**Abstract**

Many plant pathogens manipulate host preference and performance of their vectors to improve their transmission. Insect vectors themselves also develop host-race specialization which impacts preference and performance on specific host plants. Research on both phenomena is often confined to independent experiments evaluating manipulation and host-race specialization. Consequently, effects if virus infection and host-race associations on vectors are rarely considered together in the same framework. For example, *Pea enation mosaic virus* (PEMV) and *Bean leafroll virus* (BLRV) modify the preference, performance, or both, of their principal vector, the pea aphid, *Acyrthosiphon pisum* on its host *Pisum sativum,* but the viruses and aphids have multiple host species. Several host-plant species have empirically established host-race associations that alter preference and performance of pea aphids. Here, we evaluate host-plant preference and performance in a factorial experiment with five aphid biotypes, five host-plant species, and two viruses (PEMV & BLRV) compared to a sham-control. Aphid performance, measured as the rate of increase on potted plants, differed among hostplant species depending on their biotype, and their relative performance among these hostplants was altered by virus infection. Preference, measured using a free-choice bioassay in which aphids were released in an arena where they could settle on any of the five plant species, also differed among aphid biotypes but was unaffected by virus infection status. We did not observe evidence for infection status of host plants altering the performance of aphids biotypes on their matching preferred hosts. These results show that host preference and performance is deeply imprinted in aphid biotypes, and that such adaptations may become hardly reversible even in presence manipulating plant pathogens.

Keywords: insect biotypes, inter-specific transmission, Bean leafroll virus, pea aphid, Pea enation mosaic virus, virus reservoir.

# Introduction

Almost half of the emerging plant infectious diseases worldwide are caused by viruses (Anderson et al., 2004) and their success as pathogens is increasingly threatening global food security (Nicaise, 2014). Most plant viruses depend upon vectors for their transmission, typically generalist insect herbivores with piercing-sucking mouthparts (Gray and Banerjee, 1999). Evidence is accumulating that many of these plant viruses, and other vector-borne plant pathogens, alter host-plant traits or directly affect vector behavior in a manner that enhances the movement of virions from infected to healthy hostplants (Eigenbrode et al., 2018). Vector manipulation has considerable economic consequences for the emergence and spread of insect-transmitted plant viruses. It is suggested that by altering vector behavior, viruses can facilitate the movement of insect hosts to new crop varieties or species that would otherwise be avoided by the host herbivore. Similarly, increased performance of vectors on infected plants increases the risk of catastrophic outbreaks. Consequently, understanding vector manipulation and the ecological factors which impact vector manipulation is a core question in understanding insect-virus-plant pathosystems.

The “vector manipulation” hypothesis predicts plant viruses alter vector-host-plant interactions as a function of transmission mode (Heil, 2016, Ingwell et al., 2012). Most of the theory and empirical work on vector manipulation has been built upon tripartite host-virus-vector pathosystems rarely considering multiple hostplants (Eigenbrode et al., 2018). However, the persistence of pathogens in host populations that exhibit temporal or spatial structure depends on pathogen’s ability to infect more than one host plant (Ashby et al., 2014, McLeish et al., 2018). Moreover, a diverse vector diet breadth can favor virus prevalence in transient host populations by either increasing interspecific transmission rates between hosts and reservoirs (Duffus, 1971), or ensuring the maintenance of high vector populations over time even when virus hosts are scarce (Swei et al., 2011). Most plant viruses have multiple hosts, although many seem to be constrained to one or a few vector species (Power and Flecker, 2003). Likewise, many insect vectors are generalist or oligophagous herbivores that can feed and survive on several plant species (Nault, 1997, Gilbertson et al., 2015, Gadhave et al., 2020).

