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Disease progression of vector-mediated *Grapevine leafroll-associated virus 3* infection of mature plants under commercial vineyard conditions

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Abstract *Grapevine leafroll-associated virus 3* (GLRaV-3) is associated with the economically damaging grapevine leafroll disease, and is transmitted in a semi-persistent manner by several mealybug species. We performed the first controlled field study of vector-mediated inoculations with GLRaV-3 in a commercial vineyard with previously asymptomatic vines, and monitored the vines during four growing seasons. We then compared the outcome of vector-mediated inoculations in the field study to an analogous laboratory study. In the vineyard, about half of all inoculated plants became infected with GLRaV-3, fewer than in the controlled laboratory inoculations. Mealybugs had lower settling and feeding success in the field than in the laboratory inoculations. Our study suggests that laboratory studies may overestimate transmission efficiency. All successfully inoculated vineyard plants first became symptomatic one growing season after inoculations, and berry quality declined within one year after inoculations. Vineyard plants were effective virus sources one year after inoculations. Our findings generally indicate that newly symptomatic vines in commercial vineyards probably became infected during the previous growing season, and a decline in berry quality can be expected during the same year in which symptoms appear.

Keywords *Grapevine leafroll-associated virus-3* · Grapevine leafroll disease · Incubation time · *Pseudococcus maritimus* · Semi-persistent transmission · *Vitis vinifera*

Abbreviations

ANOVA	Analysis of Variance
GLRaV-3	*Grapevine leafroll-associated virus-3*
RNA	ribonucleic acid
RT-PCR	reverse transcription and polymerase chain reaction

Introduction

More than three quarters of all plant viruses are transmitted by insects (Hogenhout et al. 2008), and information regarding key biological traits of vector-borne pathogens is needed to inform effective control strategies. For example, knowledge of transmission efficiency can aid in predicting rates of pathogen spread (Purcell 1981). Another key parameter in estimating the rate of appearance of newly diseased hosts is the pathogen incubation period, the time between initial infection and when symptoms become evident. Despite the importance of transmission efficiency and incubation period with respect to the development of disease

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management strategies, data are often not available and, when available, are usually derived from research performed under artificial conditions such as greenhouse environments.

Grapevine leafroll-associated virus 3 (GLRaV-3), in the genus *Ampelovirus*, family *Closteroviridae*, is the primary virus species associated with grapevine leafroll disease in vineyards of wine growing regions worldwide (reviewed in Almeida et al. 2013). GLRaV-3 can cause interveinal reddening and downward rolling in red berried grape varieties (*Vitis vinifera* L.), inhibits photosynthesis, decreases vine lifespan, and reduces fruit yield and quality (Almeida et al. 2013). GLRaV-3 is one of the most common and detrimental viruses of grapevines, and has led to economic losses of 25 % or more (Atallah et al. 2012; Cabaleiro et al. 2013; Ricketts et al. 2015). Spread of GLRaV-3 in vineyards and vector-borne transmission in controlled laboratory studies were first documented in South Africa (Engelbrecht and Kasdorf 1990), and since then GLRaV-3 spread in vineyards and transmission by several mealybug species have been documented in wine growing regions worldwide (Cabaleiro and Segura 1997; Charles et al. 2009; Golino et al. 2008; Habili 1995; Le Maguet et al. 2012; Tsai et al. 2008).

Although multiple grape-colonizing mealybug species transmit GLRaV-3, estimates of vector transmission efficiency vary both among and within mealybug species (Almeida et al. 2013). GLRaV-3 is transmitted in a semi-persistent manner with no latent period required between acquisition and inoculation by vectors; transmission can occur after access periods of as little as one hour, and reaches a maximum after access periods of 24 hours (Tsai et al. 2008). First instar mealybugs are the most efficient vectors, and mealybugs lose the ability to transmit GLRaV-3 four days after being removed from an infected source (Le Maguet et al. 2012; Petersen and Charles 1997; Tsai et al. 2008). There is no evidence of GLRaV-3 transovarial passage (Tsai et al. 2008). While laboratory-based transmission studies have been informative, there is still a need for field-based transmission experiments to accurately estimate expected vector transmission efficiency and disease incubation time within the host. Information is lacking regarding how soon disease symptoms will appear after vector-borne infections are initiated, or how soon crop quality will be affected.

