) (Quito-Avila 2011).

RLMV is a semi-persistently transmitted member of the family *Closteroviridae*. Symptoms due solely to RLMV vary depending on the raspberry cultivar and may be asymptomatic or cause leaf mottling symptoms. Transmission studies of RLMV conducted with the closely related *A. idaei* showed a required acquisition feeding period

of at least a half hour or greater, with a persistence of 4 to 18 hours (Cadman 1951), and the acquisition times are likely to be similar for *A. agathonica*. RpLV is a novel member of the family *Reoviridae* that is transmitted in a persistent-propagative manner by *A. agathonica*, although transmission rates are low (Quito-Avila *et al.* 2012). A minimum of 15 minutes of phloem ingestion is required for *A. agathonica* to acquire RpLV, and the virus has a 6-day latent period (Quito-Avila *et al.* 2012). Strategies for control of raspberry crumbly fruit disease involve managing and monitoring for the three viruses in the complex. Because RBDV is pollen-borne and spread primarily by bees, vector control would lead to severe crumbly fruit due to poor pollination. Thus, effective control of raspberry crumbly fruit relies upon managing RLMV and RpLV and understanding the biology of the vector, *A. agathonica*.

A. agathonica, the large raspberry aphid, is native to the northern regions of North America. Morphologically, *A. agathonica* is almost identical to the other *Rubus* feeding aphids in the genus *Amphorophora*. Adult females are large, 2.5 to 4.5 mm in length with swollen siphunculi, and range in color from pale to dark green (Blackman and Eastop 2000). Many members of the genus look so similar that the most reliable way to distinguish them is their chromosome counts (Blackman *et al.* 1977). Because of the morphological similarities, host plants are often used to more quickly distinguish between species; although the reliance on host plants for identification renders the true host range of this species questionable because of the possibility of misidentifications. Known hosts of *A. agathonica* include commercial red and black raspberry (*R. idaeus* L. and *R. occidentalis* L.), and many native *Rubus*, including *R. parviflorus* Nutt., *R. ursinus* Cham. & Schltdl., *R. odoratus* L., *R. procerus* auct.non P. Muell. ex Genev., and *R. phoenicolasius* Maxim. (Blackman and Eastop 2000).

A. agathonica is monoecious, spending the full year on its primary *Rubus* host. Aphids overwinter as a shiny black oval egg on the undersides of leaves and at the base of canes (Dicker 1940, Winter 1929). In early spring at bud break, the founding stem mother

(fundatrix) hatches and begins feeding on the leaf buds. The fundatrix female is morphologically distinct from the viviparous summer forms, with dark spots along the abdomen in early instars, and single hairs that run the length of the abdomen (Kennedy 1974, Dicker 1940). Asexual reproduction continues throughout the duration of the growing season. Alate aphids may fly throughout the summer; in New York, peak aphid flight occurs in June through mid-July (Kennedy 1974). Sexual forms are produced beginning in late September and October (Winter 1929, Dicker 1940, Kennedy 1974). Males are distinct, with wings, a smaller abdomen, and heavily sclerotized thorax and genitalia. Oviparous females are apterous and lack the eyespots of nymphs that are easily seen through the abdomen of viviparous females.

Large populations of *A. agathonica* cause wilting of new leaf growth and produce honeydew that encourages fungal growth (Defrancesco 2012). However, this damage is rarely observed, and numbers required to cause this damage is undefined (Antonelli *et al.* 2004). Additionally, *A. agathonica* can be a crop contaminant of cane fruits during harvest (Kieffer *et al.* 1983). To remove *A. agathonica* and other insect contaminants from fields, a "clean-up" spray is typically applied prior to harvest. Populations of *A. agathonica* are regulated in part by generalist predators and, in the fall, an entomopathogenic fungus (Antontelli *et al.* 2004). However, because the primary reason for concern over the presence of *A. agathonica* in production fields is its role as a virus vector, the occurrence of even small populations of aphids may be enough to successfully transmit viruses within a field. The seasonal phenology of *A. agathonica* has been described in New York (Kennedy 1974) but not in the PNW. Knowledge of the seasonal abundance of *A. agathonica* in the PNW may help define periods for aphid control.

Interactions between viruses, plants, and vectors are complex. Because RLMV and RpLV rely upon *A. agathonica* for spread to new hosts, there are selective pressures on the viruses to enhance vector rates of acquisition and transmission. As a result of selection pressures, infected plants in several pathosystems have been shown to modify vector

behavior in ways that benefit virus spread (Fereres and Moreno 2009). Symptomatic plant coloration is often more attractive for vectors; while changes in volatile profiles related to infection enhance vector attraction towards infected hosts (e.g. Eckel and Lampert 1996, Alvarez *et al.* 2007, Eigenbrode *et al.* 2002, Ng and Falk 2006). Additionally, vectors often benefit from feeding on infected plants through enhanced fecundity or increased longevity, which in turn increases the potential for virus dispersal (e.g. Fereres *et al.* 1999, Blua and Perring 1992, Baker 1960, Hogenhout 2008). Models created to examine the rates of virus spread by insect vectors have predicted that preferences for infected or healthy plants impact the rates of virus spread (Sisterson 2008, McElhany *et al.* 1995). While patterns between different types of virus-vector interactions have begun to be defined (Mauck *et al.* 2012), the diversity of virus systems studied has been limited. Studies of *A. agathonica* responses to plants infected with RLMV and RpLV will expand the scope of current knowledge of vector-virus interactions and lead to greater understanding of virus epidemiology.

Aphid resistant plants have long been used to help control virus diseases of red raspberry transmitted by *A. agathonica* (North America) and *A. idaei* (Europe) (Converse and Bailey 1966, Birch *et al.* 2005, Daubeny and Anderson 1993). Inability to feed successfully on resistant plants may decrease the successful acquisition and inoculation of semi- persistently and persistently transmitted viruses. The decreased colonization of aphids on resistant plants also helps to decrease secondary transmission of viruses within a field. The gene Ag_1 was identified as a dominant resistance gene against *A. agathonica* in 1966. In 1993, a resistance-breaking biotype of *A. agathonica* was reported, but did not appear to be widely spread outside of British Columbia (Daubeny and Anderson 1993). In 2012, Dossett and Kempler (2012) provided evidence for seven different biotypes of *A. agathonica*. The resistance sources that differentiate the biotypes have not yet been fully characterized (Dossett, pers. comm). Until recently, there were no known effective sources of resistance against *A. agathonica* in black raspberry. Dossett and Finn (2010) reported the finding of three native black raspberry genotypes that have strong resistance

against these aphids. However, they were unable to conclusively determine whether the three genotypes had a unique resistance source against *A. agathonica*. Identifying and understanding the mechanisms of resistance to *A. agathonica* will help to determine the best ways to incorporate and maintain resistance in the field.

The main objectives of this research were to 1) increase knowledge of the biology and life history of *A. agathonica* in the Pacific Northwest region, 2) document interactions between *A. agathonica* and virus-infected plants and 3) evaluate resistant red and black raspberries for the mechanisms that may be responsible for resistance against *A. agathonica*.

Chapter 2:

Seasonal phenology of *Amphorophora agathonica* and spread of viruses in red raspberry in Washington

Danielle Lightle, Diego Quito-Avila, Robert Martin, and Jana Lee

Abstract:

Amphorophora agathonica (Hottes) is the primary vector of aphid transmitted viruses in red raspberry in the Pacific Northwest region of the United States. To better understand the biology of the aphid, we estimated the lower developmental threshold from laboratory data and studied the seasonal activity of *A. agathonica* in commercial fields in northern Washington state. Additionally, we monitored the spread of raspberry viruses (*Raspberry latent virus*, RpLV, and *Raspberry leaf mottle virus*, RLMV) to determine how rapidly fields became infected and whether there was a relationship between aphid presence and infection. The lower developmental threshold of *A. agathonica* was estimated to be 2.7°C. In the field, apterous and alate aphid populations began rapidly increasing at approximately 800 degree days (DD) and peaked at 1050 DD. RLMV spread rapidly, with 30 to 60% of plants in four different commercial fields testing positive after three growing seasons. There was no discernible relationship between the presence or abundance of aphids based on 10 leaves sampled per plant location, and subsequent infection with RLMV.

Introduction:

Amphorophora agathonica (Hottes), sometimes referred to as large raspberry aphid, is a common pest found in commercial red and black raspberries across the northern U.S. and Canada. Feeding damage resulting from *A. agathonica* is limited, however it is a crop contaminant and important vector of economically damaging viruses (Kieffer *et al.* 1983, Isaacs and Trefor Woodford 2007). In black raspberries, *A. agathonica* is the main vector of *Black raspberry necrosis virus* (family *Secoviridae*, genus unassigned *Secoviridae* species, BRNV), which is responsible for loss of plant vigor and decline (Halgren *et al.* 2007). In red raspberries, *A. agathonica* is responsible for transmission of *Raspberry leaf mottle virus* (family *Closteroviridae*, genus *Closterovirus*, RLMV) and *Raspberry latent virus* (family *Reoviridae*, genus unassigned *Reoviridae* species, RpLV). These viruses, when found in combination with *Raspberry bushy dwarf virus* (family unassigned, genus

Idaeovirus, RBDV), cause crumbly fruit disease and reduced plant growth, which decreases fruit quality and marketability (Martin *et al.* 2013).

A. agathonica is monoecious, a non-host alternating aphid that utilizes *Rubus* spp. as its only host. Reported hosts include commercially planted black raspberry and red raspberry (*R. occidentalis* L. and *R. idaeus* L.), as well as a suite of wild native *Rubus* (*R. parviflorus* Nutt., *R. ursinus* Cham. & Schltdl., *R. odoratus* L., *R. procerus* auct.non P. Muell. ex Genev., and *R. phoenicolasius* Maxim., (Blackman and Eastop 2000). There are scattered reports of *Fragaria× ananassa* Duschesne as also being an accepted host, although the degree to which *Fragaria* are utilized is unknown (Stultz 1968). *Amphorophora agathonica* nymphs and adults may be found on *Rubus* from early spring until late fall. It overwinters as an egg usually laid on the underside of a leaf or, rarely, on the cane itself (Winter 1929).

Aphids, with short generation times and rapid population growth, can be very efficient transmitters of plant viruses. Thus, it is important to understand the seasonal phenology of a given aphid species in order to predict when populations will be greatest and develop effective management strategies that have the greatest impact (Poehling *et al.* 2007). The seasonal phenology of *A. agathonica* has been documented in the past in New York (Kennedy and Schaefers 1974a), but the populations may have different trends in the Pacific Northwest, where the summer and winter climates are milder. By determining the lower developmental threshold of the aphid, the seasonal development through use of degree-days can be calculated and compared among growing seasons to observe when management strategies may be applied most effectively.

The research objectives for this study were to determine the lower temperature threshold and monitor the seasonal phenology of *A. agathonica*. Additionally, we monitored raspberry fields in northern Washington for infection with RLMV, RpLV, and RBDV to look for potential relationships between aphid populations and virus infection.

Materials and methods:

Determination of temperature thresholds. The raspberry cultivar used for all studies was 'Meeker' obtained as planting stock from Sakuma Brothers Inc. (Burlington, WA). Canes were planted in 10 cm pots and grown in a greenhouse set at 16°C night and 21°C day temperatures and a 16 hr photoperiod.

The aphid colony was begun with adult *A. agathonica* collected from commercial raspberry fields in Whatcom County, Washington, USA in June 2010. Ten aphid adults were used to begin the colony so the colony was not clonal. Because aphids in the colony exhibited decreased acquisition rates of plant viruses (personal obs.), the colony was restarted with field-collected aphids every October and June. Aphids were reared on 'Meeker' plants in a growth chamber under fluorescent growth lights at 22°C and a 16L:8D photoperiod. New 'Meeker' plants were added weekly to maintain plant material of consistent quality.

To determine the lower developmental threshold of *A. agathonica*, aphid development was measured at five different temperatures in growth chambers (Percival Scientific Inc., Perry, Iowa): 10, 14, 18, 22, and 26°C. A HOBO datalogger (Onset Computer Corp., Bourne, MA) recorded the temperature and humidity in each chamber. Three days prior to the study, actively growing 'Meeker' plants with 15-30 cm tall primocanes were placed into the growth chambers to acclimate. A cohort of aphid nymphs was obtained by isolating adult aphids in a petri dish with a 'Meeker' leaf. After 12 hr, aphid nymphs were removed and placed on plants in the different temperature treatments. Nymphs were caged to a terminal leaflet of a young fully expanded leaf using clip cages made from 15 ml plastic tubes that were cut into 2 cm lengths. Clip cages were attached to the leaf with a rubber-coated washer and metal hair clip. The hair clip was affixed to a binder clip on a wooden stake to reduce the stress to the petiole of the leaf. Aphids were moved to a new leaflet when leaf quality began to decline.

Cages were inspected every 24 hr for molting into the next nymphal instar, as indicated by the presence of aphid exuviae inside the clip cage. The number of days for each instar and the number of days until the first nymph born were recorded. The development rate (y) of each insect was calculated, using the x-intercept approach (Arnold 1959) as y = 1/d where d was the number of days required for the insect to molt into the next instar, and regressed against the temperature. The degree day model

$$y = a + bT$$

was fit over the linear portion of the regression, where T was the temperature at which the insect developed and a and b are regression constants. The lower developmental threshold was calculated as -a/b and the number of degree days required for development (K) was calculated as 1/b. Values of y and K were calculated for instars I-II, instars III-IV and the pre-reproductive period (time from adult to first nymph born).

Field monitoring. Aphid populations and virus infection levels were surveyed in four commercial 'Meeker' red raspberry fields located in Whatcom Co., Washington, USA. 'Meeker' is the most commonly grown cultivar in the region (WRRC 2008). Fields were located within a 7 km radius and were managed conventionally with 4-7 insecticide sprays each year. Three of the fields were planted in spring 2010 and the fourth was planted in spring 2009. In September 2010, 108 plants were flagged across the four commercial fields: 36 in one field, and 24 in each of the other three fields. Two plants were selected per row of raspberries, and rows were spaced across the width of each field.

To monitor population dynamics of *A. agathonica*, leaves were collected weekly from March to October 2011 and April to October 2012. Fifty locations were selected for weekly sampling, which represents a subset of the 120 locations where plants were flagged and tested for viruses. At each sample location, 10 leaves were collected randomly at different heights. Only fully expanded leaves near the meristem were collected because these are preferential feeding locations for *A. agathonica* (Kennedy and

Schaefers 1974a). Leaves were frozen to stop aphid reproduction until processing in the laboratory. Under a dissecting microscope, both sides of the leaves were checked and all arthropod stages present were counted and recorded (e.g. aphids, insect eggs, mites). Aphids found on the leaves were stored in 70% EtOH.

A. agathonica collected from the leaf samples were identified as a member of three different age classes: nymphal instars I-II, instars III-IV, and adults. Adults were easily distinguished by the presence of a protruding cauda and eye spots of the developing nymphs in the abdomen. Nymphs were sorted into the two ages classes based on size. Because the size of aphids may vary depending on plant quality (Kennedy 1974), aphid size was compared within each trap date to account for variation in plant quality throughout the growing season. Aphids with wings, visible wing buds, males, and oviparae (egg laying females, determined through dissection of adults) were also recorded.

Raspberry plants were sampled for viruses by collecting a young fully expanded leaf from each of the 120 flagged plants, and stored at 4°C until testing. Plants were sampled in September 2010, May 2011, September 2011, and September 2012. 66% of the plants were found during all four sampling periods; other plants that had died, were removed, or could not be found were replaced with a new plant in the same vicinity. Each sample was tested for RLMV, RpLV and RBDV by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA as initial template. RNA was extracted using a combination of the methodologies described by Halgren *et al.* (2007) and Rott and Jelkmann (2001). Briefly, 100 mg of leaf tissue was ground in extraction buffer and precipitated in isopropanol followed by resuspension in 500 ul of wash buffer and 25 ul of glass milk. The RNA was eluted in 150 ul of water and stored at -80°C until used.

RT reactions were performed using random primers as described in Halgren *et al.* (2007). 2.5 µl of the RT product were used as template for the PCR in a final volume of 25 µl.

The reaction was carried out according to the polymerase manufacturer's instructions (TaKaRa Bio Inc. Shiga, Japan). Primers developed by Tzanetakis *et al.* (2007a) and Quito-Avila *et al.* (2011) were employed for detection of RLMV and RpLV, respectively. RBDV was detected by using the degenerate primers F:AAAGACKYSCAGAAATCCGTTA and R:TGWAWARGAAGTTDGCCCATTT (Keller, unpublished). The PCR program for amplification of the targets consisted of initial denaturation for 4 min at 94°C followed by 40 cycles with denaturation for 40 s at 94°C, annealing for 25 s at 58°C (RLMV and RpLV) or 55°C (RBDV) and extension for 40 s at 72°C, with a final 7-min extension step at 72°C. To assess the RNA quality and effectiveness of the RT reaction and RNA quality, the highly conserved plant gene NADH dehydrogenase ND2 subunit (ndhB) was used as endogenous control to verify the RNA quality and RT reaction by amplification of a 721bp transcript region (Thompson *et al.* 2003; Tzanetakis *et al.* 2007b)

Analysis. The proportion of aphids in each size class (instars I-II, instars III-IV, and adult) was regressed against the accumulated growing degree days (GDD) to determine whether population composition varied throughout the growing season. Binomial logistic regressions were run to investigate the relationship between observed aphid counts at each sampling location on the probability of a corresponding plant testing positive for RLMV. A plant was counted as recently positive for RLMV if it was negative at the beginning of the growing season and positive when tested at the end of the growing season. First, to explore whether time of the season may influence the probability of infection, a logistic regression model was run using the aphid counts per location each week as predictor variables. The percentage of plants infected in the field at the beginning of the growing season was included as a covariate. A full model was fit separately for 2011 and 2012 with all sampled weeks, and non-significant weeks were removed in a stepwise process. Secondly, to investigate whether overall aphid abundance affected probability of infection, a logistic regression model combining 2011 and 2012 was fit using the maximum observed aphid count per location and the percentage of

infection in the field as the predictor variables. All analyses were carried out in SAS (PROC GLIMMIX, Ver. 9.3.2).