With respect to the potential association between a single vector species and multiple host-plant species, the “host-race specialization” hypothesis predicts local adaptations to associated plant species. Specialized vector populations exhibit higher performance on associated host-plant species and can exhibit general feeding preferences for those same hosts. Despite higher potential performance, this local adaptation has trade-off costs for herbivores that result in host breadth restrictions, host specialization, and genetic isolation (Drès and Mallet, 2002, Loxdale et al., 2011). A similar pattern has been described for plant viruses, which experience host-specific selection pressures that often lead to antagonistic pleiotropies that limit their ability to overcome host defenses across plant taxa (García-Arenal and Fraile, 2013). On the one hand, both plant pathogens and insect vectors experience a selection pressure towards host specialization, but specialization could become maladaptive when host-plant populations are transient or pathogens are restricted to a small number of insect vectors.

The pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), is an oligophagous herbivore that feeds on leaves, buds, and pods of legumes (Fabaceae). The pea aphid host range includes hundreds of plant species, including multiple cultivated legumes where it is an economic pest such as dry pea, lentil, and alfalfa (Eastop, 1971, Peccoud and Simon, 2010). The pea aphid is considered a model organism for several study fields, including the evolution of distinct biotypes in response to local plant communities (Brisson and Stern, 2006, Eigenbrode et al., 2016, Via, 1991, Peccoud and Simon, 2010). Distinct biotypes of pea aphid associated with local specializations to host plants have been reported in the United States and Europe, several of which include evidence of reproductive isolation, via pre- and post-zygotic barrier (Via et al., 2000, Via, 1999, Peccoud et al., 2008, Eigenbrode et al., 2016). The pea aphid is a vector for more than 30 plant viruses, including the *Pea enation mosaic virus* (PEMV) and the *Bean leafroll virus* (BLRV), two of the main causes of yield losses in peas, broad beans, and lentils in the northern hemisphere (Sandhi and Reddy, 2020, Skaf et al., 1999, Makkouk et al., 2012). Both PEMV and BLRV are persistently transmitted viruses that can infect numerous crop and non-crop legumes, and utilize perennial pea aphid host-plants as reservoirs when host crops are not present (Rashed et al., 2018, Clark et al. 2023). PEMV functions as a mutualistic association of two unrelated taxonomically, autonomously replicating viral RNAs (families Enamovirus and Umbravirus) ( Hull and Lane, 1973, Demler et al., 1993), while BLRV (recently reassigned to family Tombusviridae) (Walker et al., 2021) consists of a single non-segmented RNA strand (Miller, 1999). Previous work has shown that both PEMV and BLRV infections manipulate pea aphids through a “honest” syndrome that includes both increased attraction and survival of vectors in virus-infected plants (Hodge and Powell, 2010, Wu et al., 2014, Davis et al., 2017).

In the present study, we aim to disentangle the convergence of the “vector manipulation” and the “host-race specialization” hypotheses. If viruses require interspecific transmission for long-term persistence and vector biotypes (i.e., host races) prefer and perform better on different hostplants, the “vector manipulation” hypothesis predicts that vector host preference and performance should be altered in presence of infected hostplants in a manner that enhances interspecific transmission among virus hosts. In contrast, the “host-race specialization” hypothesis predicts that physiological and behavioral adaptations will reinforce preference and performance towards a narrow range of hosts (Drès and Mallet, 2002, Loxdale et al., 2011). In particular, while host preference and performance seems to be deeply imprinted in pea aphid biotypes (Via, 1991, Eigenbrode et al., 2016), the presence of pathogens that rely on interspecific transmission to persist in transient host populations might cause an evolutionary conflict with significant consequences for the evolution of host-virus-vector pathosystems. In the Pacific Northwest, USA (PNW), several pea aphid host races coexist, most of them associated with either alfalfa or pea crops (Eigenbrode et al., 2016). Some perennial or winter-hardy legumes, such as hairy vetch (*Vicia villosa*), red clover (*Trifolium pratense*), and alfalfa (*Medicago sativa*), are known to both sustain early pea aphid immigrant infestations during early spring and serve as reservoirs of PEMV or BLRV (Rashed et al., 2018, Clark et al., 2023). Here we test the hypothesis that performance and preference of pea aphid biotypes across a panel of potential host plants is altered when these plants are infected with PEMV or BLRV. We evaluate the effects of virus infection on the preference performance relationships among these hosts and aphid biotypes. We interpret the results in the context of horizontal transmission of the viruses among these hosts.