The goal of our study was to obtain information about vector-borne transmission of GLRaV-3 and subsequent disease progression under commercial vineyard conditions. We performed a controlled GLRaV-3 transmission study into mature *V. vinifera cv.* Cabernet franc vines in Napa Valley, CA USA. We used first instars of the vector *Pseudococcus maritimus* (Ehrhorn), a mealybug species that is a common vineyard pest and native to North America (Daane et al. 2012). We performed a concurrent laboratory study, with identical experimental design, to compare pathogen transmission efficiency under controlled laboratory and field conditions. In the field study, we estimated transmission efficiency and monitored time to GLRaV-3 detection via molecular diagnostics, appearance of symptoms, and effects of disease on berry quality. Our findings are key to informing sound management practices with respect to understanding spread and progression of disease in a commercial vineyard. Furthermore, we provide a previously missing link between controlled laboratory studies and realistic vineyard conditions.

Materials and methods

Virus, plant, and insect materials

Virus-infected dormant cuttings of *V. vinifera* cv Cabernet Sauvignon were used as source of GLRaV-3 in our transmission experiments. Foundation Plant Services at the University of California, Davis provided accession LV89-01 from their Virus Source Vineyard, which is known to be infected with genetic variant group III of GLRaV-3, *Grapevine virus B* (GVB), and *Grapevine fleck virus* (GFkV). This accession was chosen because genetic variant group III of GLRaV-3 is common in Napa Valley (Sharma et al. 2011). Plant cuttings were cut to three buds each, treated with RootBoost (GardenTech, Lexington, KY) rooting hormone, planted in 1:1 vermiculite: perlite, and kept on a mist bench for 6 weeks, until a few leaves were produced and roots were approximately 2.5 cm long. Cuttings were then removed from the mist bench and transplanted to 10 cm pots with a growth medium consisting of 2:1:1 SuperSoil: perlite: sand (Rod McClellan Company, Elk Grove, CA), and kept in the greenhouse until used as virus sources in transmission experiments. GLRaV-3 infection was confirmed by molecular diagnostics prior to use for source material in transmission studies. Virus-free dormant *V. vinifera* cv

Pinot noir cuttings were also provided by Foundation Plant Services, collected during winter dormancy 2011, and propagated in the same manner as the virus-infected source cuttings.

We used *Ps. maritimus* as the mealybug vector, which is difficult to maintain in insect colonies; therefore we relied on field collections for experimental inoculations. To obtain virus-free first instar *Ps. maritimus* mealybugs, third instar females were collected from a vineyard in Pope Valley, CA in May 2011, and allowed to mature and oviposit in the laboratory. The third instar females were collected from underneath the bark of the trunks and cordons of mature grapevines and placed into gel capsules for transport to the laboratory. The mealybugs were immediately transferred to 100 mm petri dishes, each containing one piece of 70 cm Whatman filter paper. The mealybugs were kept in darkened conditions at 25 °C: 20 °C, 16: 8 h day: night temperatures- females were removed and discarded after oviposition. The filter papers containing ovisacs were pinned to the underside of the aforementioned infected source plants, which were then kept in a growth chamber until the first instar mealybug crawlers hatched. Approximately 72 h after hatching on the infected source plants, mealybugs were transferred to mature vines in the vineyard and to uninfected vines in the laboratory, for a 48 h inoculation access period. The timing of hatching led us to perform field inoculations on 18 July 2011, which coincided with the emergence of the new *Ps. maritimus* generation in Napa Valley. Twenty replicate source vines were propagated and used, with one to five recipient test vines inoculated per source plant in each inoculation experiment (field and laboratory). All recipient test vines were treated with an insecticide upon completion of the inoculation access period.