Results:

Aphid monitoring. The number of days aphids spent in each development stage are shown in Table 2.1. The lower developmental threshold from birth through the pre-reproductive period was calculated as 2.7°C (Table 2.2, Fig. 2.1). The threshold remained fairly consistent throughout the stages of nymphal development, although was lower (1.2°C) for the pre-reproductive period (adult to first nymph born, Table 2.2). The development time, K, was approximately 250 degree days from birth until development into a reproductively mature adult.

GDD were calculated for each growing season using the calculated lower developmental threshold of 2.7°C and a biofix of January 1 each season for examining aphid populations on a standardized scale. In 2011 and 2012, the timing of aphid appearance and population growth was very similar. Aphids were first detected as early as 350 GDD. However, aphid populations increased most rapidly beginning at approximately 800 GDD and peaked between 1000 and 1100 GDD (Fig. 2.2a). Population numbers decreased sharply after this peak because of the application of insecticides for routine pre-harvest clean-up sprays common in raspberry production. After this peak, aphid populations increased and decreased marginally throughout the latter half of the growing season, but never attained the high populations seen early in the growing season.

Winged *A. agathonica* were collected at two main periods. The first coincided with the period of largest population growth between 800 and 1000 GDD (Fig. 2.2b). A second, smaller peak in winged morphs was observed near the end of the growing season (approximately 2000 GDD). 15 to 40% of aphids collected during the end of the growing season were males.

Throughout the growing season, adult aphids comprised 10% of the overall aphid population on average. Young nymphs (instar I-II) accounted for the majority of the aphids collected, averaging 62% of the aphids at each collection point, while older nymphs (instar III-IV) made up 26% (Fig. 2.3). There was no effect of time within the growing season or year on the age-structure of the populations (Table 2.3). The remaining 2% collected were sexual aphid morphs collected at the very end of the growing season.

Virus monitoring. None of the three viruses was detected in any of the newly planted raspberry fields, indicating that the growers were using clean planting stock and that nurseries were doing a good job of virus control during the plant propagation cycles. The virus with the highest rate of spread was RLMV. One year after planting, fields had an infection rate of 0 – 20% (Fig. 2.4). By three years after planting, 30 – 60% of the raspberry plants tested positive for RLMV. The infection rates for RpLV and RBDV were much lower. RpLV was not detected in any of the fields tested during the first two years. Two fields had plants that tested positive for RpLV in year 3, with only 3-4% of the plants infected, while a 4-year old field had 8% of plants infected. RBDV was not detected in any of the fields until year 3. At year 3, infection rates were approximately 15%, and increased to 37% in the 4-year old field.

Relationship between aphid presence and virus infection. The virus incidence in a given field in the prior year was not a significant predictor of the probability of infection in subsequent growing seasons (Table 2.4). In 2011, the aphid counts at two out of twelve weeks were correlated with the probability of a given plant becoming infected with RLMV. The collection on July 5, 2011 (1076 GDD) was negatively associated with RLMV infection (Table 2.4), with the odds of infection being 1.15 times lower with each additional aphid counted. This week corresponded to the highest numbers of aphids collected, as well as the peak flight of the alate adults. Unfortunately, the numbers of alate aphids at each site was not recorded in 2011, so the influence of alate vs. apterous

aphids could not be examined further. In 2011, aphid counts on August 14, 2011 (1660 GDD) were positively associated with RLMV infection (Table 2.4), with odds of infection increasing 1.28 times with each additional aphid counted. This collection corresponds with the 2nd greatest peak in aphid counts in 2011. In 2012, none of the aphid counts during the twelve weeks was a significant predictor of the probability of a plant testing positive for RLMV. Finally, there was no correlation between the maximum number of aphids detected at a given site, and the probability of a plant in that area becoming infected during the two-year period (Table 2.4).

Discussion:

Amphorophora agathonica is a pest of *Rubus* across North America, but this is the first study of *A. agathonica* biology in the Pacific Northwest region. Previous work has surveyed the seasonal phenology of this aphid, with the most detailed work occurring at the New York State Agricultural Experiment Station in Geneva, New York (Kennedy and Schaefers 1974a). The major difference between the aphid phenology in New York and Washington were the periods of aphid flight. Anticipating and controlling alate aphids is important because these aphids may act as primary vectors in newly planted or previously uninfected fields. In New York, a large number of alate aphids were counted in June with subsequent survey dates turning up no detectable numbers of alate individuals (Kennedy 1974). However, in this study, we observed two periods where alate aphids were frequently caught: at about 1000 GDD (approximately late June or early July) and lesser numbers during a second period at about 2000 GDD (early September).

The levels of RLMV in the four three-year-old commercial fields surveyed averaged 50% infection at three years of age. Five-to seven-year-old commercial fields surveyed throughout northern Washington in 2011 ranged from 60 to 100% infection (Quito-Avila 2011). When RLMV is found co-infecting plants with RBDV, RBDV titers increase 400 fold (Quito-Avila and Martin 2012) and therefore RLMV control may be the most important factor in limiting the spread of RBDV and the impact of crumbly fruit disease

in red raspberry. Rates of RpLV were much lower in our surveyed fields, with infection levels in 3-4 year old fields remaining under 10%, although other surveys conducted in 5-7 year old fields showed rates of RpLV at up to 80% (Quito-Avila 2011). *A. agathonica* is an inefficient transmitter of RpLV (Quito-Avila *et al.* 2012), thus spread of RpLV is likely dependent on high populations of *A. agathonica*.

Integrated management decisions for aphid control should ultimately be based upon accurate timing and population threshold levels. The relationship between aphid population levels and odds of virus infection were not readily apparent from our data. In one instance, a plant tested positive for RLMV when there were 8 aphids sampled in that location over the entirety of the growing season. One reason the aphid counts may have not been a significant factor in the constructed models is the relatively small sample size at each location (10 leaves per week). In a study on cereal aphids, population growth in individual plots was unable to be tracked when aphid densities were low, whereas data pooled over all plots were more accurate (Jarosik *et al.* 2002). Increasing the sample size in each location will give a better estimate of true aphid population densities and the relationships with virus spread; however, the sample size needed may be impractical for field sampling programs.

Establishment of treatment thresholds is difficult in systems where the vector transmits a virus. Even with well-studied aphid vectors, such as cereal aphids that transmit Barley yellow dwarf virus (BYDV), no relationship has been related between aphids and virus incidence levels in barley and wheat (Poehling *et al.* 2007). The raspberry cropping system presents an additional difficulty because the canes are perennial. As a result, the amount of initial inoculum present in a field increases from year to year until the field is replanted. In this system, further work is needed to better time insecticide applications to prevent infestation or large populations from developing.

The largest aphid population counts occurred around the calendar date that corresponded to 1050 GDD in both growing years, followed by a rapid population decline. This decline is expected from the pre-harvest "clean-up" insecticide spray that is routinely applied in raspberry production to remove contaminant pests such as leafhoppers, leafrollers and spiders (DeFrancesco 2012). The consistency observed between years allows for predictions of when aphid populations will begin to increase and peak. Future work should look at applying insecticides earlier than the pre-harvest spray in order to prevent the large aphid population peak observed between 800 and 1000 GDD (approximately the month of June), and determine whether aphid control during this period decreases the prevalence of RLMV.

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Table 2.1. Mean number of days (± SD) in each life stage at constant temperatures. Parentheses following the days represent the number of aphids at each temperature.

Development	**Average days of development (± SD) at constant temperature (°C ± SD)**				
Stage	9.28 ± 1.7	13.75 ± 0.7	17.55 ± 0.6	21.49 ± 0.1	25.22 ± 0.5
Nymph to adult	31.2 ± 6.4 (24)	18.5 ± 2.4 (22)	14.8 ± 3.1 (27)	11.10 ± 1.4 (23)	9.23 ± 1.4 (21)
Pre-reproductive	6.85 ± 2.6 (21)	3.57 ± 1.1 (21)	2.30 ± 0.7 (27)	2.00 ± 0.8 (23)	2.12 ± 0.9 (17)
Birth to first nymph	38.14 ± 8.5 (21)	22.28 ± 2.8 (21)	17.43 ± 3.7 (27)	13.39 ± 1.7 (23)	11.24 ± 1.4 (17)

Table 2.2. Lower developmental threshold, generation time (K) and the regression equation for *A. agathonica* at each development stage.

Development Stage	**Lower threshold (°C)**	**K**	**Regression equation**	**R^2**
Nymph to adult	2.9	204	y = -0.0145 + 0.0049T	0.88
Pre-reproductive	1.2	38.9	y = -0.032 + 0.0257T	0.36
Birth to first nymph	2.7	250	y = -0.0109 + 0.004T	0.88

Table 2.3. Effect of time (degree days) and year on the proportion of the population each stage comprises.

Stage	**Factor**	**F value**	**p value**
1^{st}- 2^{nd} instar	Degree Day	2.66	0.116
	Year	0.83	0.371
3^{rd}-4^{th} instar	Degree Day	0.28	0.599
	Year	1.17	0.291
Adult	Degree Day	0.71	0.407
	Year	0.01	0.922

Table 2.4. Model estimates from binomial logistic regression models to predict the probability of plants testing positive for RLMV, (probability of infection). The percentage of plants positive in the field at the beginning of the growing season (% prior infection) was included as a covariate in all models.

Year	Factor	Estimate	df	F value	p value
2011[1]	% Prior infection	0.025 ± 0.77	34	0.43	0.516
	Aphid count on July 5, 2011	- 0.13 ± 0.07	34	4.23	0.047
	Aphid count on August 14, 2011	0.27 ± 0.14	34	3.88	0.057
2012[2]	% Prior infection	- 0.10 ± 0.08	22	1.59	0.221
2011 &	% Prior infection	0.027 ± 0.02	64	1.91	0.629
2012[3]	Max. aphid number	0.012 ± 0.03	64	0.24	0.172

[1]Probability of infection = % Prior infection + July 5 Count + August 14 Count + Error

[2]Probability of infection = % Prior infection + Error

[3]Probability of infection = % Prior infection + Max. weekly aphid count + Error

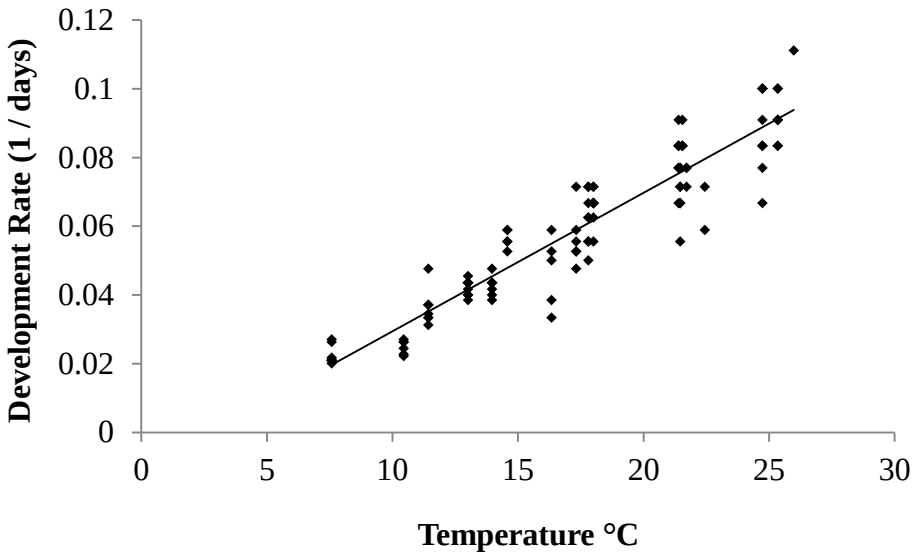

Figure 2.1. Development rate of aphids from birth to first nymph deposited when reared at constant temperatures. Regression equation: $y = -0.0109 + 0.004T$; $R^2 = 0.88$.

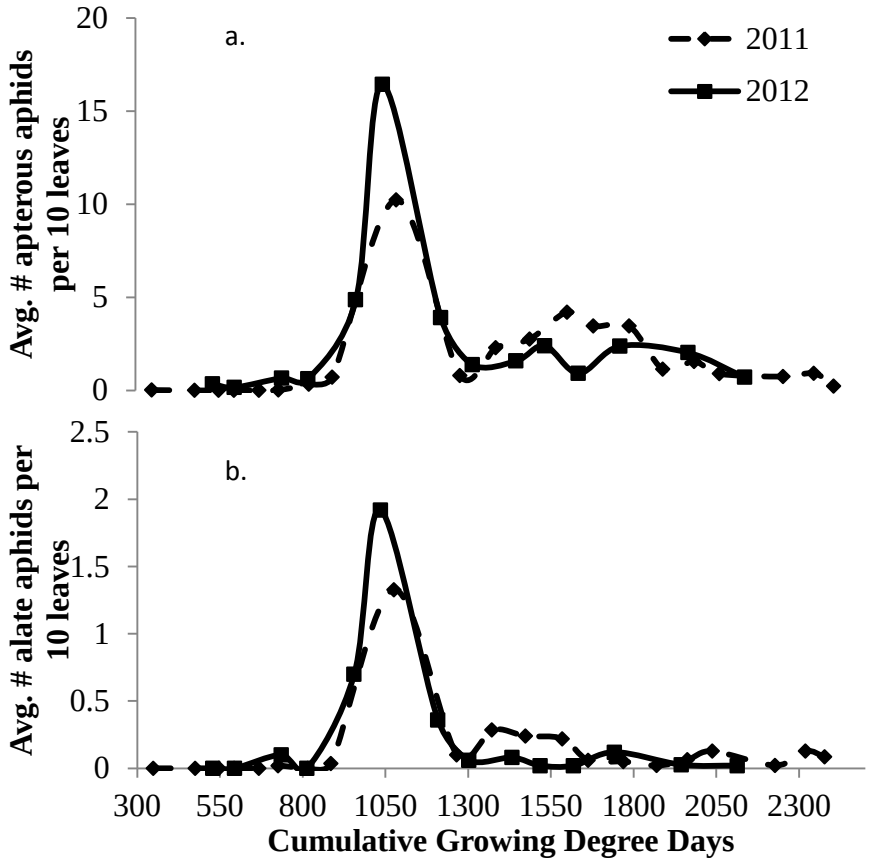

Figure 2.2. a. Average number of aphids of all ages counted per 10 leaf- collection in 2011 (dotted line) and 2012 (solid line). b. Average number of aphids with wings or wingbuds counted per 10-leaf collection in 2011 (dotted line) and 2012 (solid line). Degree days were calculated from Jan 01 above a 2.7°C lower threshold.

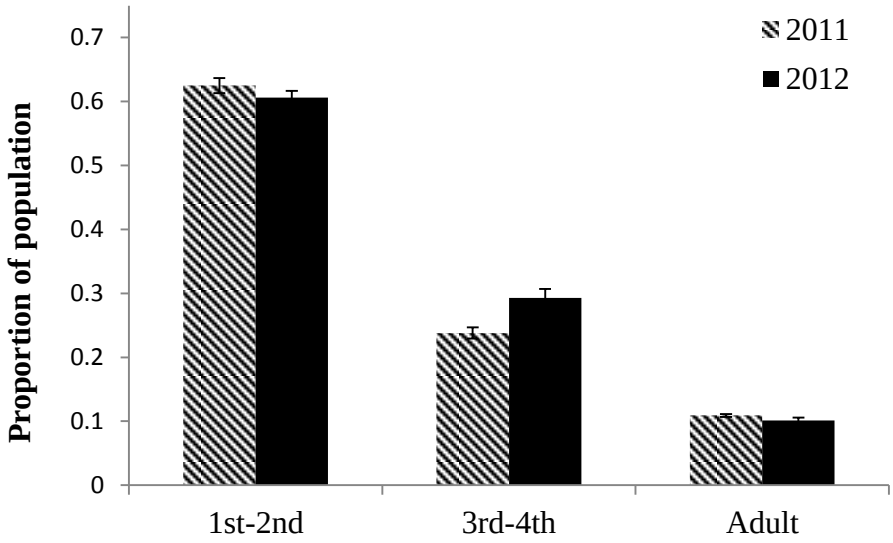

Figure 2.3. Proportion of different age categories of the total aphid population collected throughout 2011 and 2012. There was no difference in the population structure between years.

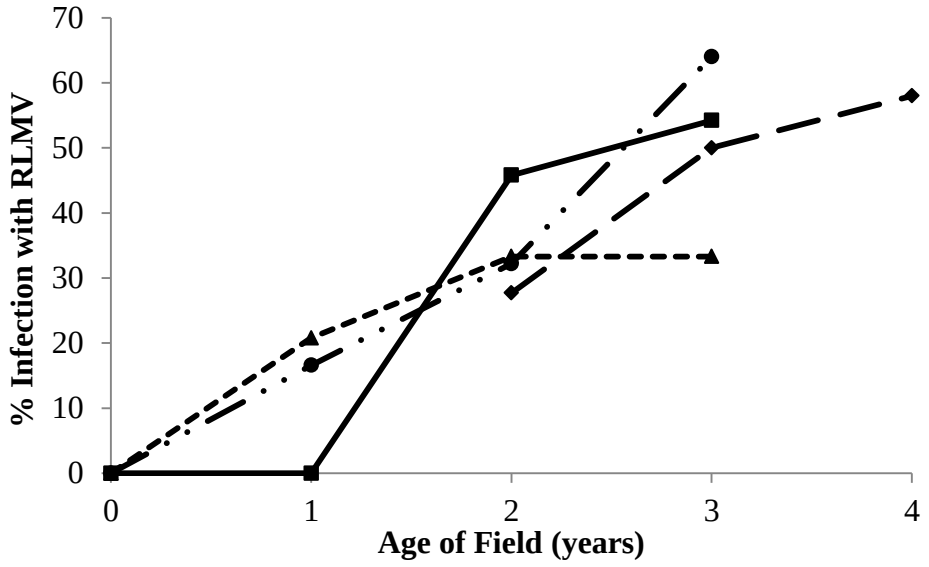

Figure 2.4. Rate of infection with RLMV of four different commercial 'Meeker' raspberry fields in Whatcom Co., Washington, USA.