# Materials and methods

## Aphid and Virus Colonies and Treatments

We established five clonal colonies of pea aphid, *Acyrthosiphon pisum,* from apterous reproductive aphids. We collected these aphids from pea, vetch, clover, or alfalfa fields near Moscow ID (46.7325° N, 116.9992° W, 786 m.a.s.l.) or near McMinnville OR (45.2108° N, 123.1945° W, 40 m.a.s.l.) in 2017. Two aphid genotypes we collected exhibited better performance on alfalfa, labeled AL-PK-ID and AL-GN-OR. We consequently referred to these genotypes as “alfalfa” biotypes in all experiments. Similarly, two genotypes collected from pea, PA-GN-ID and PA-GN-OR and one collected from clover, CL-PK-OR. We consequently referenced these as “pea” biotypes in all experiments as well. Prior field surveys in Washington state suggest pea aphids are found feeding on introduced species of vetch (*Vicia*) and clover (*Trifolium*) in addition to cultivated dry peas (Clark et al., 2023). We maintained aphid colonies at the Manis Entomological Laboratory greenhouses at the University of Idaho in individual 60- by 60- by 60-cm mesh tents (BugDorm 2120F; BioQuip, Rancho Dominguez, CA). We reared these colonies at 20 ± 2°C, with a photoperiod of 18:6 (L:D) h, and 50% relative humidity. For at least 20 generations prior to use in experiments, these colonies were reared on potted fava bean plants (*Vicia faba* L.), which is a universal host for pea aphid (Peccoud et al., 2014). We confirmed colonies were genetically separate using 12 autosomal microsatellite loci [described in Eigenbrode et al. (2016)].

Our experiments included the host-plant species of pea (*Pisum sativum* L.), red clover (*Trifolilium pratense* L.), common vetch (*Vicia sativa* L.), alfalfa (*Medicago sativa* L.), lentil (*Lens culinaris* Medik.), and fava bean (*Vicia faba*). We grew these host-plants for experiments in conditions identical to those for maintaining insect colonies. For experimental treatments comparing viruses effects to non-infected plants, we used infective aphids to inoculate plants with either *Pea enation mosaic virus* (PEMV) or *Bean leaf roll virus* (BLRV). We sourced infective colonies of pea aphid reared on *V. faba* infected, where both viruses are maintained under controlled conditions. We originally collected virus isolates from commercial fields of alfalfa (BLRV) or pea (PEMV) near Moscow ID. For bioassays we grew plants in 15-cm diameter pots filled with commercial soil mix (Sunshine Mix #1; SunGro Horticulture, Bellevue, WA, USA). Fifteen days after emergence we inoculated plants by placing five aphids, from an infectious colony of either BLRV or PEMV, in a clip cage (5 cm. diam) onto a leaf from the top node of the test plant. Following a 3-day inoculation access period, we removed aphids using a soft bristled paintbrush. We maintained these plants to be aphid-free until they were used in experiments. To control for the effects of aphid feeding on the test plants, we performed a ‘sham’ inoculation with non-infective aphids using the same timings and clip cages as virus treatments.

## Host settling bioassay

To evaluate preference behavior in aphids, we quantified host settling with a multiple host-plant choice test. We constructed a circular arena approximately 30 cm in diameter with a flat foam core floor and enclosed with a clear plastic wall coated with Fluon (PTFE-30). To hold the stems of test plants, we cut evenly spaced notches around the perimeter. To provide some control over difference in plant sizes, we germinated hosts at different times based on known growth rates in greenhouse conditions. For each trial, we released 50 apterous aphids into the center of the arena. After 24 hours, we counted the number of aphids on each test plant. Mortality was only 1.6% for aphids tested across all trials. We conducted five replicate trials for each aphid biotype × virus treatment (PEMV, BLRV, sham) followed by an additional five replicate trials in a second time block.