Experimental design and inoculations

The experimental field inoculations were located in three rows of a vineyard block of *V. vinifera* cv. Cabernet Franc clone 01 grafted to 110R rootstock, obtained from Duarte Nursery and planted in Oakville, Napa Valley, CA in 1994. No vines in the experimental area were symptomatic for grapevine leafroll disease prior to our experimental inoculations. To confirm initial GLRaV-3-free status prior to inoculations, three petioles were collected from each experimental vine in July 2011 before inoculations were performed, for diagnostic testing (described below). The block consisted of 8315 vines planted at 588 vines per hectare. Row spacing was 1.8 m, and vine spacing was 1.5 m, with a vertical shoot positioning trellis system and bilateral pruning. Row direction was northwest-southeast. Drip irrigation was provided using one 3.8 - $L{\cdot}h^{-1}$ emitter every 1.5 m. A minimum of five buffer vines were left untreated at each end of the rows. Experimental vines were spaced every third vine, and treatments were fully randomized. The three treatments included inoculations with no leaf cages, inoculations using mesh leaf cages, and negative controls for which no experimental manipulation was performed. Each treatment included 30 replicate vines, for a total of 90 experimental vines. The experiment comprised an area including 360 total vines, including the 90 experimental vines plus the spacer vines. The spacer vines were monitored periodically throughout the study for symptoms of grapevine leafroll disease. A survey for any signs of mealybugs was performed in October 2012. On 11 October 2012, 15 months post-inoculation, a commercial testing service (Agri-Analysis LLC, Davis, CA USA) collected and analyzed material from some vines that were symptomatic for grapevine leafroll disease in the experiment and tested for a broad panel of known grape pathogens: GLRaV-1, GLRaV-2, GLRaV-2 strain Red Globe, GLRaV-3, GLRaV-4, GLRaV-4 strain 5, GLRaV-4 strain 6, GLRaV-4 strain 9, GLRaV-7, *Syrah virus 1*, *Grapevine virus A*, GVB, *Grapevine virus D*, *Grapevine fanleaf virus*, *Xylella fastidiosa*, GFkV, *Rupestris stem pitting-associated virus*, *Rupestris stem pitting-associated virus strain Syrah*, and *Grapevine red blotch-associated virus*.

The vineyard block was not treated with insecticide prior to inoculations during the 2011 growing season. For inoculations, ten *Ps. maritimus* first instar insects were gently moved with a paintbrush from leaves of infected source plants onto the underside of one fully expanded mid-height leaf, located on a vertical cane growing from a middle spur on the south cordon of each grapevine. For the caged treatment, a cloth mesh cage was placed over the inoculated leaf and secured at the petiole using a twist tie. For the uncaged treatment, no covering was used on the inoculated vine. The experimental area was commercially treated with spirotetramat insecticide on 20 July 2011, after a 48 h inoculation access period. After inoculations the experimental area was managed following standard commercial practices.

Sample collection and monitoring

Three months after inoculations, the petiole of the inoculated leaf was collected on 14 October 2011 for diagnostic testing. In the instance where that petiole had fallen off the vine or could not be found, a petiole near the inoculated leaf was collected; inoculated petioles were missing from 9 of 60 inoculated vines. Immediately following the first appearance of symptoms in 2012 and 2013, petioles were collected from each experimental vine and tested for presence of GLRaV-3. Petioles were collected from each experimental vine in September 2014, and tested for the presence of GLRaV-3, GVB, and GFkV. On each sampling date, three petioles were collected from each vine and pooled for diagnostic testing. If a vine had symptomatic leaves at the time of sample collection, symptomatic leaves were preferentially collected over asymptomatic leaves.

During each growing season in 2011 through 2014 (Years One-Four), experimental vines were surveyed regularly for visible leafroll disease symptoms, beginning immediately after inoculations. On each survey date vines were marked as either asymptomatic or symptomatic, with surveys beginning in May and continuing through October. Shortly after symptoms first emerged in 2012, a detailed symptom survey of each symptomatic vine was performed to determine possible variation in disease symptom severity among vines and if there was an association between location of inoculation and initial appearance of symptoms within vines. For this survey, the position of each spur and the number of symptomatic and asymptomatic leaves on each spur were recorded.

In Year Two, berry quality of all vines was measured three times during the weeks immediately preceding commercial harvest. Degrees Brix (soluble solids), pH, and titratable acidity ($g \cdot L^{-1}$ tartaric acid) were measured on 31 August, 21 September, and 3 October 2012, and harvest was 4 October 2012. In Year Three, berry quality of a randomly selected subset of 30 vines was measured on 28 August and 14 September, and harvest was 14 September 2013. The 30 vines were evenly divided between uninfected negative controls, uninfected and infected vines from the caged inoculation treatment, and uninfected and infected vines from the uncaged inoculated treatment.

For berry quality analysis, on each sampling date approximately 200 berries were collected from each vine to minimize variance in measurements (Kasimatis and Vilas 1985). Within each grapevine, berries were collected from the top, middle, and bottom of each harvestable cluster of grapes and pooled for laboratory analysis. All samples were processed by Constellation Laboratories in California, USA. Total soluble solids as °Brix were measured using an Atago refractometer, and pH was measured using an Orion pH meter. Titratable acidity of the juice was measured via direct titration with 0.1 N NaOH, using phenolphthalein as an indicator.