Chapter 3:

Raspberry viruses affect the behavior and performance of *Amphorophora agathonica* in single and mixed infections

Danielle Lightle and Jana Lee

Abstract

Pathogens may alter their hosts which consequently may affect transmission efficiency by their vectors. We examined the effects *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (RpLV), alone and in a co-infection in raspberry plants on the behavior and performance of its vector, *Amphorophora agathonica*. Longevity was increased in aphids feeding on all infected plant treatments compared to healthy plants, but aphid fecundity only increased in the co-infection treatment. In a two-way choice study between infected and healthy plants, aphids showed no difference in preference between plants after 30 minutes of exposure. After 24 hours, aphids significantly preferred to settle on plants infected with RLMV over healthy; but healthy plants over plants infected with RpLV. There were no differences in settling preferences between healthy and co-infected plants. An electrical penetration graph (EPG) study of feeding behavior showed no differences in feeding by aphids feeding on plants infected with RLMV and RLMV+RpLV when compared to healthy controls. Our results are consistent with past findings that infected plants impact vector performance and behavior, but also highlight the need to further investigate greater virus diversity and effects of mixed infections.

Introduction

Vector-borne pathogens have a close relationship with their vector, which is their primary means for transmission to a new host. Thus, it follows that pathogens may alter the host in ways that increase transmission efficiency (Thomas et al. 2005). In plant- pathogen systems, changes may occur through manipulation of the plant (host manipulation) or through manipulation of the vector (vector manipulation). Vector manipulation has been demonstrated in *Rhopalosiphum padi*, where aphids infected *in vitro* with *Barley yellow dwarf virus* (BYDV, Luteoviridae) were subsequently more attracted to healthy wheat plants over infected plants (Ingwell et al. 2012). Host manipulation studies frequently focus on how infected plants influence the vector performance (e.g. fecundity or longevity) or vector behavior (e.g. initial attraction and settling preferences). Several

hypotheses have been proposed to explain host manipulation by a pathogen, including adaptive manipulation (Poulin 2000) and behavioral manipulation (Thomas et al. 2005).

Mauck et al. (2012) found that changes in vector behavior or performance were related to the mode of virus transmission. Non-persistently transmitted viruses are rapidly acquired from a plant and extended periods of feeding on an infected plant are often associated with decreased rates of virus transmission (Wang and Ghabrial 2002). Plants infected with non-persistent viruses are typically equally or more attractive to vectors than healthy plants; however,vectors are more likely to desert infected plants than vectors on healthy plants. Fereres et al. (1999) showed that *Rhopalosiphum maidis* (Fitch) was equally attracted to soybean infected with *Soybean mosaic virus* (SMV, Potyviridae), but remained on infected plants for less time than aphids on healthy soybean. In the field, aphids added to plants infected with *Zucchini yellow mosaic virus* (ZYMV, Potyviridae) were more likely to emigrate than aphids added to healthy plants (Blua and Perring 1992). Increased emigration from infected plants in favor of healthy plants is modeled to increase the rate of pathogen spread (Sisterson 2008).

Acquisition of semi-persistently and persistently transmitted viruses is increased with longer durations of ingestion by the vector. Semi-persistent viruses are non-circulative and bind to the vector's stylets or foregut (Uzest et al. 2007, Ng and Falk 2006). While semi-persistent viruses may be acquired very quickly, acquisition rates of some semi-persistent viruses increase after periods of longer ingestion (Palacios et al. 2002). Persistent viruses are acquired from the phloem of the host plant and are circulated through the vector to the salivary glands, where they may be inoculated into a new host. Persistent viruses may be divided into two categories, those that simply circulate through the host (persistent- circulative) and those that replicate within the insect as well as the plant (persistent- propagative).

Because increased ingestion time corresponds to increased rates of virus acquisition, host manipulation predicts that plants infected with semi-persistently and persistently transmitted viruses will have greater attraction and settling rates of aphids over those on healthy plants. Additionally, performance outcomes, such as longevity or fecundity, may be increased on plants infected with semi-persistent and persistent viruses (Mauck et al. 2012). *Myzus persicae* Sulz. and *Aphis fabae* Scop were more attracted to sugar-beet leaves infected with the semi-persistent beet yellows virus (BYV, *Closteroviridae*) than to healthy leaves. Both species also reproduced more quickly and had greater longevity on BYV plants than healthy ones (Baker 1960). Plants infected with the persistent *Potato leaf roll virus* (PLRV, *Luteoviridae*) are consistently more attractive to vectors than healthy plants and are associated with increased growth rates, longevity and fecundity (Srinivasan et al. 2006, Castle et al. 1998, Eigenbrode et al. 2002, Castle and Berger 1993). The adaptive value of increased settling and improved performance may be greater crowding on host plants, which results in increased vector migration to new potential hosts (Gildow 1980; 1983, Zhang et al. 2000).

Mauck et al. (2012) found strong support among the published literature for their hypothesis that virus transmission type predicts the direction of changes in vector performance or behavior. However, they identified several short-comings of this conclusion, among which include a relative low diversity in the virus families examined, few studies that focused on both vector performance and behavioral changes, and little attention to naturally occurring (non-agricultural) systems or mixed viral infections. Additionally, studies of semi-persistent and persistent- propagative viruses are under-represented in the literature.

The objectives of this study were to examine the effects of singly infected and mixed infected host plants on the performance, attraction and settling behavior of an aphid vector. The model system that we used was two viruses that co-infect red raspberry (*Rubus idaeus* L.). *Raspberry leaf mottle virus* (RLMV) is a semi-persistently transmitted

member of the family *Closteroviridae*. *Raspberry latent virus* (RpLV) is a persistent-propagative transmitted virus in the family *Reoviridae*. Both viruses are transmitted by the aphid *Amphorophora agathonica* Hottes. We hypothesized that 1) single and mixed infections of RLMV and RpLV would improve the performance of *A. agathonica*, and that 2) aphids would show increased attraction toward and settling on infected plants over healthy plants. Lastly, we monitored the feeding behavior of aphids on healthy and infected plants to determine if changes in feeding behavior may explain differences in aphid attraction and settling behavior.

Materials and Methods

***Plants and insects*.** The raspberry cultivar used was 'Meeker', which was obtained as plugs from North American Plants (McMinnville, OR). Plugs were planted individually in 10 cm pots with 8 g/gal of 21-2-11 N-P-K fertilizer (Apex, Boise, ID). Plants were grown in a greenhouse at 21°C daytime and 16°C nighttime temperatures and a photoperiod of 16:8 (L:D) until large enough for grafting.

Virus source plants were single infected RLMV and RpLV plants that had previously been collected from 'Meeker' plantings in production fields in Washington, U.S. A co-infected plant (RLMV+RpLV) had previously been generated by graft inoculation. The new 'Meeker' plants were grafted with the single or mixed infection plant treatments, and healthy controls were mock inoculated. Two months after grafting, plants were tested using RT-PCR to ensure the grafts were successful.

Ten adult *A. agathonica* were collected from a commercial raspberry field in Whatcom Co., WA in September 2012 and maintained as a single colony on 'Meeker' plants in a growth chamber (Percival Scientific, Perry, IA). Aphids were reared at 21°C and a 16:8 (L:D) photoperiod. Plants were replaced weekly to maintain high quality.

Aphid performance on infected plants. An aphid cohort was obtained by isolating reproductively mature adults on a 'Meeker' leaf in a Petri dish. After 24 h, the nymphs were caged individually on a fully expanded leaf on either an healthy plant, a plant infected with RLMV or RpLV, or a plant co-infected with RLMV+RpLV. Clip cages were made from 15 ml plastic tubes cut into 2 cm lengths and attached to the leaf with a rubber coated washer and metal clip. The metal clip was supported on a wooden stake to reduce stress to the leaf petiole. Aphids were checked daily for the presence of nymphs until they died. When nymphs were present, they were counted and removed from the cage. Aphids were moved to a newly expanded leaf when leaf quality declined. Seven aphids were caged to each treatment and the study was replicated three times. Differences in the pre-reproductive period (days from birth to first nymph born), fecundity, and longevity between treatments were compared using a generalized linear mixed model with replicate as a random factor (PROC GLIMMIX). Tukey's HSD was used to correct for multiple comparisons. All analyses were conducted in SAS 9.2.3.

***RpLV effects on aphid performance*.** To clarify whether changes in aphid performance on RpLV plants were due to virus-induced changes on the plants or to replication of the virus within the aphid, we examined differences in healthy vs infected aphid performance. Clonal aphid nymph pairs were obtained by isolating adults individually in a 24 well plate, where each well contained moistened filter paper and a 'Meeker' leaf disc. Nymphs born within a 24 h period to the same adult were considered to be genetically identical pairs. After 24 h, one half of the pair was caged to a healthy plant, while the other half was caged to a plant with RpLV. Aphids fed for 5 d, which is long enough for successful acquisition of RpLV (Quito-Avila et al. 2012). The aphids were then caged individually to the same healthy plant. Data collected was the same as the performance on infected plants. Eight aphid pairs were tested per replicate. The study was replicated three times. Differences in the pre-reproductive period, fecundity, and longevity between treatments were compared using a paired t-test (PROC MIXED).

Aphid attraction and settling. The assay design was modified from Srinivasan et al. (2006) and Castle et al. (1998). Two treatment plants, one healthy and one infected (either RLMV, RpLV or RLMV+RpLV) were placed on opposite sides of the test arena, which consisted of an 14 cm Petri plate placed on a stage (Fig. 3.1). The youngest fully expanded leaflet from the test plant was inserted into the Petri plate and held into place using parafilm. All possible exits from the arena were sealed off using parafilm.

Fifteen late instar nymphs and adult *A. agathonica* were held in a small Petri plate for 1 hr prior to the beginning of the assay. After 1 hr, the aphids were added to the edge of the test arena, equidistant from the two test leaves. Aphids were free to probe and walk on the test leaves. The number of aphids on each leaf was counted at 30 min and 24 hr after introduction.

The study was replicated sixteen times per infected plant treatment, using a new plant pair for each replicate. Because there was low correlation between aphid choice at 30 min and 24 h, each time point was analyzed separately. The proportion of aphids selecting each leaf was analyzed using a generalized linear mixed model with a binomial distribution (PROC GLIMMIX, SAS 9.2).

Feeding behavior. Aphid feeding behaviors on healthy plants and plants infected with RLMV and RLMV+RpLV were monitored using an AC/DC EPG system (Backus and Bennett 2009). Young adult aphids were starved for 0.5 hr, during which time they were connected to an electrode using 0.05 µm gold wire approximately 12 mm long. 40 mV DC current was applied to the plant through a copper electrode inserted into the soil at the base of the plant. When the aphid fed on the plant, the circuit was complete and the voltage change was measured using a DI-710 (Dataq, Akron, Ohio). Recordings began in the afternoon (1500 to 1700 hours) and continued for 24 hr.

Data were exported to The Observer (Noldus, Waginangen, Netherlands) and scored for the number and duration of pathway behaviors (C; salivation and other behaviors occurring in the plant epidermis and mesophyll), potential drops (PD; cell punctures), xylem ingestion (G), phloem salivation (E1), and phloem ingestion (E2). Calculation of variables was done using a new SAS program designed to calculate variables matching those produced in the Excel program of Sarria et al. (2009; Ebert pers. comm.). Variables were analyzed using iteratively optimized generalized linear models (PROC GLIMMIX) with the Kenward-Rogers degree of freedom adjustment (Littell et al. 2006).

Results

Performance. All three infected plant treatments (RLMV, RpLV, and RLMV + RpLV) significantly increased the longevity of aphids ($F_{3,73} = 7.47$, $p < 0.001$, Fig. 3.2a). Aphid fecundity increased on all three infected plant treatments, but only significantly so on the mixed infection treatment ($F_{3,70} = 2.95$, $p = 0.038$, Fig. 3.2a).There were no differences in the pre-reproductive development time of the aphids feeding healthy and infected plants ($F_{3,73} = 2.46$, $p = 0.07$, Fig. 3.2a). Aphids infected with RpLV but developing on healthy plants did not show any changes in their development time, fecundity, or longevity compared to healthy aphids ($p > 0.1$, Fig. 3.2b).

Attraction and settling. Thirty minutes after addition to the two-way choice arena, aphids did not show a significant attraction to either RLMV, RpLV, or RLMV+RpLV plants over healthy ones (RLMV: $F_{1,15} = 1.87$, $p = 0.2$; RpLV: $F_{1,15} = 0.75$, $p = 0.4$; RLMV+RpLV: $F_{1,15} = 0.05$, $p = 0.8$; Fig. 3.3a). After 24 hr, aphids significantly preferred RLMV plants to healthy plants ($F_{1,15} = 4.54$, $p = 0.05$). However, the opposite was true when aphids were given a choice between RpLV and healthy plants. Aphids significantly preferred to settle on healthy over RpLV-infected plants after 24 hr ($F_{1,15} = 4.89$, $p = 0.04$). There was no difference in the proportion of aphids that settled on either treatment when exposed to RLMV+RpLV and healthy plants ($F_{1,15} < 0.01$, $p = 0.9$; Fig. 3.3b).

Electronic monitoring. There were no significant differences in any of the calculated parameters relating to pathway behaviors, phloem salivation or ingestion, or xylem ingestion between aphids feeding on healthy plants and plants infected with RLMV or RLMV+RpLV (Table 3.1).

Discussion

Based on our results, RLMV, RpLV, or RLMV+RpLV infected plants enhanced the performance of *A. agathonica* compared to healthy plants. While the pre-reproductive development rate was not changed, aphids on all three infected plant treatments had increased longevity. In all infected treatments, aphids had elevated fecundity compared to healthy plants; however it was only in the co-infection treatment where aphids had significantly increased fecundity over aphids feeding on healthy plants. Reasons for improved performance may relate to the amino acid composition and concentration of the phloem sap of the infected plants. *Barley yellow dwarf virus* infected wheat had decreased amino acid concentration that correlated with poor performance by the aphid *Sitobion avenae* (Fiebig et al. 2004). Raspberry plants co- infected with RLMV and *Black raspberry necrosis virus* (BRNV) showed an overall increase in amino acid concentrations, however the closely related vector *Amphorophora idaei* Börner had longer developmental times on infected plants over the healthy raspberry controls (McMenemy et al. 2012). The authors hypothesized that the high levels of the amino acid glutamate in RLMV+BRNV infected plants may have reduced plant suitability (McMenemy et al. 2012, Chen et al. 1997). Because McMenemy et al. (2012) did not identify the amino acid composition of plants infected singly with RLMV, we cannot directly compare our data; however, it is clear that different combinations of co-infections (RLMV+BRNV vs RLMV+RpLV) have different performance outcomes for aphids in raspberry.

Increased performance on plants infected with RpLV is because of changes in the plant, rather than a fitness effect of RpLV replication within *A. agathonica*. The extent to which

persistent- propagative transmitted plant viruses affect vector fitness is unknown. The persistent- propagative *Tomato yellows leaf curl virus* (TYLCV, *Geminiviridae*) reduces the fitness of its whitefly vector *Bemisia tabaci* (Rubinstein and Czosnek 1997). However, the closely related *Tobacco curly shoot virus* (TbCSV, *Geminiviridae*) increased *B. tabaci* longevity by 18-fold (Jiu et al. 2007; Hogenhout et al. 2008). Few examples of fitness effects of persistent- propagative viruses may be recorded because the viruses evolved in insects and moved secondarily to plants (Power 2000, Nault 1997). Our data show no evidence of a loss or gain of aphid fitness as a result of RpLV propagation within *A. agathonica*.

The vast majority of prior studies support the hypothesis that aphid attraction and settling is enhanced on hosts infected with semi-persistent and persistent viruses (Mauck et al. 2012). Our data showed no evidence for greater initial attraction by aphids after 30 minutes of exposure on infected plants. The mechanism driving differential attraction is in large part due to changes in the volatile profiles of infected plants. Infected plants typically do not produce novel compounds, but rather elicit exaggerated amounts of attractive compounds already produced by the host plant (Mauck et al. 2010). McMenemy et al. (2012) found increased attraction by *A. idaei* to RLMV+BRNV infected raspberry that could be partly attributed to enhanced amounts of (Z)-3-hexenyl acetate. Again, the co-infection combination of RLMV+BRNV used in that study resulted in different behavioral outcomes than we observed with RLMV and RpLV alone and in combination. Further study on the mechanisms of these virus interactions would be valuable for understanding how mixed infections affect vector performance behavior.

After the attraction phase, an increased or neutral preference for settling by aphid vectors is typically observed among plants infected with semi-persistent and persistently transmitted viruses (Mauck et al. 2012). True to this pattern, *A. agathonica* preferred RLMV-infected plants at 24 hours compared to healthy plants. Increased settling has been observed for another *Closterovirus*: *Beet yellows virus* infected sugarbeets were

preferred by four different species of aphids in the greenhouse (Baker 1960, Macias and Mink 1969). *A. agathonica* preferred to settle on healthy over RpLV infected plants, placing this experiment with a small minority of studies that failed to find a positive or neutral settling preference for a persistent virus (Power 1996, Mauck et al. 2012). To our knowledge, RpLV is the first *Reovirus* and the first double-stranded RNA virus to be studied for changes in vector behavior. Incorporating a greater diversity of viruses into research on behavioral preferences of vectors will help to discern whether RpLV is an outlier or whether virus family and genome type play a large role in the behavioral effects.