## Aphid performance bioassay

We quantified aphid performance in a no-choice bioassay. We placed eight pea aphids on individual plants infected with PEMV, BLRV, or sham inoculated among each isolated biotype. We counted the total number of aphids present on each plant after 10 days. In this assay, we completed two total replicates for each combination of aphid biotype × plant species × virus infection status (PEMV, BLRV, sham) across two separate time blocks. In a third time block, three additional replicates were completed across all plants, with only two additional replicates for alfalfa and lentil due to low germination rates. In all, six fully factorial replicates were available for data analysis.

## Statistical analyses

All analyses were completed in R version 4.2.2 (R Development Core Team, 2022). Three models were used to evaluate the outcomes of bioassays. First, we modeled the effects of aphid biotype, plant species, and virus status on the total number of aphids counted on a host plant at the end of each assay (“performance”). All models fitted to these count data followed a negative binomial distribution using the glm.nb function in the MASS package in R (Venables and Ripley, 2002). Second, we modeled the effects of aphid biotype, plant species, and viral status on the number of aphids that moved towards a host plant by the end of each bioassay (“preference”). Third, we completed a linear model examining the relationship between preference and performance with an interaction term of virus status using pooled results from both types of bioassays. We ran this model to specifically test the hypothesis that virus status modifies the known correlation between preference and performance among aphid biotypes. In this third model, the predictor variable aphid counts were log-transformed to meet linear regression assumptions. Both the second and third models were normally distributed and used the glm function in base R.

For the first two models, we employed a stepwise model selection approach. We started with a fully specified generalized linear model (GLM) including the third-order interaction term between aphid biotype, plant species, and virus status, as well as all underlying second and first-order terms. Given the relatively small size of the dataset to this large model, we sought to reduce model complexity and avoid overfitting via stepwise regression. After running these fully specified models, the stepAIC function in the MASS package was applied to each model (Venables and Ripley, 2002). When applied to a GLM, this function sequentially reduces the number of parameters to produce a model with the lowest AIC. Such an approach allowed for consistent, unbiased strategy to avoid overfitting across both types of aphid count data. Both full and reduced models had significance tests (P-values and critical values) completed using the car package (Fox and Weisberg, 2018). Post-hoc tests *via* Tukey HSD were calculated using the emmeans package (Lenth, 2023). Unless otherwise indicated, bar plots present mean and standard error of the means.

# Results

## Biotype, Plant, and Virus effects on aphid performance

Aphid counts on each host plant after one week were significantly different among host-plant species following patterns expected from prior research on pea aphid performance. The effect of host-plant species was highly significant (Table S1, P < 0.001, 𝛘2 = 23.75, DF = 5), with aphid performance being relatively lower on red clover and relatively higher on hosts like fava bean and lentil (Fig 1). There was a significant first-order effects of virus status which significantly impacted aphid abundance (Fig S1, Table S1, Virus effect, P = 0.04, 𝛘2 = 6.66, DF = 2). Examination of this virus main effect, when averaging across all host plant species, showed a slight increase in overall aphid abundance among PEMV infected plants compared to BLRV and sham plants. Virus status of host plant also significantly impacted aphid performance, with non-additive affects based on host-plant species (Tables S1, Plant:Virus interaction, P < 0.001, 𝛘2 = 32.16, DF = 9). For example, aphids performed better on alfalfa plants exposed to BLRV compared to a sham (Fig. 2, Tukey HSD). Conversely, aphids performed more poorly on hairy vetch exposed to BLRV compared to PEMV and sham plants (Fig. 2, Tukey HSD). The remaining host-plant species did not have significant differences in aphid performances among virus exposure status (Fig. 2, Tukey HSD).

Aphid performance differed depending on host-plant species identity (Table S1, Biotype:Plant interaction, P < 0.001, 𝛘2 = 57.31, DF = 5). As predicted, the “alfalfa” biotypes performed better on alfalfa compared to pea (Fig 1, Tukey HSD). Conversely, the “pea” biotypes performed better on hairy vetch and pea compared to the “alfalfa” biotypes (Fig 1, Tukey HSD). We observed no differences in aphid performance among the remaining host plants, red clover, fava bean, and lentil (Fig 1. Tukey HSD). Given that analysis of deviance tables were calculated using Type II Wald-𝛘2 tests (Fox and Weisberg, 2018), results suggest the Biotype:Plant interaction term statistically accounts for the effect of biotype on aphid performance (Table S1, P = 0.5, 𝛘2 = 0.45, DF = 1).