Concurrent laboratory inoculation study

To compare field inoculations in an established commercial vineyard with controlled laboratory studies, a laboratory experiment including the same treatments was performed, using the same collection of *Ps. maritimus* and same known infected source plants. *Ps. maritimus* first instars were moved from known infected source plants to uninfected recipient test plants on 15 July 2011 and allowed a 48 h inoculation access period, after which plants were treated with a contact insecticide. Mealybugs on half of the recipient test plants were caged on the underside of one leaf using clip cages, as described by Tsai et al. (2008), and mealybugs remained uncaged on the other half of the recipient test plants. Thirty replicate recipient test plants were used for each treatment, caged and uncaged, for a total of 60 recipient test plants. Additionally, a replicate of 30 uninoculated plants was kept in the greenhouse following inoculations to assure that all GLRaV-3 infections were due to our experimental inoculations. Following inoculations, plants were kept in a greenhouse with 16:8 light: dark using supplemental lighting as needed, and 25 °C: 22 °C day: night temperatures, with regular fertilizer and insecticide treatments. Because disease symptoms under greenhouse conditions are not reliable for diagnosis of GLRaV-3, we used molecular testing to confirm infection with GLRaV-3. Four months after inoculations, at which time new GLRaV-3 infections can be reliably detected using molecular diagnostics, three petioles per inoculated test plant were collected and stored at −80 °C until testing for GLRaV-3 (Tsai et al. 2008).

Inoculated field vines as virus source

To test whether the newly infected field vines could be a source of GLRaV-3 one season after mealybug inoculations, a transmission experiment was performed in the laboratory from cuttings of these newly infected field

vines. *Ps. maritimus* were not used because of the abovementioned difficulty in obtaining virus-free first instars for transmission experiments. Instead we used first instars of *Planococcus ficus*, which are easily maintained in colonies and therefore can be ready for use in transmission studies at any time. Furthermore, *Pl. ficus* is a known vector of GLRaV-3 (Blaisdell et al. 2015; Engelbrecht and Kasdorf 1990). Field cuttings were collected on 4 October 2012 and the stem bases were placed in flasks of water. First instar *Pl. ficus* were allowed a 24 h acquisition access period on the field cuttings, then transferred to the underside of a leaf of virus- free *V. vinifera* cv. Pinot noir recipient test vines; ten insects per recipient test vine were confined using a leaf cage (as described in Tsai et al. 2008) for a 24 h inoculation access period. Following inoculations, plants were treated with a contact insecticide and then kept in a greenhouse for four months until petiole sample collection for diagnostic detection of GLRaV-3.

For this experiment, a randomly selected subset of experimental field vines of each treatment was tested as a potential GLRaV-3 source. In total, nine symptomatic vines were tested; five from the caged inoculation treatment and four from the open inoculation treatment, and seven recipient test vines were inoculated in the laboratory from each symptomatic field vine. One of these 63 recipient test vines died before petiole sample collection to test for infection with GLRaV-3. Eleven total asymptomatic field vines were tested as a negative control: three from the caged inoculation treatment, three from the open inoculation treatment, and five uninoculated negative control vines. There were no symptomatic negative control vines in the field experiment. For each asymptomatic field vine, three replicate recipient test plants were inoculated, for a total of 33 recipient test vines from asymptomatic field vines. Additionally twenty uninoculated test vines were included with the recipient test vines in the experiment as negative controls, for a total of 116 experimental and control test plants.

Diagnostic testing

To verify infection status of source plants, RNA extractions were performed on 100 mg of petioles from all infected source plants using Qiagen Plant Mini Kits (Qiagen, Valencia, CA). To test all experimentally inoculated plants for infection with GLRaV-3, total RNA was extracted and denatured using tissue from three petioles of each plant sampled following Sharma et al. (2011). For all plant samples, one step RT-PCR was performed using Qiagen kits, followed by fragment analysis as described in Sharma and others (2011) and denatured following Sharma et al. (2011), A fluorescent primer pair CP was used, sequence F: 5' GAA CTG AAA TTA GGG CAG ATA TA 3', R: 5' AAR AAC TTG TCT GGA TCY TT 3', which amplifies a 320 bp fragment of GLRaV-3 (Sharma et al. 2011).