Plants co-infected with multiple viruses are known to undergo competition or synergistic interactions. In many cases, one virus increases its titers when co-infecting a plant over the titers observed when infecting the plant alone (Quito-Avila and Martin 2012, Wintermantel et al. 2008). RpLV and RLMV do not appear to experience a synergistic or antagonistic interaction during co-infection because titer levels of both viruses remain at similar levels to titers when singly infected (Quito-Avila 2011). Interestingly, the mixed virus combination RLMV+RpLV showed no significant differences in settling behaviors in a two way choice, despite a positive preference for RLMV alone and a negative preference for RpLV alone. Too few mixed virus systems have been studied for aphid preference to hypothesize whether the co-infected plant was equally as attractive to healthy plants because the effect of virus infections 'cancelled' each other out, or because there are novel changes occurring due to the co-infection. Regardless, our data show that RpLV gains a competitive advantage when it is found in combination with RLMV. In a single infection of RpLV, aphids had a preference for healthy plants, which would ultimately decrease the likelihood that RpLV would be acquired. However, deterrent effects of RpLV infection appear to be mitigated by co-infection with RLMV, with aphids showing no significant preference for healthy or co-infected infected plants. While transmission of RpLV may be enhanced with co-infection, RLMV experiences a competitive disadvantage when in combination with RpLV. Aphids were more likely to

settle on singly infected RLMV plants but then only equally likely to settle on the RLMV+RpLV plants as healthy plants. Since RpLV is a propagative virus in *A. agathonica*, perhaps the preference for healthy plants is advantageous to the virus. In this case, once the virus is acquired by the aphid, the aphid should be able to transmit the virus to healthy plants for the remainder of its life after the latent period has been met. Thus, a preference for healthy plants would result in more plants becoming infected with the virus over the lifespan of the aphid.

We hypothesized that differences in the attraction and settling behaviors of aphids on infected plants may be explained by plant changes that affect feeding behavior. However, no feeding differences were observed between RLMV and RLMV+RpLV as compared to healthy plants. EPG studies conducted with *A. gossypii* feeding on plants infected with *Zucchini yellows mosaic virus* (ZYMV) found greater numbers of probing events but decreased numbers of phloem contacts compared to healthy controls (Blua and Perring 1992). Plants infected with PLRV were found to enhance *M. persicae* feeding at the mesophyll level because there were decreased incidences of stylet penetration difficulties (waveform F) and fewer short test probes (Alvarez et al. 2007). Both these studies found that feeding differences occurred only when symptoms were obvious. Therefore the feeding differences observed in those studies may be because of the structural changes occurring in the leaves as a result of infection. RLMV and RpLV produce no obvious visual symptoms in 'Meeker', and may explain why feeding behaviors were not different than on healthy plants.

We have shown that infection of raspberries with RLMV, RpLV and RLMV+RpLV increase *A. agathonica* performance relative to healthy plants. Despite a positive performance change for aphids on all infected plant treatments, aphids were less likely to settle on RpLV infected plants. Our results illustrate the need for research on a wider diversity of plant virus families, as well as the effects of virus co-infections, which are common in the field. Continued understanding of the complex relationships between

virus infection and vector transmission will illuminate the evolutionary forces at play, as well as improve the understanding of virus epidemiology and disease management.

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Table 3.1. Number and duration (mins) of the EPG feeding variables (mean ± SE). Waveform definitions: E1 – phloem salivation; NP – non-probing (stylets withdrawn from plant); C – pathway behaviors; E2 – phloem ingestion; G – xylem ingestion; PD – potential drops (cell punctures).

Parameter	**Healthy**	**RLMV**	**RLMV + RpLV**	**F**	**df**	***P***
Time to first probe	226.12 ± 294.87	205.86 ± 228.90	137.52 ± 161.65	0.29	2,50	0.75
Time to first E1	296.52 ± 63.70	392.56 ± 79.77	403.94 ± 86.78	0.87	2,50	0.42
Total dur. NP	701.71 ± 186.94	550.32 ± 138.68	587.91 ± 156.62	0.35	2,50	0.70
Total Dur. C	284.25 ± 30.59	352.72 ± 28.94	315.93 ± 30.59	1.33	2,50	0.27
Total Dur. E1	45.69 ± 14.28	38.41 ± 11.35	46.33 ± 14.47	0.20	2,50	0.82
Total Dur. E2	872.08 ± 467.49	665.28 ± 316.87	911.50 ± 488.62	0.30	2,46	0.74
Total Dur. G	76.77 ± 24.85	85.09 ± 38.94	89.34 ± 28.91	0.07	2,7	0.94
No. of C events	19.25 ± 3.10	21.10 ± 3.21	19.01 ± 3.06	0.16	2,50	0.85
No. of PD events	257.67 ± 27.67	282.58 ± 26.17	263.18 ± 27.67	0.24	2,50	0.79
No. of E1 events	10.96 ± 2.52	8.88 ± 1.93	7.35 ± 1.69	1.06	2,50	0.35
No. of E2 events	16.01 ± 4.66	8.96 ± 2.25	9.59 ± 2.79	1.80	2,46	0.18
No. of G events	2.26 ± 0.77	3.37 ± 2.48	2.69 ± 0.92	0.53	2,7	0.61

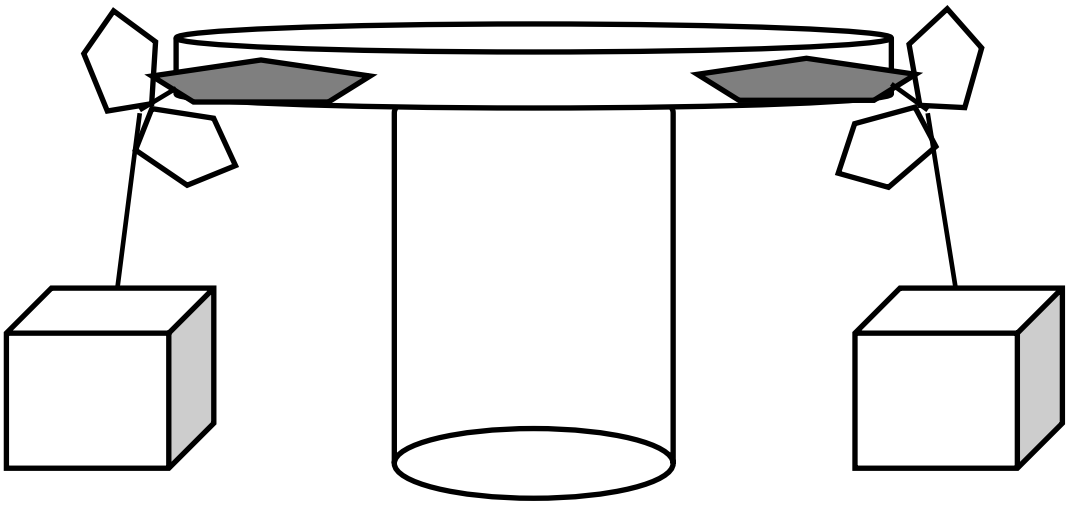

Figure 3.1. Experimental setup for the attraction and settling assay. A Petri dish arena was set on top of a stage and the terminal leaflet of the two test plants inserted. Leaves remained attached to the test plant. Aphids were added to a central location. Assay design was modified from Srinivisan *et al.* (2006) and Castle *et al.* (1998).

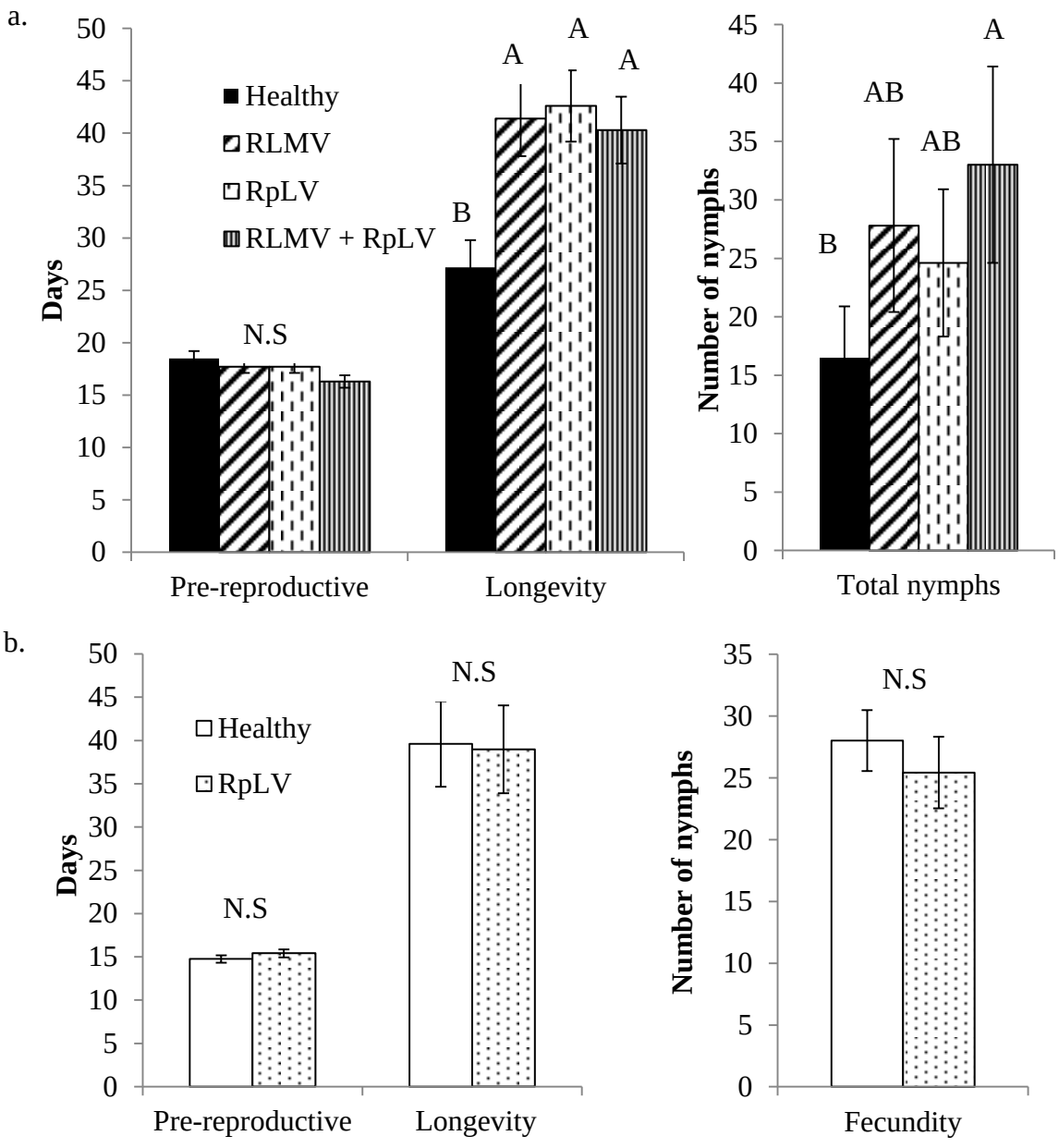

Figure 3.2. a) Aphid performance on healthy and infected plant treatments (means ± SE). Different letters above the means indicate significant differences between treatments ($p < 0.05$). b) Aphid performance when healthy or infected with RpLV when feeding on healthy plants (means ± SE).

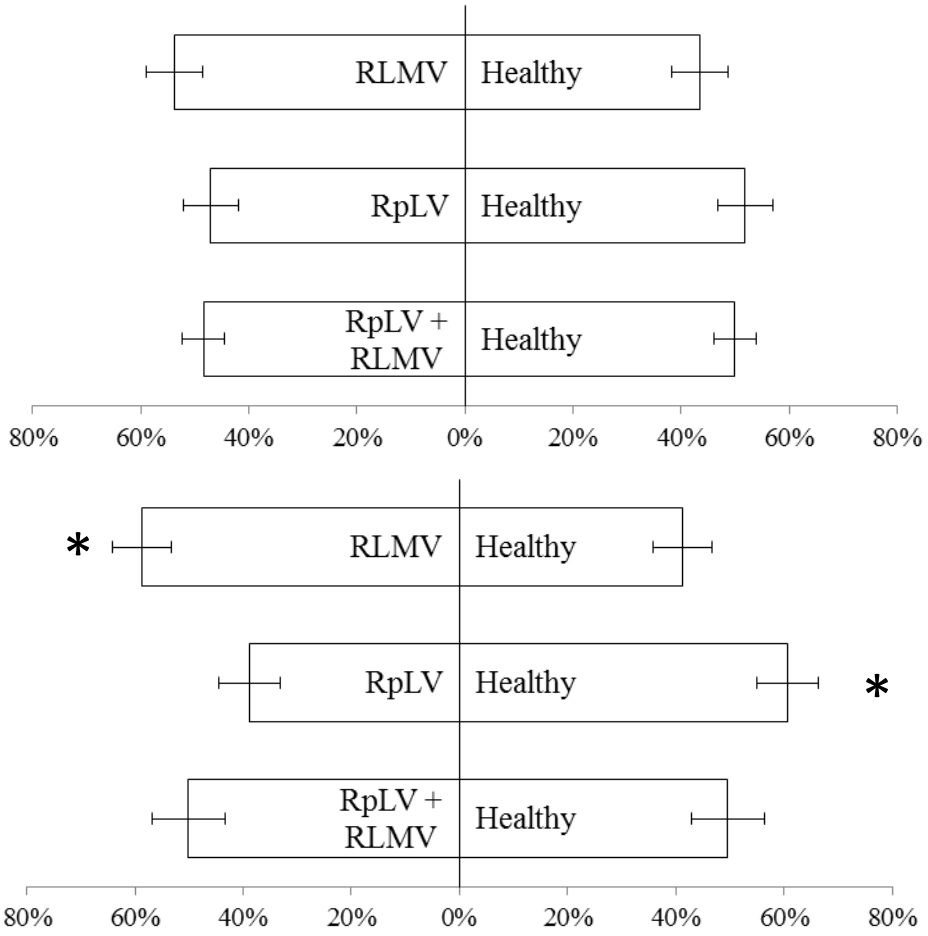

Figure 3.3. Proportion of aphids (mean ± SE) on the leaf of each treatment at a) 30 minutes and b) 24 hours after aphids were introduced to the arena. An asterisk indicates a significant difference ($p < 0.05$) in the proportion of aphids making that selection.

Chapter 4:

Location of the Mechanism of Resistance to *Amphorophora agathonica* (Hemiptera: Aphididae) in Red Raspberry

Danielle Lightle, Michael Dossett, Elaine Backus and Jana Lee

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Abstract

The aphid *Amphorophora agathonica* Hottes is an important virus vector in red (*Rubus idaeus* L.) and black (*Rubus occidentalis* L.) raspberries in North America. Raspberry resistance to *A. agathonica* in the form of a single dominant gene named Ag_1 has been relied upon to help control aphid-transmitted plant viruses; however, the mechanism of resistance to the insect is poorly understood. Aphid feeding was monitored using an electrical penetration graph (EPG) on the resistant red raspberry 'Tulameen' and compared with a susceptible control, 'Vintage'. There were no differences in pathway feeding behaviors of aphids as they moved toward the phloem. Once in the phloem, however, aphids feeding on resistant plants spent significantly more time salivating than on susceptible plants, and ingested significantly less phloem sap. This suggests that a mechanism for resistance to *A. agathonica* is located in the phloem. Reduced ingestion of phloem may result in inefficient acquisition of viruses and is a likely explanation for the lack of aphid-transmitted viruses in plantings of resistant cultivars.

Introduction

Aphid transmitted viruses are an important problem for red (*Rubus idaeus* L.) and black (*Rubus occidentalis* L.) raspberry production in North America. Viruses such as *Raspberry leaf mottle virus* (RLMV), *Raspberry latent virus* (RpLV), and *Rubus yellow net* (RYNV) in red raspberry and *Black raspberry necrosis virus* (BRNV) in black raspberry, cause a decline in cane health and fruit quality, resulting in a shortened life of the infected plantings(Halgren *et al.* 2007, Tzanetakis *et al.* 2007a, Quito-Avila *et al.* 2011). In North America, the most important vector of these viruses is the large raspberry aphid, *Amphorophora agathonica* Hottes (Hemiptera: Aphididae). *A. agathonica* is distributed throughout the United States and Canada and colonizes only *Rubus* species (Blackman and Eastop 2000).

Host plant resistance has long been recognized as an effective method for reducing virus spread (van Emden 2007). Indeed, this practice has played a major role in reducing the

spread of viruses in resistant red raspberry by *A. agathonica* in North America and by the closely related *A. idaei* Börner (Hemiptera: Aphididae) in Europe (Isaacs and Trefor Woodford 2007). Resistance to *A. agathonica* is conferred by the Ag_1 gene, which was originally found in cv. Lloyd George and has since been used widely in breeding (Daubeny 1966). Ag_1, a single dominant gene, has been effective for over fifty years and resistance has not been overcome by the most common biotype of *A. agathonica*, although an Ag_1-breaking biotype of *A. agathonica* has been reported in British Columbia (Daubeny and Anderson 1993). Despite reliance upon this gene, the mechanism of resistance is not entirely understood.

Kennedy and Schaefers (1974b) presented evidence that Ag_1 plants were resistant through antixenosis, or aphid non-preference for the host, which led to host rejection and eventual aphid death. In choice trials, aphid colonies became established only on susceptible cultivars, while in no-choice trials, aphids experienced decreased survival and high desertion rates on resistant cultivars compared to susceptible cultivars (Kennedy and Schaefers 1974b). However, phloem contact was not entirely avoided because histological studies of stylet sheath pathways showed that aphids on resistant plants reached the phloem sieve elements (Kennedy 1974). Furthermore, Kennedy and Schaefers (1975) showed that the ingestate from resistant plants was more dilute than on susceptible plants based on honeydew and whole-body homogenate analysis. They hypothesized that in addition to antixenotic mechanism, there was a nutritional role to the resistance against *A. agathonica*. Yet, questions about the resistance mechanism of Ag_1 remain. It is unlikely that a substantial nutritional deficit exists in Ag_1 raspberries because several other aphid species, including *Aphis rubicola* Oestlund (Hemiptera: Aphididae) and the closely related *A. idaei*, will readily feed on Ag_1 plants without an impact on survival or ability to colonize these plants (Kennedy *et al.* 1973, Kennedy and Schaefers 1975).