## Biotype, Plant, and Virus effects on aphid preference

Aphid preference for host plants was determined using total counts of aphids settling on a respective host plant in a behavioral assay. Aphid preference differed significantly among target hosts (Table S2, P < 0.001, 𝛘2 = 111.77, DF = 5), and this preference varied according to aphid biotype grouping (Tables S2, P < 0.001, 𝛘2 = 51.78, DF = 5). Matching results from aphid performance, the "alfalfa” biotypes were more attracted to alfalfa hosts compared to the “pea” biotypes (Fig 3, Tukey HSD). Likewise, the “pea” biotypes preferred hairy vetch and red clover (Fig 3, Tukey HSD). Paradoxically, we did not see a preference for pea plants by the “pea” biotypes (Fig 3, Tukey HSD).

All terms including virus exposure status were dropped from final model using our stepwise AIC approach. Consequently, there was no statistical evidence for the virus exposure status of these target host plants altering aphid preference (Table S2), nor did we observe any evidence that virus status influenced the effects of aphid biotype group or plant species identity (Table S2).

## Preference by performance relationship

We examined the relationship between the number of aphids settling on host plants (metric of preference) and the total number of aphids found on infested host plants after one week (metric of performance). We observed a positive relationship between aphid preference and aphid performance (Table S3, P < 0.001, 𝛘2 = 21.71, DF =1). As such, aphid movement to host plants for a given trial was higher under similar conditions by which aphid performance was higher (Fig 4). However, we did not observe any evidence that this relationship was modified for plants exposed to BLRV or PEMV (Table S3, Log Aphid Count:Virus interaction, P = 0.57, 𝛘2 = 1.13, DF =2). Across all infection statuses, aphid preference increased in trials where aphid performance was higher (Fig 4).

# Discussion

*How did hypotheses and predictions match up with outcomes?*

Host specialization and manipulation by plant viruses is already established as an ecological condition modifying pea aphid, virus, and host-plant interactions (Eigenbrode et al., 2016, Davis et al., 2017, Wu et al., 2014). Taken together, both hypotheses tested in our experiments predict biologically significant chances to vector preference and vector performance depending on infection status of a host plant. We found context-dependent support for the virus manipulation hypothesis with respect to aphid reproduction. Plants infected with BLRV virus exhibited different apparent aphid counts at the end of the experiment, with aphid abundance being higher on BLRV alfalfa and lower on BLRV infected vetch. While we did not observe any changes in aphid abundance among PEMV infected host plants at a per-species level, across all host-plant species aphids were slightly more abundant on PEMV infected plants. However, no changes to preference behavior among infected or sham plants were reported under any of the statistical models considered. Such results support the assertion that viruses may be manipulating aphids by altering their fitness on certain host plants, but without concomitant behavioral changes we only have partial support for the virus manipulation hypothesis. Conversely, we found multiple lines of evidence that pea aphid performance and preference fit the predictions of the host-race association hypothesis with some caveats for PEMV-infected plants.

*What does it mean that evidence for virus manipulation was only seen in performance? (Bring up lack of virus detection in assayed plants as a reason for cautious interpretation)*