To test samples for infection with GVB, the following primer pair was used: F: 5′ GTG CTA AGA ACG TCT TCA CAG C 3′, R: ATC AGC AAA CAC GCT TGA ACC G, which amplifies a 460 bp fragment of a putative RNA binding protein (Gambino and Gribaudo 2006). For GFkV the following primer pair was used: F: 5' TGA CCA GCC TGC TGT CTC TA 3', R; 5' TGG ACA GGG AGG TGT AGG AG 3', which amplifies a 179 bp fragment of the coat protein region (Gambino and Gribaudo 2006). The two primer pairs were multiplexed into one RT-PCR reaction, with each primer's concentration 0.5 μmol. The same extraction and denaturing procedure describe above was used. Samples were tested using the abovementioned Qiagen OneStep RT-PCR kits, with thermal cycler conditions as follows: reverse transcription 50 °C for 30 min, initial activation 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. RT-PCR products were visualized using agarose gel electrophoresis and ethidium bromide staining (Gambino and Gribaudo 2006; Voncina et al. 2011). The Foundation Plant Services LV-89-01 was used as an infected positive control, and known virus-free Pinot noir was used as an uninfected negative control for all diagnostic assays.

Data analyses

For each field and laboratory experiment, proportions of resulting successful inoculations from replicate source plants were compared using a Pearson chi-square test; proportions of successful inoculations did not differ, and therefore infected source plants were pooled for further analyses. A chi-square test revealed that caged and uncaged treatments did not differ in the field or laboratory studies, and data from caged and uncaged treatments were therefore pooled for all analyses. For each transmission experiment, proportions of recipient test plants that became infected with GLRaV-3 in each treatment were compared using chi-square tests. We calculated the estimated probability of transmission by

a single insect following Swallow (1985). The Swallow estimator can be used to estimate the probability that one insect will transmit a pathogen based on the number of insects used per recipient test plant, the number of recipient plants tested, and the proportion of recipient test plants that become infected. For the detailed symptom survey in Year Two on symptomatic vines only, we tested for a difference in the proportion of leaves that were symptomatic among spurs, using a generalized linear model with a Gaussian distribution; proportion data were arcsine-transformed prior to analysis to better meet the assumptions of the model. All above analyses were conducted using R Version 3.2.0. To assess the effects of GLRaV-3 infection on berry quality, °Brix, pH, and titratable acidity of symptomatic and asymptomatic vines were compared using a repeated measures ANOVA, using SPSS Version 23. We found no effect of GVB infection on any of the variables measured in our field experiment; therefore the four vines that became infected with both GVB and GLRaV-3 were included with GLRaV-3-infected vines in our analyses.

Results

Field symptoms developed in Year Two

Visual monitoring for symptoms of grapevine leafroll disease determined that symptoms did not develop until Year Two (Fig. 1). No vines became symptomatic during Year One, when mealybug inoculations were performed. During Year Two, five vines first showed symptoms on 22 June, and 29 vines developed symptoms by 6 July 2012. In Year Three, symptoms first appeared on 29 May and the same 29 vines were symptomatic by 3 June. During Year Four, symptoms first appeared on 28 May, and the same 29 vines were symptomatic by 14 June. No spacer vines in the experimental area became symptomatic for grapevine leafroll disease during the study, and no mealybugs were found from the survey in October of Year Two.

Diagnostic testing and disease symptoms reached concordance in Year Three

In October of Year One, the petiole of the inoculated leaf was collected from each vine and tested for the presence of GLRaV-3 using RT-PCR. Twenty of 60 inoculated vines tested positive for GLRaV-3. In June of Year Two, 24 of 60 inoculated vines tested positive, and in Year Three, 29 of 60 vines tested positive (Fig. 2). There was 100 % concordance between the 29 symptomatic vines and the 29 vines that tested positive for GLRaV-3. Fifteen caged vines and 14 uncaged vines became infected with GLRaV-3. The caged and uncaged treatments did not significantly differ from each other in number of vines that tested positive for GLRaV-3 ($\chi^2 = 0.04$, df = 1, $P = 0.842$). No negative control vines developed grapevine leafroll disease symptoms or tested positive for GLRaV-3. We found no spread beyond our experimental inoculations during the four years that we monitored this site, which corresponded to our finding of no active mealybug populations at the site during that time. GLRaV-3 was the only pathogen detected in the