Insight on potential mechanisms of resistance can be obtained by studying the feeding behaviors of aphids on resistant and susceptible plants. The electrical penetration graph (EPG) technique has been invaluable in measuring the feeding behavior of aphids and other hemipterans (Walker 2000). In EPG, the insect is wired into an electrical circuit with a host plant. The insect's stylets then act as a switch, completing the circuit when the stylets are inserted into the plant. Changes in output voltage over time, known as waveforms, represent different activities, such as phloem salivation or ingestion, within the plant. The objective of this study was to compare the feeding behavior of *A. agathonica* on resistant and susceptible hosts using EPG to determine which plant tissues were most important for resistance.

Materials and Methods

***Plants and insects*.** The raspberry cultivars selected for this study were the aphid resistant 'Tulameen' and susceptible 'Vintage,' a new release from the USDA Horticultural Crops Research Unit and Oregon State University cooperative breeding program. 'Tulameen' was selected for aphid resistance conferred by gene Ag_1 (Daubeny and Anderson 1991, Daubeny and Kempler 2003). 'Vintage' and 'Tulameen' plants were obtained as hardened-off tissue culture plugs from Sakuma Brothers (Burlington, WA). Randomly sampled individuals of each cultivar tested negative for the presence of all known aphid-transmitted raspberry viruses. Plugs were grown individually in 10 cm pots (Dura-Pot, Lake Oswego, OR) of Sunshine Professional Growing Mix (Sun Gro Horticulture, Bellevue, WA) amended with 8 g/gal of 21-2-11 NPK fertilizer (Apex, Boise, ID). The plants were grown in a greenhouse at 16:8 L:D and 21ºC daytime and 15.5ºC nighttime temperatures, and used when they were approximately 30 cm tall.

Six apterous parthenogenic female *A. agathonica* were collected from commercial red raspberry fields in Whatcom Co., Washington in July 2010 and offspring from these females were combined into a single colony. This aphid colony was maintained in a Percival growth chamber at 18 ± 2°C under fluorescent growth lights at 16:8 L:D on

virus-free ‘Meeker’ red raspberry grown from root stock (Sakuma Brothers, Burlington, WA) in 12.5 cm pots (Dura-Pot, Lake Oswego, OR) of Sunshine Professional Growing Mix (Sun Gro Horticulture, Bellevue, WA) amended with 8 g/gal of 21-2-11 N-P-K fertilizer (Apex, Boise, ID). Aphids in the study were used within 3 d of molting into the adult stage unless noted otherwise.

Aphid performance on ‘Tulameen’. Because Ag_1-breaking clones of *A. agathonica* have been reported (Daubeny and Anderson 1993), ‘Tulameen’ plants were tested to verify that they were resistant to the aphid clones used in this study. Two ‘Tulameen’ plants were placed in one mesh aluminum cage (35.5 cm x 35.5 cm x 35.5 cm) and two ‘Vintage’ plants were placed in a second cage. Twenty adults and 20 nymphs were evenly distributed in each cage. The plants and aphids were maintained in this insect cage in a greenhouse at 16:8 L:D and 21ºC daytime and 15.5ºC nighttime temperatures. After 2 wk, the aphid populations in each cage were counted. This procedure was replicated three times.

Aphid settling behavior. To test whether resistant plants may affect aphid settling behaviors or tendency of an aphid to immediately leave the plant (Pelletier and Giguere 2009), aphids were observed for differences in behavior on ‘Tulameen’ or ‘Vintage’ leaves. Adult aphids were starved in a Petri dish for 1 h to be consistent with the 1 h handling time aphids underwent in the electronic monitoring protocol (see below), then placed individually on a tri-foliate leaf cutting from a resistant or susceptible plant. Leaf cuttings were obtained by excising the leaf with a razor blade and immediately submerging the petiole in water, which reduces the likelihood that the resistance properties are lost (Kennedy and Schaefers 1974c). The aphids’ position (on top of or under leaf, on stem, desertion of plant) and activity (walking, settled) was recorded every 5 min for 1 h. Observations on aphids in each treatment were replicated 15 times.

***Electronic monitoring*.** The EPG system used for this study was the AC-DC EPG developed by Backus and Bennett (2009). Prior to the beginning of monitoring, adult aphids were starved for 1 h, during which time the aphids were immobilized and attached to the insect electrode via a 1-2 cm long, 25.4 µm diameter gold wire using silver conductive glue (1 part school glue: 1 part water: 1 part silver flake by weight). A second copper electrode was inserted into the soil at the base of the plant. Direct current (DC) signal (40 mV) was applied to the plant and data was collected using a giga-Ohm (10^9) input resistor. The data sample rate was 100 Hz. EPG recordings were acquired using a DI-710 and Windaq Acquisition Software (Dataq Instruments Inc., Akron, OH).

Recordings began every evening at 1800 h and lasted for 12 h. Recordings were conducted overnight to reduce interference resulting from lab activities during the daytime. The plants and aphids were set up in the laboratory in a metal Faraday cage to reduce extraneous electrical noise. The temperature ranged from 20-24°C and ambient light was provided. Each aphid and plant was used in only one recording. Electronic monitoring for each insect-plant combination was replicated 20 times for each treatment. Waveform data was imported into The Observer XT version 10.0 (Noldus Information Technology, Wageningen, The Netherlands) and each waveform event (an uninterrupted performance of a behavior during a probe) was coded as one of the common DC system aphid waveform names (Tjallingii 1988). The waveforms scored were as follows: non-probing, C, potential drops, G, F, E1, and E2. Waveform C encompasses waveforms A, B & C which represent initial stylet penetration, sheath salivation, and intercellular movement through the epidermis and mesophyll, respectively. These behaviors are commonly referred to as pathway phase and were scored together for simplicity. Potential drops (pd) represent intracellular punctures made by the stylets as they travel between parenchyma and mesophyll cells. Waveform G is correlated with ingestion from xylem. Together E1 and E2 comprise phloem phase; E1 represents salivation into the phloem sieve elements and E2 represents ingestion from the phloem sieve elements. The waveform F, signifying penetration or stylet difficulties by the aphid, was rarely observed

(n = 3 on ‘Vintage’, n = 2 on ‘Tulameen’; $P>0.1$; data not shown) and was pooled with pathway behaviors because it was typically bordered on both sides by the C waveform.

***Statistics*.** Statistical variables for EPG, whose names were adapted from Backus *et al.* (2007), were calculated using an automated Excel workbook (Sarria *et al.* 2009). All variables concern the durations or numbers of behaviors, either as a mean per insect or a mean of a mean, (i.e. mean per event per insect) because each insect was a statistical unit. Individual aphids that did not perform a certain behavior were excluded. Differences in EPG variables between ‘Vintage’ and ‘Tulameen’ were tested using ANOVA mixed models (PROC GLIMMIX), with treatment as a fixed factor and the night tested (i.e. block) as a random factor. Degrees of freedom were calculated with the Kenward-Rogers adjustment as recommended for use in mixed models by Littell et al (2006). The GLIMMIX models were iteratively optimized using the protocol of Littell *et al.* (2006). Differences between treatments were calculated using Wald’s F test. For the aphid settling observations, differences in aphid position (top or underside of leaf, or off leaf) at each 5 min time interval were tested with a Chi-square analysis, and the number of times an aphid changed locations on the leaf was tested with an ANOVA (PROC GLIMMIX). All statistics were conducted using SAS 9.2.3 (SAS Institute 2008) with $\alpha = 0.05$.

Results

***Aphid performance on ‘Tulameen’*.** No aphid colonies formed on ‘Tulameen’ plants in the greenhouse in any of the three replicates, whereas aphids on the corresponding ‘Vintage’ plants formed colonies of over 50 aphids on each plant. ‘Tulameen’ was thus considered to be resistant to the aphid clones used for the EPG study. Additional observation of *A. agathonica* derived from the same clones on ‘Tulameen’ in a no-choice situation also failed to maintain a colony (M.D., unpublished data).

***Aphid settling behavior*.** There were no significant differences in aphid position or movement on ‘Tulameen’ leaf cuttings versus ‘Vintage’ leaf cuttings. At each 5 min time

interval, there was no significant difference in the number of aphids that moved to the top or bottom of the leaf ($P > 0.1$ for each interval). At 1 h, 80% of aphids on resistant leaves and 72% of aphids on susceptible leaves had moved to the underside of the leaf and settled with their rostrum against the leaf and held their antennae back, an indication of settling. There was no difference in the number of times aphids changed location on the plant (susceptible = 2.0 ± 0.58, resistant = 1.8 ± 0.39; F = 0.08; df = 1,27; $P > 0.5$). Only one aphid on a susceptible plant deserted the leaf altogether.

***Electronic monitoring*.** Three aphids on 'Vintage' and two aphids on 'Tulameen' did not probe during the entire recording period; data from these aphids were discarded from analyses. The proportion of recording time that aphids spent performing different feeding behaviors is shown in Fig. 4.1; when combined, all probing behaviors accounted for only 38% of the time spent on 'Tulameen' compared with 56% on 'Vintage'. Accordingly, the mean duration of probing per insect was significantly lower on 'Tulameen' (Table 4.1). Despite this, the number of probes per insect was greater on 'Tulameen', including a greater number of short probes (less than 3 min in duration) (Table 4.1). There were no differences in the number of xylem ingestion events or the duration of xylem ingestion between the two cultivars (Table 4.1; waveform G).

There were no differences in the mean durations (per event or per insect) of pathway behaviors; nor were there differences in the mean potential drops per insect (Table 4.1; waveform C, pd). In contrast, there was a significantly higher number of pathway waveform events per insect on 'Tulameen' (Table 4.1). Aphids on 'Tulameen' were able to reach the phloem more quickly than aphids on 'Vintage' (Table 4.1; variable shortest duration C before E1).

There were significant differences in the variables relating to phloem phase behaviors. For example, there was no difference in the number of aphids that reached the phloem, with 10 of 18 aphids on resistant plants and 12 of 17 on susceptible plants reaching the

sieve elements (χ^2= 0.85, df = 1, *P*> 0.1). However, aphids spent 77% of phloem phase salivating when they were on ‘Tulameen’, while aphids on ‘Vintage’ spent only 17% of the time salivating (Fig. 3. 2). Aphids on ‘Tulameen’ were significantly more likely to perform a single salivation event, withdraw from the sieve elements without ingesting phloem sap, and not re-enter a sieve element in that probe (Table 4.1; single E1). Additionally, the mean durations of phloem salivation, both per event and per insect, were significantly higher on ‘Tulameen’ (Table 4.1).

On ‘Vintage’, 12 out of 17 aphids ingested from the phloem, while significantly fewer aphids (4 out of 18) feeding on ‘Tulameen’ were able to successfully do so (χ^2= 6.61, df = 1, *P* = 0.011). An average aphid was significantly less likely to engage in sustained phloem ingestion over 10 min long on ‘Tulameen’ (Table 4.1; sustained E2). The mean durations of phloem ingestion, both per event and per insect, were also significantly greater than on ‘Vintage’ than on ‘Tulameen’ (Table 4.1; waveform E2).

Discussion

The differences in feeding behaviors recorded between aphids on ‘Tulameen’ and ‘Vintage’ largely appear to be confined to factors within the phloem sieve elements. Aphids on ‘Tulameen’ salivated for much longer into phloem sieve elements and often did not initiate phloem sap ingestion. One role of aphid salivation into the phloem is to prevent sieve tube occlusion through the release of Ca^{2+} binding proteins (Will *et al.* 2009). An induced defense by the raspberry plant may play a role in preventing the proteins contained in aphid saliva from interfering with callous deposition and wound repair (Tjallingii 2006). In the aphid-resistant melon line ‘TGR-1551,’ *Aphis gossypii* displayed extremely long salivation durations, often without entering into passive phloem ingestion (i.e. longer than 10 min) (Garzo *et al.* 2002). ‘TGR-1551’ resistance is controlled by the *Agr* gene, which belongs to a group of NBS-LRR resistance genes important in plant defenses against diseases and wounding (Garzo *et al.* 2002). A similar

phenomenon may be occurring in raspberry; thus, future studies of Ag_1 should include identifying the class of protein that the gene encodes.

This study did not find evidence of a resistance mechanism located outside of the phloem (Sarria *et al.* 2009), based on the EPG waveforms for 12 h, as well as the observed behavior of aphids on excised leaves for 1 h. In the settling study, aphids were just as likely to move to the underside of the 'Tulameen' leaves and begin probing as were aphids on 'Vintage' leaves, suggesting that a pre-penetration factor is not a significant deterrent to probing. Additionally, there was no difference in the amount of time spent in pathway activities or the duration of potential drops. In fact, aphids feeding on resistant plants reached the phloem in a significantly shorter time than those on susceptible plants (Table 4.1).

Past research has suggested that one mechanism for resistance may be that the phloem sap is nutritionally deficient for *A. agathonica* survival (Kennedy and Schaefers 1975). Their evidence was that, while aphid stylets reached the phloem sieve elements on resistant plants, the honeydew collected and analyzed was much more dilute than from aphids feeding on a susceptible control. The data from our study also suggests that aphids were able to easily locate the phloem; however, instead of ingesting, more time was spent salivating and aphids were unable to engage in sustained phloem sap ingestion. A possible explanation for the dilute honeydew measured by Kennedy and Schaefers (1975) is that their aphids may have engaged in xylem ingestion because the aphids were caged on the resistant plant for four days before the honeydew was analyzed. Xylem sap is a very dilute source of nutrition, yet xylem ingestion is common in aphids that are starved and may serve as an easy-to-access water source (Powell and Hardie 2002). While the aphids in our study rarely engaged in xylem ingestion (1-2% of the 12 h study time), they might have engaged in this behavior more if they had been EPG-monitored for longer.

Vector resistance is one of the best methods for controlling plant viruses, but the location of the resistance mechanism is important in determining how the spread of different types of viruses will be affected. Non-persistent viruses could be acquired and inoculated by *A. agathonica* on Ag_1resistant plants because aphids will readily probe. Additionally, spread may increase because the aphids may become restless and desert the unsuitable host in search of another. The main viruses currently of concern (RLMV, RpLV, RYNV and BRNV) are phloem-limited and transmitted both semi-persistently and persistently, whereas non-persistent viruses are not a research focus.

Because aphids feeding on Ag_1-resistant plants are able to access and salivate into phloem sieve elements, it is probable that they could inoculate plants with both semi-persistent and persistent viruses, should they be viruliferous. However, because there is very little phloem ingestion occurring on Ag_1 resistant plants, common strains of *A. agathonica* are inefficient at acquiring persistent or semi-persistent viruses from an infected aphid-resistant plant. This hypothesis is supported by Stace-Smith (1960) who found that aphids feeding on resistant raspberry plants infected with BRNV, a semi-persistent virus, were unable to acquire and inoculate the virus to aphid-susceptible indicator plants efficiently. Also, because aphids do not develop colonies on resistant plants, there would be little secondary virus spread within a field.

The interactions between plant defenses and aphid saliva are poorly understood in many systems, including raspberry. Further research into these interactions would help explain why *A. agathonica* salivation apparently was unsuccessful in overcoming phloem sieve element defenses (Will *et al.* 2009), preventing initiation of phloem sap ingestion. Future research should also focus on the mechanism of resistance in raspberry containing resistance genes Ag_2 and Ag_3, as well as resistance genes recently reported in black raspberry (Daubeny and Stary 1982, Dossett and Finn 2010). Thorough knowledge of the mechanism of each gene will facilitate breeding of cultivars that pyramid multiple

defensive mechanisms to slow the advance of new *A. agathonica* biotypes and maintain the effectiveness of resistant red raspberries for virus control.

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Table 4.1. Mean and standard error of pathway and xylem behaviors performed by *A. agathonica* on resistant 'Tulameen' and susceptible 'Vintage' red raspberry. Durations are reported in minutes. Means followed by different letters are significantly different .