Vector-borne plant viruses often rely on transmission among alternate hosts, especially when hosts are annual or transient (Elena et al., 2009, Wilke et al., 2006). Consequently, it may be adaptive for viruses to manipulate vectors to facilitate this horizontal transmission. In order for virus manipulation of vectors to be manifested, some changes to aphid behavior in response to host plants should be observable. For example, it is anticipated that host plants infected with PEMV would trigger different aphid preference behaviors compared to sham-inoculated plants. However, preference behaviors among aphids when presented with PEMV or BLRV plants did not differ. It is possible that virus manipulation in this system is only manifested in the form of changes to aphid performance, or that a more reliable infection status of hosts is required. In this experiment, plants we exposed to infective aphids for a brief time period, and we were not able to validate that every single plant was symptomatic or had detectable levels of viral titer at the time of completing bioassays. While pea aphids can detect a wide range of plant volatiles, facilitating host-plant recognition (Shih et al., 2023, Robertson et al., 2019), is plausible these host-plant signals below detectable thresholds. Finally, our study examines plant-originating mechanisms (Bruce et al. 2005) rather than comparing behavioral differences in viruliferous vectors compared to non-viruliferous vectors. For example, beet leafhoppers carrying BCTV (Beet curly top virus) have increased probing behavior (Lee et al. 2022), while Bird-cherry oat aphids carrying BYDV (barley yellow dwarf virus) prefer perennial grasses relative to other hosts. Such vector-originating mechanisms are not evaluated in our experimental framework, suggesting future work should also consider comparisons of viruliferous and non- viruliferous aphids.

*What does it mean that we saw predictable effects of pea and alfalfa biotypes? Similarly, what does it mean that viruses did not disrupt host race associations?*

Many generalist insect herbivores exhibit higher performance on single species of hosts, and these often are host-plant species they could have fed on for multiple generations (Garrido et al., 2012) Such mechanisms have long been predicted to be incipient evolution of dietary specialization, generated by selective pressures to better utilize host-plant resources (Van Zandt and Mopper, 1998). Pea aphids form clonal associations with both crop and non-crop hosts, with some populations exhibiting local adaptation (Caillaud and Via, 2000). Host specialization implies performance trade-offs that often match host-plant preference and reduce the diversity of plants that insect herbivores can effectively exploit (Berlocher and Feder, 2002). For example, Eigenbrode et al. (2016) observed “pea” clones have dramatically reduced performance on alfalfa compared to competing “alfalfa” clones. Our results corroborate this finding; however we did not find evidence for a similar trade-off for “alfalfa” biotype aphids with respect to pea. Furthermore, due to similar mechanisms by which host-race associations modify preference and performance of vectors, virus infection status of host plants was predicted to alter host-race associations. However, neither factorially-designed bioassays show a disruptive effect, suggesting these two mechanisms may be operating independently. Prior work on aphid performance among multiple host plants infected with PEMV where infected plants can have positiveor negative effects aphid reproduction (Clark and Crowder, 2021), but multiple pea aphid biotypes were not considered. To resolve this issue, further work would need to look at the plant response to infection and changes to nutritional quality over longer time periods.

*How does this compare to other systems looking at these two hypotheses? What is the takeaway given this comparison?*

Correlations between pea aphid’s host performance and preference and reproductive isolation have been established as a function of local adaptations or recent co-evolution (Via et al., 2000, Hawthorne and Via, 2001, Queller and Strassmann, 2018). By facilitating higher reproductive output on specific host-plant species, host-race associations can exacerbate outbreaks of insect herbivores in agroecosystems or increase the likelihood of outbreaks occurring in the future. Locally adapted herbivore may be more likely to break through plant defenses (Scriber 2002, Garrido et al., 2011). [Use alfalfa example?] Similar predictions are made for virus manipulation. [Mauck study here or rob’s Oecologia paper?]. Conversely, vector manipulation may actually slow viral epidemics within crops by encouraging viruliferous vectors movement into non-crop plants (Shoemaker et al. 2019).

**Author contributions**

SDE originated experimental design and hypotheses. REC and SDE completed bioassays. REC completed all statistical analyses and data visualization under advisement by DWC and SDE. DFR, DWC, REC and SDE wrote and revised the manuscript.

**Acknowledgments**

Greenhouse work and bioassays in this experiment would not have been possible without the help of multiple student researchers at Washington State University (WSU) and University of Idaho. WSU students include J. Wallis, W. Troutman & L. Dean. Both behavioral and performance assays were completed thanks to help from Ying Wu (University of Idaho). Experimental work completed on this project by R.E.C. was supported by USDA-NIFA award 2017-67013-26537, while data analysis and technical writing by R.E.C. and D.F. R. weresupported by USDA-NIFA award 2002-67013-36422.

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