Parameter	**'Tulameen'**	**'Vintage'**	**df**	***P* value**
Duration of probing per insect	286.37 ± 47.53a	416.80 ± 48.10b	1, 21.06	0.0112
Number of probes	7.77 ± 1.27a	5.17 ± 0.90b	1, 33	0.0045
Number of short probes[a]	2.87 ± 0.78a	1.81 ± 0.53b	1, 33	0.0361
Number of G events[b]	0.32 ± 0.14	0.12 ± 0.08	1, 33	0.2310
Duration of G per event[c]	55.08 ± 24.71	84.82 ± 38.05	1, 3.98	0.6226
Duration of G per insect[d]	68.90 ± 34.39	90.53 ± 45.19	1, 4.16	0.7743
Number of C events[b]	10.23 ± 1.38a	6.14 ± 0.92b	1, 14.38	0.0042
Duration of C per event[c]	26.77 ± 5.02	29.49 ± 5.53	1, 33	0.7513
Duration of C per insect[d]	196.27 ± 33.70	146.24 ± 25.57	1, 22.32	0.1709
Number of pd events[b]	96.95 ± 28.94	71.84 ± 21.63	1, 11.73	0.4448
Shortest Duration of C event before E1	39.67 ± 0.71a	61.33 ± 1.04b	1, 20	0.0335
Number of E1 events[b]	1.86 ± 0.60	3.00 ± 0.92	1, 20.04	0.2953
Number of single E1 probes[e]	1.15 ± 0.33a	0.27 ± 0.13b	1, 33	0.0071
Duration of E1 per event[c]	14.23 ± 3.13a	4.16 ± 0.91b	1, 20	0.0030
Duration of E1 per insect[d]	52.53 ± 10.61a	19.49 ± 3.94b	1, 20	0.0232
Number of E2 events[b]	0.48 ± 0.24a	2.23 ± 0.85b	1, 25.92	0.0210
Number of sustained E2 events	0.32 ± 0.16a	1.89 ± 0.60b	1, 15.79	0.0078
Duration of E2 per event[c]	18.91 ± 7.12a	104.58 ± 39.42b	1, 14	0.0114
Duration of E2 per insect[d]	23.75 ± 45.29a	312.08 ± 41.35b	1, 20	0.0001

[a]Defined as probes less than 3 min in duration
[b]Waveform events; defined in text
[c]Waveform duration per event per insect; Backus *et al.* 2007
[d]Waveform duration per insect; Backus *et al.* 2007
[e]Defined as a single E1 event in a probe, without entering E2 or another phloem sieve element

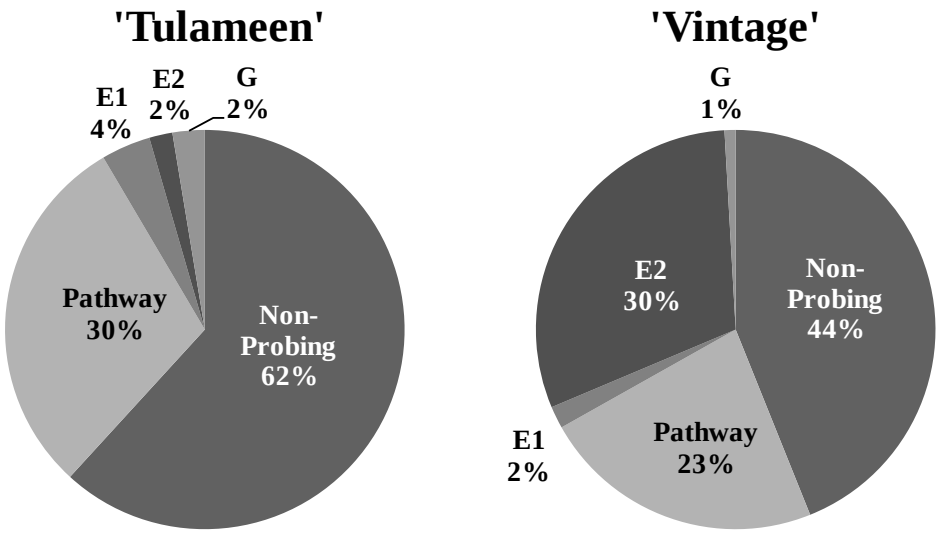

Figure 4.1. Proportion of time (%) spent performing each feeding behavior by aphids on resistant 'Tulameen' (n=18) and susceptible 'Vintage'(n=17) during 12 h of EPG monitoring. Waveform definition: non-probing – stylets withdrawn from plant; pathway – stylet activities in epidermis & mesophyll including cell punctures (potential drops); E1 – salivation into phloem sieve elements; E2 – ingestion from phloem sieve elements; G – ingestion from xylem.

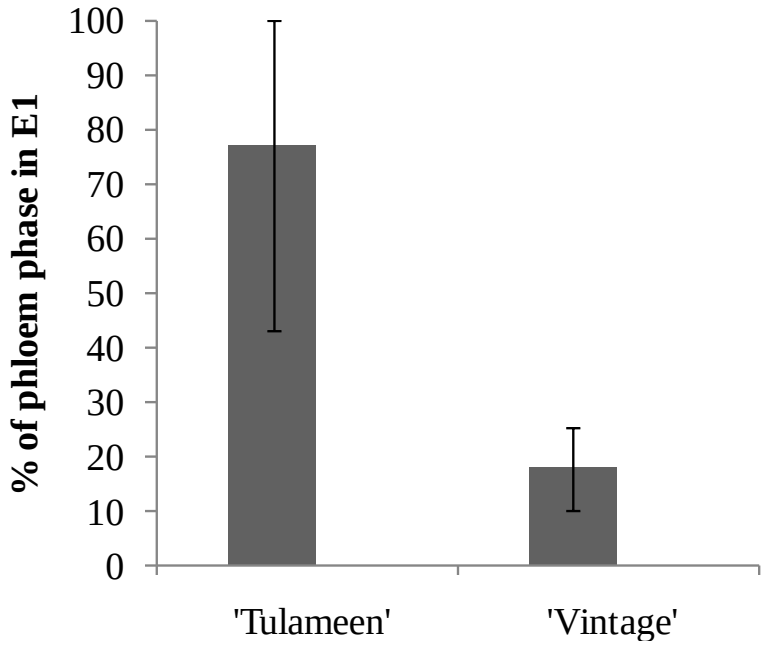

Figure 4.2. The percentage of the phloem phase (E1 + E2) spent salivating (E1) by aphids on susceptible ‘Vintage’ and resistant ‘Tulameen’ plants. Aphids on ‘Tulameen’ spent a significantly greater proportion of time salivating ($P = 0.024$). Waveform definition: E1 –salivation into phloem sieve elements; E2 – ingestion from phloem sieve elements.

Chapter 5:

Effects of three novel resistant black raspberry selections on *Amphorophora agathonica* feeding behavior and performance

Danielle Lightle, Michael Dossett, Tim Ebert, Chad Finn, and Jana Lee

Abstract

Host plant resistance is an effective and inexpensive management approach for insect pests. Recently, three new sources of resistance (ORUS 3778-1, ORUS 3817-1, and ORUS 4109-1) against the aphid *Amphorophora agathonica* were identified in black raspberry. We studied stages of host plant acceptance: host plant attraction, parturition (deposition of nymphs), nymph survival, and feeding behavior, to identify the location of the plant resistance mechanism. Aphids were more attracted to the susceptible control than to ORUS 3778-1 and ORUS 3817-1, while equally attracted to the control and ORUS 4109-1. Parturition occurred on the resistant selections, but fewer nymphs were deposited on resistant lines relative to the susceptible control. Nymphs survived only an average of 3.3-3.6 days on resistant selections. There were differences in feeding behavior between the susceptible control and the resistant selections, but no differences between the three resistant selections. The tissue responsible for resistance appears to be the phloem sieve elements. Aphids had a reduced probability of salivation into the phloem sieve elements, and only one aphid each on ORUS 3778-1 and ORUS 4109-1 successfully ingested from the phloem. Because feeding behavior of *A. agathonica* did not differ between resistant selections, independent confirmation that resistance is conferred by unique genes should be obtained before pyramiding these sources together.

Introduction:

Plant resistance is considered to be an inexpensive and effective method for control of insect pests, and is often one of the few options available for control of vectors of virus diseases (van Emden 2007). Resistant plants may act through antixenosis (lack of colonization), antibiosis (lowered colony growth), or tolerance (no economic losses caused by pest). Antixenotic mechanisms may act against the insect at several stages during the colonization process. Resistance located at the plant surface, such as waxy leaves or surface cues, may decrease the attraction of aphids to these plants relative to a susceptible cultivar (Shepherd *et al.*1999). Mechanisms located in the plant tissues may be a result of different chemical composition that results in non-preference or toxicity

(Givovich and Niemeyer 1995); mechanical difficulties in maneuvering through the plant tissue, for example, through increased levels of callose (Shinoda 1993); or inability to ingest from the phloem sieve elements (Tjallingii 2006).

Amphorophora agathonica Hottes, or large raspberry aphid, is an important pest in red and black raspberry (*Rubus idaeus* L. and *R. occidentalis* L.) cropping systems in the Pacific Northwest region of North America. The primary concern of *A. agathonica* is its ability to vector several viruses of economic importance. In red raspberry, *A. agathonica* is the primary vector of the raspberry mosaic virus complex and the crumbly fruit disease complex, while in black raspberries, severe field decline has been attributed to *Black raspberry necrosis virus* (BRNV) (Martin *et al.*2013; Halgren *et al.*2007). While at least seven sources of resistance to *A. agathonica* have been identified in red raspberry (Daubeny and Stary 1982, Dossett and Kempler 2012, Daubeny 1966), the only sources which appear to remain effective against all seven biotypes have yet to be fully characterized (Dossett and Kempler 2012).

Three selections of black raspberry (ORUS 3778-1, ORUS 3817-1 and ORUS 4109-1) with strong antixenotic resistance against *A. agathonica* were recently identified (Dossett and Finn 2010). Because these are the only known sources of resistance against *A. agathonica* in black raspberry, maintaining durability of these genes when they are deployed in the field is a priority. Multiple sources of resistance against *A. agathonica* in red raspberry have been broken, resulting in multiple aphid biotypes (Dossett and Kempler 2012). One technique for preventing pests from overcoming resistance sources is to pyramid resistance genes that confer different mechanisms of resistance, making it less likely that the insect pest will be able to overcome both types at one time (Porter *et al.*2000). Thus, knowledge of the mechanism of resistance against *A. agathonica* in these selections will aid in maintaining their durability in the field.

The objectives of this study were to characterize how the newly identified resistance sources in the black raspberry selections ORUS 3778-1, ORUS 3817-1, and ORUS 4109-1 affect the behavior of *A. agathonica* at various stages of host acceptance (Fig. 5.1). We examined host plant acceptance through attraction and settling assays, induction of parturition (deposition of nymphs), and nymph survival. Additionally, we used the electrical penetration graph (EPG) to examine differences in feeding behavior on resistant and susceptible plants to identify the plant tissues important for resistance. The electrical penetration graph (EPG) has been a valuable tool for localization of the plant tissues responsible for resistance against piercing-sucking insects such as aphids (Van Helden and Tjallingii 2000). The EPG system works by wiring the plant and insect into an electrical circuit and applying a small DC current to the plant. When the insect stylets are inserted into the plant tissue, the circuit is completed; the resulting change in voltage over time, known as waveforms, have been correlated to specific feeding behaviors in different plant tissues (Walker 2000, Tjallingii and Hogen Esch 1993, Tjallingii 2006). Changes in feeding behaviors on resistant plants relative to the feeding observed on a susceptible control helps to reveal the plant tissue(s) in which the resistance mechanism is located.

Materials and methods:

Plants and aphids. The three recently identified aphid-resistant black raspberry selections were ORUS 3778-1, ORUS 3817-1, and ORUS 4109-1 (selections available from the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR; PI 658505, PI 658506 and PI 659143, respectively). All plants were propagated in tissue culture, and approximately 35 plants of each selection were planted individually in 10 cm pots. Plants were maintained in a greenhouse with temperatures set at 22°C day and 17°C night and a 16 hr photoperiod. Plants were trimmed back frequently to maintain fresh tissue growth. Each selection tested negative for the presence of common aphid transmitted viruses (*Raspberry leaf mottle virus*, *Raspberry latent virus*, and *Black raspberry necrosis virus*); however all ORUS 4109-1 were infected with *Raspberry bushy dwarf virus* (RBDV,

pollen-borne). The susceptible Munger, a standard commercial cultivar, was used for comparison. All ‘Munger’ plants were obtained from North American Plants (McMinnville, OR) as small plugs from tissue culture. Plugs were transplanted into 10 cm pots and maintained in identical conditions as the resistant selections.

The aphid colony was begun from multiple adult *A. agathonica* collected from commercial raspberry fields in Whatcom Co., Washington, USA. Because of the potential for genetic drift of *A. agathonica* reared continuously in colony (e.g. decreased acquisition of aphid transmitted viruses, personal observation), aphids were collected twice per year from the field and the colony was restarted. Thus, the aphids used for the feeding behavior study were collected in September 2011, and the aphids used for all remaining bioassays were collected in June 2012. Colonies were not clonal and contained a mix of aphid biotypes A and B (Dossett and Kempler 2012). Aphids were reared on ‘Meeker’ red raspberry in a growth chamber set at 22°C and a 16 hr photoperiod. Plants were watered as needed and replaced weekly to maintain high plant quality.

Short term attraction assay. The assay design was modified from Srinivisan *et al.* (2006) and Castle *et al.*(1998). Two treatment plants, one ‘Munger’ and one resistant plant (ORUS 3778-1, ORUS 3817-1, or ORUS 4109-1) were placed on opposite sides of the test arena, which consisted of a 14 cm Petri plate placed on a stage (Fig. 5.2a). On each side of the plate, the youngest fully expanded leaflet from the test plant was inserted and held into place using parafilm. Leaves were not detached from the test plant. All possible exits from the arena were sealed off using parafilm.

Twenty late instar nymphs and adult *A. agathonica* were held in a small Petri plate for 1 hr prior to the beginning of the assay. After 1 hr the aphids were added to the edge of the test arena, equidistant from the two test leaves. Aphids were free to walk on and probe the test leaves. The number of aphids on each leaf was counted at 10 min intervals for 2 hr, for a total of 12 observations. The study was replicated 15 times per resistant

selection, using a new plant pair for each replicate. The proportion of aphids selecting a leaf over time was compared using a repeated measures generalized linear mixed model with a binomial distribution (PROC GLIMMIX, SAS 9.3.2).

Settling assay. One plant of each treatment ('Munger' and the three resistant selections) was randomly placed around the test arena (Fig. 5.2b), which consisted of a 14 cm Petri plate with a 16 mm hole drilled into the center to accommodate a 15 mL conical tube. The top of the 15 mL tube was flush with the bottom of the arena. The youngest fully expanded leaf of each plant was inserted into the arena equidistant from each other and held into place using parafilm. The test leaves remained attached to the plant. All possible exits from the arena were sealed using parafilm.

Thirty late instar and adult aphids were added to the 15 mL conical tubes. Aphids were not starved beyond the handling period required to count them, approximately 0.5 hr. The tube was inserted into the test arena and uncapped. Aphids were able to climb upwards from the tube into the test arena, and free to walk on and probe each leaf. Aphids remained in the arena for 18 hr. At the end of the assay, the test leaves were removed from the plants and the numbers of aphids on the leaf of each treatment plant were counted. The assay was replicated 20 times using different plants for each replicate. The proportion of aphids that settled on each leaf was compared using a generalized linear mixed model with a binomial distribution (PROC GLIMMIX). Differences between treatments were tested using differences in least-squared means, with a Tukey-Kramer adjustment for multiple comparisons.

Parturition assay: A cohort of aphids was obtained by isolating reproductively mature aphids on a 'Meeker' red raspberry leaf in a Petri dish for 24 hr. After 24 hr, the nymphs were collected and placed on an uninfested 'Meeker' plant isolated in a growth chamber. When the aphids reached reproductive maturity, determined by the presence of new aphid nymphs on the plant, the adult aphids were caged individually to either one of the three

resistant black raspberries or 'Munger' using a 15 mm diameter clip cage. Cages were supported using wooden stakes to reduce stress to the petiole. At each 24 hr interval for three days, the number of nymphs laid by each aphid was recorded and nymphs were removed from the clip cage. The assay was replicated three times, with seven aphids per treatment per replicate. The number of nymphs deposited each day was compared using a repeated measures generalized linear mixed model fit with treatment, time and the treatment x time interaction (PROC GLIMMIX).

Nymph survival: A cohort of 1st instar nymphs was obtained by isolating reproductively mature adults as described above. After 18 hr, all nymphs were collected and clip-caged individually to either one of the resistant selections or 'Munger'. Nymphs were checked every 24 hr and the number that died were recorded until no more nymphs survived on the resistant plants. The assay was replicated three times, with six aphids per treatment per replicate. The number of days nymphs survived in each treatment was analyzed using a Kruskall-Wallace one way ANOVA (PROC NPAR1WAY). Significant differences between treatments were analyzed pairwise using a Wilcoxon rank-sum test with a Bonferroni correction to control experiment-wise error rates in multiple comparisons.

Electronic monitoring of feeding. The feeding behavior of aphids using EPG was conducted as described in Lightle *et al.*(2012), using the AC-DC EPG system (Elaine A. Backus and Bennett 2009). Adult aphids were starved for 1 hr, during which time they were attached to an insect electrode using a 2 cm length of 25.4 µm-diameter gold wire with silver conductive glue (1:1:1 by weight, school glue: silver flake: water). Study plants had a copper electrode inserted into the soil at the base of the plant. The plant/aphid system was placed in a metal Faraday cage to reduce extraneous electrical noise. A 40 mV direct current (DC) signal was applied to the plant, and data were collected using a giga-Ohm (10^9) input resistor. Recordings were acquired at a 100 Hz sample rate using a DI-710 and Windaq Acquisition Software (Dataq Instruments Inc., Akron, OH). Recordings began at 18:00 and lasted for 12 hr. Four recordings were done

each night, using one plant of each resistant selection and the susceptible control, 'Munger'. The study was replicated 23 times, with each plant used for only one recording.

Recordings were imported into The Observer XT (Ver. 10, Noldus Information Technology, Wageningen, The Netherlands) and scored for the duration of the common aphid waveform behaviors (Tjallingii 1988). The behaviors scored were: Pathway (C; salivation and formation of the stylet sheath in the epidermis and mesophyll), potential drops (PD; cell punctures during which salivation and gustatory tasting occur), stylet derailment (F; stylet penetration difficulties, may occur in any tissue), xylem ingestion (G; ingestion from the xylem elements), phloem salivation (E1; salivation into the phloem sieve elements), and phloem ingestion (E2; ingestion from the phloem sieve elements). Analysis of EPG waveforms used sequential and nonsequential variables, whose names were from Sarria *et al.*2009. These variables were calculated from the raw data using a program in SAS written to mimic the output from an Excel program (Sarria *et al.*2009). The SAS program was validated using the Sarria workbook, and the results have been checked against the results of that workbook. The advantage of the SAS program was that it allowed calculation of the variables and subsequent analysis of those variables as a single process. EPG variables were analyzed using generalized linear models (PROC GLIMMIX, SAS 9.2). GLIMMIX were run with a Kenward-Rogers degree of freedom correction and iteratively optimized (Littell *et al.*2006). Differences between treatments were tested using least square means.

Results:

Plant preference. The short term settling preference of aphids choosing between susceptible 'Munger' and each resistant selection are shown in Fig. 5.3. Time was not significant in the host preference of aphids in any assay. When the resistant hosts were ORUS 3778-1 or ORUS 3817-1, aphids significantly preferred to settle on 'Munger' leaves ($F_{1,14} = 6.14$, $p = 0.03$ and $F_{1,14} = 4.86$, $p = 0.04$, respectively). However, there was

no significant difference in the proportion of aphids that selected between ‘Munger’ and ORUS 4109-1 ($p = 0.2$). After 18 hr, aphids exposed to all four treatments significantly preferred to settle on ‘Munger’ ($F_{3,54} = 11.78$, $p < 0.001$; Fig. 5.4). However, a small proportion of aphids did remain on each resistant selection.

Parturition and nymph survival. There was no interaction between treatment and time in the number of nymphs that adults deposited, so the interaction term was removed from the model. Adult aphids deposited significantly more nymphs on days 2 and 3 than on day 1 in all treatments ($F_{3,80} = 14.37$, $p < 0.01$). However, aphids feeding on ‘Munger’ deposited significantly more nymphs than aphids on any of the resistant selections ($F_{1,183} = 20.06$, $p < 0.01$, Table 5.1).

The survival of nymphs was significantly lower on resistant plants than on ‘Munger’ (Table 5.1). Monitoring of aphids was discontinued at day 11, when the nymph mortality on all resistant selections was 100%, while 94% of nymphs on ‘Munger’ were still alive.

Electronic monitoring of feeding. Some aphids did not probe throughout the duration of the experiment, but the number of non-probing aphids did not differ between treatments. The non-probing aphids were removed from further analysis, and the final numbers analyzed were 22, 20, 19 and 21 aphids in ORUS 3778-1, ORUS 3817-1, ORUS 4109-1, and ‘Munger’ treatments, respectively.

There was no difference in the amount of time that elapsed before aphids first probed on a plant ($F_{3,76} = 0.51$; $p = 0.67$). The total and mean duration of probing per insect in pathway behaviors (C) was not significantly different on resistant and susceptible plants. However, aphids feeding on any of the resistant selections spent a higher percentage of the total probing time in pathway behaviors compared to aphids on ‘Munger’ (percent of probing time in C, Table 5.2). Among the pathway behaviors, there was no difference in the average duration or total number of potential drops per insect between any treatments.

Significantly fewer aphids feeding on resistant plants engaged in phloem salivation (E1) than aphids on 'Munger' ($\chi^2 = 10.4$, $p = 0.015$). On the resistant selections, only 7, 8, and 7 aphids on ORUS 3778-1, ORUS 3817-1, and ORUS 4109-1, respectively, engaged in E1, compared to 16 aphids feeding on 'Munger'. The mean duration of E1 per insect did not differ between treatments (Table 5.2). However, aphids feeding on resistant plants were unlikely to ever ingest from the sieve elements (phloem ingestion, E2). Only one aphid feeding on ORUS 3778-1 and on ORUS 4109-1 successfully ingested from the phloem, while no aphids feeding on ORUS 3817-1 ingested. By comparison, 14 aphids of the 16 that engaged in E1on 'Munger' successfully ingested from the phloem. Because few aphids engaged in E2 on resistant plants, no further analyses were able to be done with ingestion behaviors (Table 5.3).

Aphids on resistant plants were marginally more likely to engage in xylem ingestion (G) than aphids on susceptible plants (No. of G, Table 5.2). Aphids on resistant plants ingested from the xylem for at least twice as long as aphids on 'Munger' (Total Dur. of G, Table 5.2). Stylet penetration difficulties (F) were rarely observed on any of the plants tested.

Discussion

Host plant acceptance by aphids includes a number of successive behaviors, including attraction, parturition, and sustained phloem ingestion (Powell *et al.*2006, Fig. 5.1). First, aphids must be attracted to the plant. Alate aphids likely use a combination of visual cues, such as color, and leaf volatile cues in the field (reviewed in Pettersson *et al.*2007). Aphids in our choice studies were equally attracted to 'Munger' and ORUS 4109-1 in the short term attraction assay, while preferring 'Munger' over the resistant selections ORUS 3778-1 and ORUS 3817-1. Aphids also preferred 'Munger' after 18 hr in a four-way choice test; however, only 40% of the aphids settled on 'Munger' while the remaining 60% were roughly evenly distributed between the three resistant options. Thus, while

‘Munger’ was preferred in most cases, there was not a clear repellent factor, such as a volatile or leaf surface cue, that discouraged settling on the resistant plants entirely. A second step in the host acceptance process is the induction of parturition. Tosh *et al.* (2002) showed that aphids will begin to deposit nymphs shortly after probing in the mesophyll tissues of the plant, and well before the phloem ingestion stage has been reached on a potential host plant. While it appears to be counter-intuitive to begin reproduction on a potentially unsuitable host, early reproductive decisions may translate into a large fitness advantage to parthenogenic species such as aphids (Mackenzie and Guldemond 1994, Tosh *et al.*2002). The exact cues used by aphids to decide whether or not to induce parturition are unknown, though they may comprise of primary or secondary metabolites (Powell *et al.*2006). Those cues appear to have been present in the three resistant black raspberries studied, as aphids did deposit nymphs on resistant plants, although at a lower rate than aphids on the susceptible ‘Munger’. Resistant plants proved to be unsuitable hosts for aphid nymphs, as the nymph mortality study showed that nymphs survived an average of only 3.3 to 3.6 days on resistant plants.

The last stage in plant acceptance is successful feeding. Early pathway behaviors occur in the epidermis and mesophyll tissues of the plant, and include secretion of gelling saliva to create a stylet sheath pathway, as well as numerous intercellular punctures, where it is hypothesized that aphids ingest a small amount of the cell sap content (Tjallingii 2006). In the three resistant black raspberries tested, there is little evidence that a resistance mechanism is located at the surface of any of the resistant plants as aphids did not take longer to make an initial probe on a plant.

Results suggest a mechanical impediment located in the mesophyll of these resistant plants. Aphids on all three resistant selections made significantly more test probes (short probes < 3 min long) than on ‘Munger.’ Many short test probes might indicate the presence of a repellant or distasteful chemical (Campbell *et al.*1986). Because gustatory analysis occurs during potential drops, a change in potential drop frequency may indicate

a toxin or distasteful element. However, repellency may not be occurring in this study because the potential drop frequency or duration was not different among susceptible and resistant plants. Instead, the presence of a mechanical barrier may explain why more short probes were made without affecting potential drops. Mechanical barriers, such as increased callose or methylated pectin in the middle lamella, have been observed in resistant melon and sorghum (Shinoda 1993, Dreyer and Campbell 1987).

Results also suggest a resistance factor is located in the phloem sieve elements. Aphids were less likely to salivate in the sieve elements (E1) in resistant plants, and we postulate that aphids probably could reach but did not always recognize the sieve elements. Aphids probably could reach the sieve elements on resistant plants, as aphids on resistant plants did not take longer in pathway behaviors prior to engaging in E1 than aphids feeding on 'Munger' (Dur. of shortest C before E1, Table 5.1). In fact, aphids feeding on 'Munger' took marginally longer to reach E1 within a probe than aphids on the resistant plants (p = 0.06). One explanation for this behavior is that the aphids were unable to recognize that the vascular bundle had been reached (Tjallingii 2006, Alvarez *et al.*2006). Aphids engaging in E1 do not represent the first time that aphids encounter the sieve elements, as aphids puncture the sieve elements during pathway behaviors (Tjallingii and Hogen Esch 1993). The exact cues aphids use to determine that a sieve element has been punctured or is an acceptable feeding cell are unknown, but may involve pH or sugar composition (Hewer *et al.*2010; 2011). Failure to recognize the sieve element may account for the decreased incidence of E1 on resistant plant treatments.

Further evidence that resistance resides in the phloem sieve elements is that only two aphids on resistant plants successfully ingested, out of 22 aphids across the three resistant treatments that salivated. Failure to transition into ingestion is commonly seen in plants with a phloem-based resistance factor (Tjallingii 2006, Lightle *et al.*2012). Aphids must overcome the plants' defense system, for example sieve plate occlusion, which seeks to repair the puncture wound to the sieve elements through various proteins and callose

deposition (Will *et al.*2009, Walling 2008). Interestingly, aphids attempting to overcome a plants' defense response will frequently salivate for a significantly longer period of time before withdrawing the stylets (e.g. Lightle *et al.*2012, Tjallingii 2006, Garzo *et al.*2002) but extended salvation was not observed in this study.

Previously, Dossett and Finn (2010) identified these three black raspberry selections as new sources of resistance against *A. agathonica,* though it was not possible to attribute the resistance to three different genes. This study revealed no significant differences in the feeding parameters between aphids on any of the resistant selections. In a short term attraction assay, aphids selected ORUS 4109-1 as often as 'Munger'; however, ORUS 4109-1 was infected with *Raspberry bushy dwarf virus* (RBDV). Virus-infected plants are known to have an impact on aphid behavior, with aphids frequently being more attracted to infected plants over healthy ones through visual changes and volatile profiles (Fereres and Moreno 2009, McMenemy *et al.*2012). While studies have focused on the interactions with aphid-transmitted viruses, a non-aphid-transmitted virus such as RBDV may have a similar effect because RBDV titers increase when co-infected with *Raspberry leaf mottle virus* (Quito and Martin 2012). The preference for 'Munger' over ORUS 3778-1 and ORUS 3817-1 were relatively small and may be a result of other differences between the plants rather than a deterrent due to resistance.

Few sources of effective resistance against *A. agathonica* are available, so preservation of these genes is a priority. In Europe, the closely related aphid *Amphorophora idaei* Börner, which also colonizes raspberry, has successfully broken every source of major gene resistance available (Birch *et al.*2005). A recent study of *A. agathonica* identified seven different biotypes in the Pacific Northwest region, five of which are capable of colonizing plants with the most commonly deployed red raspberry gene Ag_1 (Dossett and Kempler 2012, personal observation). It is therefore extremely likely that deploying each of the black raspberry resistance sources individually will result in new *A. agathonica* biotypes that will rapidly overcome these genes. Because there were no feeding

differences between the resistant treatments, further genetic study will be required to state unequivocally whether the novel resistance in these black raspberry selections differ from each other. However, if genetic analyses confirm that resistance is conferred by different sources, pyramiding the resistance genes may prolong durability in the field.

When reliable resistant plants are developed, these vector resistant plants are one of the most effective management tools for preventing the spread of plant viruses in the absence of resistance to the virus itself (van Emden 2007). A recent study of *Rhopalosium padi* (L) feeding behavior on *Hordeum bulbosum* L. found decreased phloem salivation and ingestion on resistant plants, which also prevented inoculation of *Barley yellow dwarf virus* (Schliephake *et al.*2013). Similarly, these black raspberry selections are likely to be effective in the field at preventing the spread of viruses through decreased acquisition and serial spread, and low colonization due to the high nymph mortality rates. Provided the genes remain durable, these selections have potential for effective virus and vector control.

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Table 5.1. Number of days aphid nymphs survived on resistant black raspberry selections and the number of nymphs adults laid on resistant selections (mean ± SE). Different letters indicate significant differences between treatments.

Treatment	Nymph longevity (d)	Nymphs laid / day
ORUS 3778-1	3.33 ± 0.61	0.35 ± 0.37b
ORUS 3817-1	3.74 ± 0.60	0.52 ± 0.37b
ORUS 4109-1	3.62 ± 0.86	0.63 ± 0.38b
'Munger'	*	1.42 ± 0.37a

* The longevity of nymphs on 'Munger' is not reported because the study was artificially ended when all nymphs died on resistant selections. After 11 days, 94% of the nymphs reared on 'Munger' plants were still alive.

Table 5.2. Number and duration (mins) of the EPG feeding variables (mean ± SE). Variable definitions: E1 – phloem salivation; C – pathway behaviors; PD – potential drops; G – xylem ingestion; see text for detailed explanation.

Parameter	'Munger'	ORUS 3778-1	ORUS 3817-1	ORUS 4109-1	df	F	*P*
No. of E1 events[a]	9.95 ± 2.91a	0.59 ± 0.23b	0.90 ± 0.33b	0.68 ± 0.28b	3,78	17.08	**<0.001**
No. of single E1 events	0.47 ± 0.23	0.45 ± 0.22	0.90 ± 0.24	0.53 ± 0.24	3,78	0.74	0.53
Mean dur. of E1[b]	3.47 ± 0.90	5.14 ± 2.02	7.76 ± 2.86	6.91 ± 2.72	3,34	2.00	0.13
Total dur. of E1[c]	50.86 ± 25.94	13.50 ± 10.41	22.72 ± 16.38	16.15 ± 12.45	3,34	2.41	0.08
Contribution of E1 to phloem phase	25.98 ± 4.50a	87.18 ± 22.95b	100.0b	95.3 ± 24.09b	3,34	10.62	**<0.001**
Total dur. single E1	17.54 ± 15.07	13.81 ± 12.81	24.83 ± 19.96	12.20 ± 10.49	3,24	0.40	0.75
Time from start of recording to first E	264.00 ± 23.38a	612.05 ± 81.97b	439.43 ± 55.03b	478.38 ± 64.06b	3,34	11.98	**<0.001**
Time from start of 1st probe to first E	206.05 ± 40.79a	622.82 ± 186.40c	289.83 ± 81.14ab	438.16 ± 131.13bc	3,34	4.76	**<0.001**
No. of probes to first E1	4.44 ± 0.72a	12.43 ± 2.49b	8.37 ± 1.68b	7.86 ± 1.70b	3,34	5.63	**0.003**
Dur. of shortest C before E1	63.04 ± 8.03	37.68 ± 7.26	48.18 ± 8.68	39.09 ± 7.53	3,34	2.63	0.06
No. of C events	8.14 ± 1.79	13.00 ± 1.75	14.85 ± 1.84	13.00 ± 1.88	3,78	2.53	0.06
% of probing time in C	44.73 ± 4.42a	81.84 ± 7.90b	93.56 ± 9.47b	77.80 ± 8.08b	3,78	11.80	**<0.001**
Total dur. of C	161.80 ± 27.71	167.63 ± 28.05	163.78 ± 28.74	148.71 ± 26.71	3,78	0.09	0.96
Mean dur. of C	24.45 ± 5.01	16.91 ± 3.38	14.38 ± 3.02	15.06 ± 3.24	3,78	1.88	0.14
No. of PD events	133.90 ± 21.17	130.41 ± 21.47	123.05 ± 21.26	119.42 ± 21.18	3,78	0.09	0.96
Mean dur. PD	8.02 ± 0.15	8.14 ± 0.14	8.06 ± 0.15	7.82 ± 0.15	3,78	0.68	0.56
No. of G events	0.09 ± 0.07	0.50 ± 0.15	0.65 ± 0.18	0.63 ± 0.18	3,78	2.28	0.09
Total dur. of G	17.48 ± 12.48	93.49 ± 31.71	44.84 ± 15.21	72.38 ± 22.21	3,27	2.78	0.06
Total dur. probing	403.12 ± 71.48a	221.77 ± 38.42b	198.13 ± 36.00b	224.40 ± 41.84b	3,78	3.23	**0.027**
No. of probes	5.76 ± 1.75a	11.95 ± 1.70b	13.30 ± 1.79b	11.68 ± 1.84b	3,78	3.64	**0.016**
No. of short probes[d]	2.28 ± 0.56a	6.36 ± 1.33b	6.10 ± 1.34b	5.37 ± 1.22b	3,78	4.19	**0.008**
Total dur. of non-probing	316.88 ± 30.70a	498.20 ± 47.15b	521.83 ± 51.80b	492.55 ± 50.16b	3,78	5.76	**0.001**
No. of non-probing periods	6.94 ± 1.57a	13.16 ± 2.90b	15.43 ± 3.57b	14.16 ± 3.36b	3,78	3.78	**0.014**
Mean dur. of non-probing periods	81.76 ± 23.86	88.73 ± 25.30	78.86 ± 23.58	79.10 ± 24.27	3,78	0.06	0.98

[a]Mean number of events per insect (Backus *et al.* 2007).
[b]Mean durations per event per insect.
[c]Total durations per insect.
[d]Defined as probes < 3 min. in length.

Table 5.3. Summary of phloem ingestion behaviors by aphids monitored using EPG (mean ± SE). Durations are reported in minutes. No analyses were conducted because of rare occurrences on resistant selections.

Parameter	**'Munger'** Mean ± SE	**ORUS 3778-1**	**ORUS 3817-1**	**ORUS 4109-1**
No. aphids that performed E2	14	1	0	1
Mean dur. of E2[a]	26.44 ± 12.31	25.75	--	9.27
Total dur. of E2[b]	266.58 ± 139.36	77.26	--	18.55
Mean no. E2 events[c]	12.14 ± 3.20	3	--	2

[a]Mean durations per event per insect (Backus *et al.*2007).
[b]Total durations per insect.
[c]Mean number events per insect.

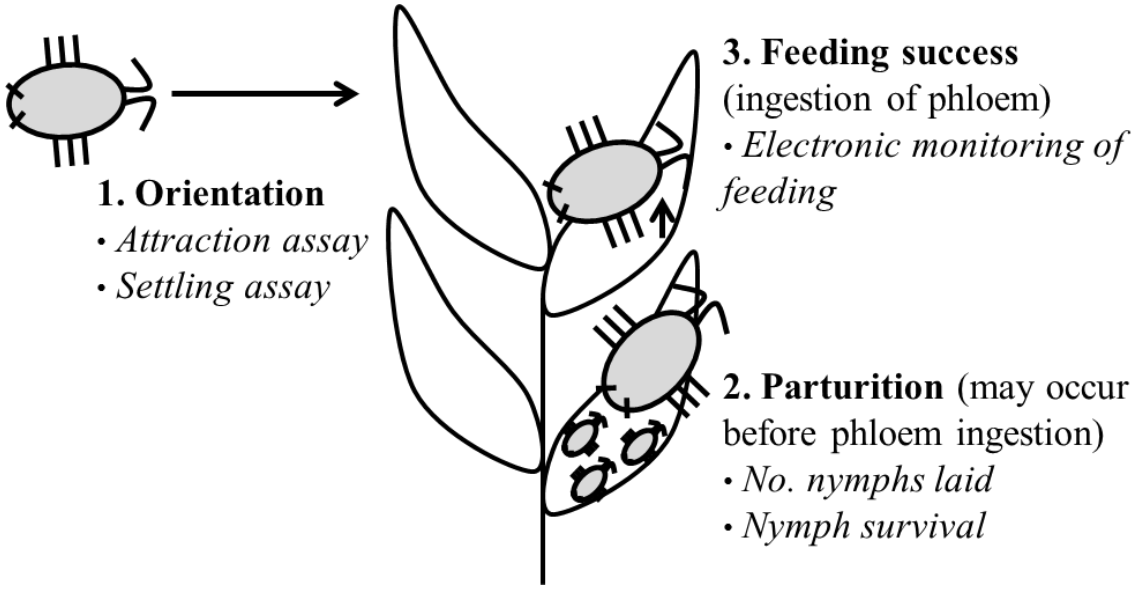
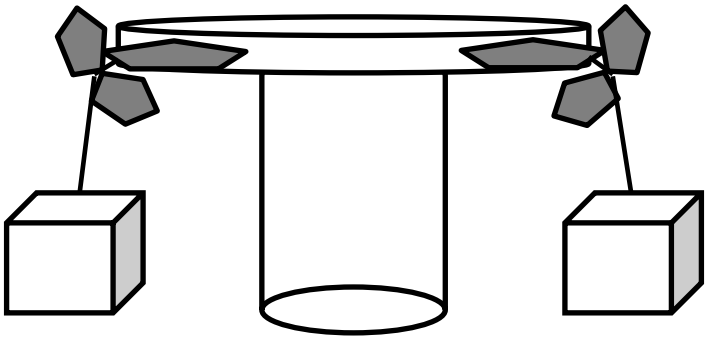


Figure 5.1. Stages of host plant acceptance by aphids (reviewed in Pettersson *et al*.2007). Major stages are in bold; experiments that examined each stage of plant acceptance by raspberry aphid are italicized.

a.



b.

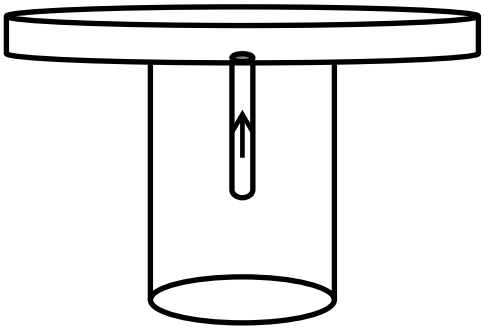


Figure 5.2. Experimental setup for the short term attraction (a) and settling assay (b). a) Petri dish arena is set on top of a stage and the terminal leaflet of the two test plants inserted. Leaves remained attached to the plant. Aphids were added to a central location. b) Assay was as in (a) except there were four test plants, and aphids were introduced via the 15mL conical tube inserted into a hole in the middle of the arena. Assay design was modified from Srinivisan *et al.* (2006) and Castle *et al.* (1998).

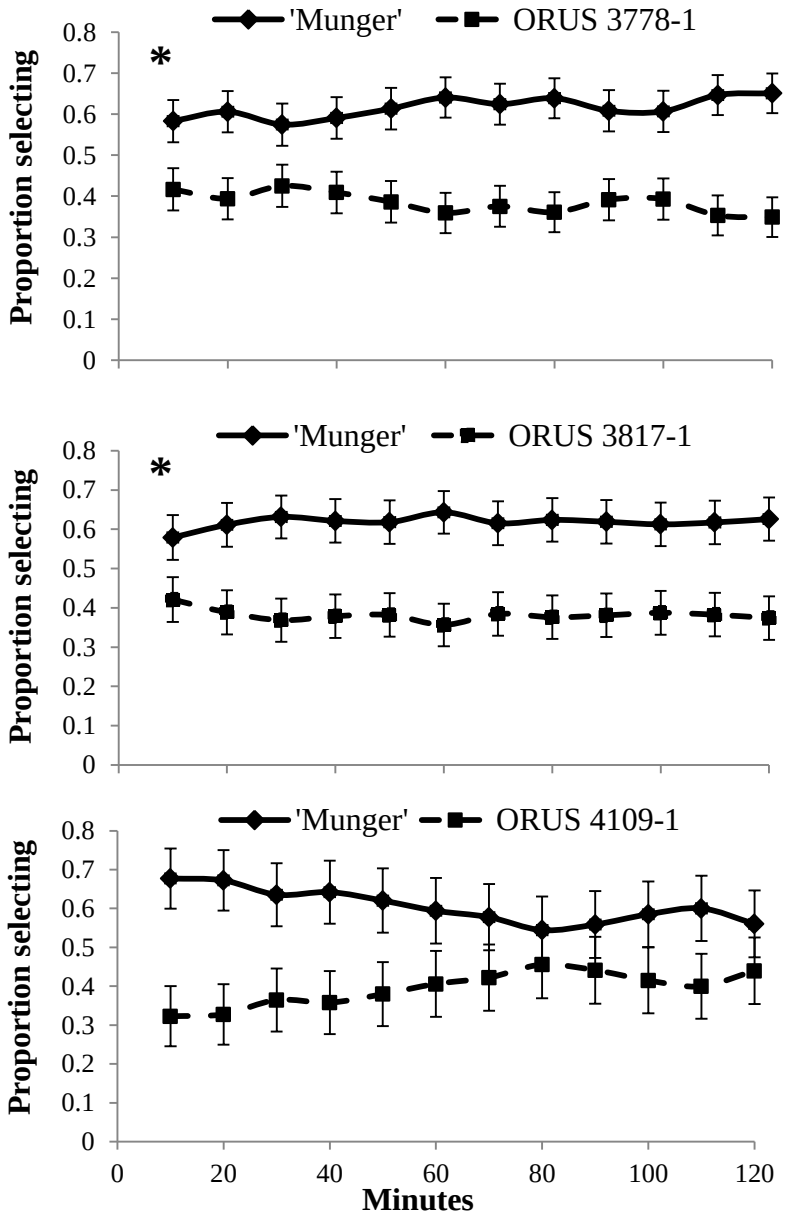


Figure 5.3. Proportion of aphids (mean + SE) that selected either ‘Munger’ or one of the resistant black raspberry selections over the duration of the 2 hr short term attraction assay. Time was not significant in any of the models. An * indicates a significant preference for ‘Munger’.

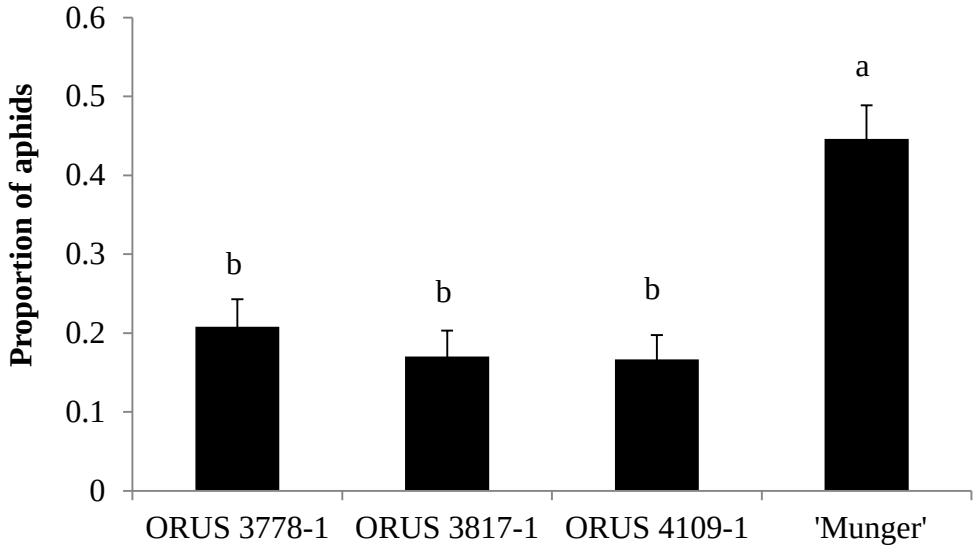


Figure 5.4. The proportion of aphids (mean + SE) that selected ‘Munger’ or resistant black raspberry selections after 18 hr. Letters denote significant differences between treatments.

Chapter 6:

Conclusion

Danielle Lightle

The PNW is the country's leading producer of red and black raspberries, but the virus diseases common in these plantings are responsible for decreased fruit quality and shortened replanting intervals. The virus complex responsible in red raspberry involves the viruses *Raspberry bushy dwarf virus* (RBDV), *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (RpLV). Because RBDV is transmitted in the pollen by bees, control is generally impractical unless RBDV resistant cultivars are planted. However, RLMV and RpLV are transmitted by the aphid *Amphorophora agathonica*, and are better targets for control through vector control.

The seasonal phenology of *A. agathonica* has been studied at the New York State Agricultural Experiment station in the 1970s (Kennedy and Schaeffer 1974a). In New York, aphid populations peak in July, with alate aphids common in June through mid-July but rarely later in the season. However, New York has a continental climate whereas the PNW has a Mediterranean climate, which has more mild temperatures in summer and the winter. Accordingly, *A. agathonica* differed in its seasonal abundance when compared to populations in New York. Aphids in the PNW peaked in June (approximately 1000 growing degree days using 2.7°C as a lower developmental threshold). Like the New York populations, alate aphids were also most abundant in June. However, in this region there was a second period of aphid flight observed in late August at approximately 2000 growing degree days. Alate aphids are the source of virus inoculum into newly planted fields, and control of both flight periods may be important for virus management.

Interactions between vectors and infected plants have been well documented (recently reviewed by Mauck et al. 2012). Common behavioral changes in vectors include increased attraction to infected plants and increased performance when feeding on infected plants. Changes that benefit the vectors are predicted to occur in order to increase virus transmission. Epidemiology models that have incorporated differential attraction and settling behaviors of vectors have found that preference for the rarer plant

status (healthy or infected) will increase the rate of virus spread (McElhany *et al.* 1995, Sisterson 2008). We found that *A. agathonica* were more likely to settle on plants infected with RLMV but less likely to settle on plants with RpLV. If disease symptoms can be largely mitigated by control of only one of the two viruses, RpLV may be the best target for control because of its longer latent period, low transmission efficiency, and decreased preference for settling by *A. agathonica*.

One of the most effective methods for virus control is through the use of vector-resistant plant cultivars. Red raspberry was thought to have a powerful resistance gene, Ag_1, and one biotype of *A. agathonica* (Daubeny 1996, Daubeny and Anderson 1993) that could successfully overcome this gene. However, work by Dossett and Kempler (2012) revealed that there were in fact at least seven biotypes of *A. agathonica* in British Columbia and several previously uncharacterized resistance sources against *A. agathonica*. Feeding studies of 'Tulameen', which carries the gene Ag_1 revealed that the location of the resistance mechanism is in the phloem sieve elements. The gene may encode a protein that acts as a recognition factor of proteins in the aphid saliva. Because Ag_1 – breaking biotypes of *A. agathonica* are already widespread in British Columbia, further deployment of Ag_1 in this region will probably be only a short term solution for vector control. However, the combination of Ag_1 with the newly recognized sources of resistance in red raspberry may be effective at prolonging the usefulness of aphid resistance for control of viruses transmitted by *A. agathonica.*

Black raspberries are also susceptible to virus diseases, primarily caused by *Black raspberry necrosis virus* (BRNV) in the PNW. Since BRNV is transmitted non-persistently by *A. agathonica*, resistant plants are less effective at preventing virus transmission because only a short feeding period is required for inoculation. However, secondary spread of viruses within a field is less likely because resistant plants prevent colonization by the aphid. Three novel selections of black raspberry were recently identified: ORUS 3778-1, ORUS 3817-1, and ORUS 4109-1 (Dossett and Finn 2010).

We found that aphids feeding on all three resistant selections were unlikely to successfully salivate in the phloem sieve elements during a 12 hour experimental period. While this feeding behavior is not as commonly observed as an extended period of salivation, such as seen with Ag_1, failure to salivate may be due to the aphids inability to recognize the phloem sieve elements (Tjallingii 2006). Unfortunately, the feeding behavior of the aphids did not differ between the three resistant selections, giving little evidence that the three selections contain different resistance sources. However, it is possible that there are different genes that result in failure to salivate in the phloem tissue and pyramiding of these genes may be beneficial. Genetic mapping is currently underway that will provide evidence as to whether the resistance sources are conferred by different genes.

A. agathonica is an economically important insect in red and black raspberry because of its role as a virus vector. Control strategies should focus on improved timing of pesticide applications using the seasonal phenology data as a means for predicting when aphid populations will peak. Resistant plants are also an important means for control of aphid transmitted viruses. It is currently possible to combine different resistance sources from red and black raspberry using conventional breeding, however, the timeline is very long due to the large number of backcrosses needed to get a plant with 'red raspberry' or 'black raspberry' type fruit. Future genetic technologies may make these efforts possible and help to speed up the process of moving resistance genes between species of *Rubus* or moving resistance genes through direct genetic manipulation. Lastly, *A. agathonica* and raspberry viruses are an excellent model system for studying interactions between vectors and infected plants. The diversity of viruses that *A. agathonica* transmits, as well as the commonality of co-infection in field plantings, provides a broader understanding of vector-virus interactions. An increased understanding of the evolutionary forces acting upon viruses, infected plants, and vectors will benefit not only raspberry production systems, but all agricultural systems where virus control is important.

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Appendix A: Pan trap catches of *Amphorophora agathonica*

Objective: Monitor flight periods of *A. agathonica* in commercial raspberry fields using yellow pan traps.

Methods: Flying alate aphids were monitored using yellow pan traps from 2010 through 2012. The traps were mounted in commercial 'Meeker' raspberry fields in Whatcom County, Washington, USA. In both years, pan traps were baited with soapy water and emptied weekly. Samples were held at 4°C until processed in the laboratory. Aphids were identified under a microscope using an alate aphid key by Pike *et al.* (2004). Identification was made only to the generic level.

2010: Sixteen pan traps were mounted in one single raspberry field. Traps were rectangular, measured 30 x 12.5 cm, and filled with approximately 2.5 cm of water. Traps were mounted in rows above the highest trellis wire. Canes were cut back throughout the growing season to ensure visibility of the trap. Pan traps were set 15-April until 6-October, but removed during harvest in July to prevent damage by the machine harvester.

2011-2012: Two commercial 'Meeker' raspberry fields were monitored using six pan traps in each field (twelve traps total). The traps were different from those used in 2010. Traps were yellow bowls, 12.5 cm in diameter, and filled with approximately 2.5cm of water. Pan traps were mounted to the anchor poles at the ends of rows that support the trellis wires. Traps were set from April until September, but removed during harvest in July to prevent damage by the machine harvester.

Results: Trap dates were standardized across growing seasons by using aphid growing degree days (GDD) (Chapter 2). Average number of aphids caught per trap are shown in Figure A.1. Aphid trap numbers were similar early in the season. However, much greater numbers of aphids were caught at the end of the season in 2010. This may be because of

the placement of the traps within the rows during 2010, as opposed to the ends of the rows in 2011 & 2012. Thus, the late season trap numbers may represent more localized aphid movement as opposed to longer distance migration. Additionally traps were not set as late in the season in 2011 & 2012 because of rainfall, which may have missed the peak flight time. Numbers during harvest are unknown because traps were not set during this period.

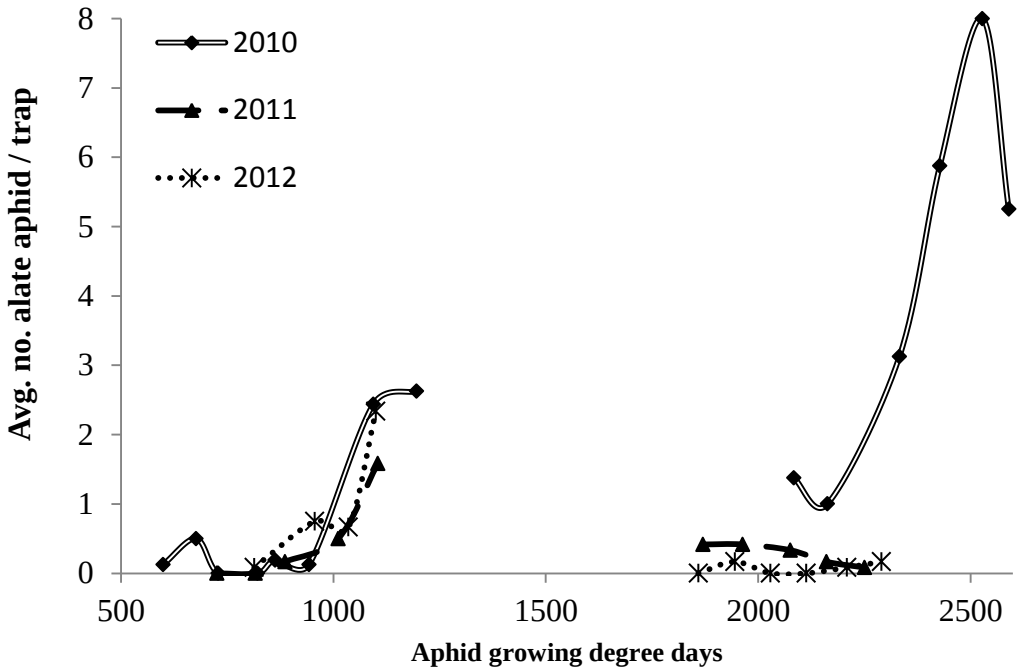

Figure A.1. Average number of *A. agathonica* trapped per pan trap during 2010 to 2012 growing seasons. Data was not collected during harvest, so no averages are included during this period.