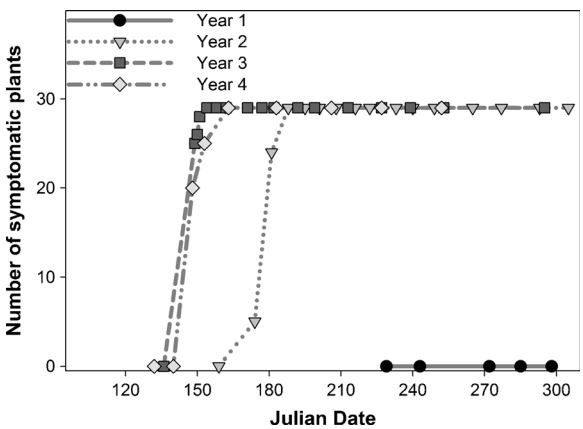

Fig. 1 Number of experimental vines that showed visible grapevine leafroll disease symptoms over the course of four growing seasons. Vines were inoculated in Year One, Julian Date 199

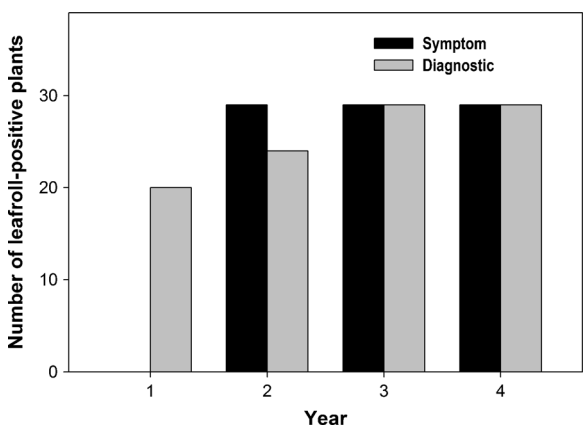

Fig. 2 Number of experimental vines that tested positive for GLRaV-3 using diagnostics or showed visible disease symptoms following inoculations

symptomatic vines that were tested for the full panel performed by a commercial service, and no viruses were detected in the asymptomatic vines.

Early disease symptoms were not localized within vines

On symptomatic vines, the proportion of leaves that were symptomatic on each spur ranged from 0 to 33 %. On all 29 symptomatic vines, the spur position with the lowest mean proportion of symptomatic leaves was 6 south (mean = 4 %, SE = 1 %), and the spur position with the highest proportion of symptomatic leaves was 2 north (mean = 12 %, SE = 4 %) (Fig. 3). However, the proportion of leaves that were symptomatic did not significantly differ among spurs (F = 1.503, df = 11/17, $P = 0.13$). Of the 29 symptomatic vines, 13 were inoculated on the second spur south of the central trunk, four on the third spur south of the second trunk, four on the fourth spur south of the second trunk, one vine on the third spur north of the trunk, and the inoculation marker was lost from the remaining seven vines.

Berry quality was affected in Year Two

Berry quality was affected by GLRaV-3 infections in Years Two and Three (Fig. 4, Table 1). In Year Two, °Brix was significantly lower in the infected vines, increased over the three sampling times preceding harvest, and there was a significant interaction between time and vine infection status (Table 1). At the final time point, immediately before harvest, °Brix was lower in the infected vines than in the uninfected vines (F = 162.169, df = 1/88, $P < 0.0001$) (Fig. 4a). In Year Two, pH was significantly lower in the infected vines, increased with time, and there was no significant interaction between sampling time and vine infection status (Fig. 4b, Table 1). Titratable acidity decreased during the sampling period preceding harvest, and vine infection status affected the change in titratable acidity during the sampling time. Infected vines had higher titratable acidity than uninfected vines (Table 1). For the first two sampling periods, titratable acidity differed between infected and uninfected vines (31 Aug: F = 20.924, df = 1/88, $P < 0.0001$; 21 Sept: F = 7.603, df = 1/88, $P = 0.007$), but at the final sampling time point, immediately before harvest, there was no significant effect of vine infection status on titratable acidity (F = 0.885, df = 1/88, $P = 0.350$) (Fig. 4c).

In Year Three, berry quality data were collected on a subset of 30 vines, on two dates leading up to harvest. Degrees Brix was reduced in the infected vines, increased between the two sampling dates, and there was no significant interaction between sampling date and vine infection status (Fig. 4d, Table 1). In Year Three, pH was significantly higher in uninfected vines, increased between sampling dates, and there was no significant interaction between sampling time and vine infection status (Fig. 4e, Table 1). Titratable acidity was significantly higher in infected vines, decreased between sampling points, and there was a significant interaction between time and vine infection status (Fig. 4f, Table 1). Similar to Year Two, titratable acidity

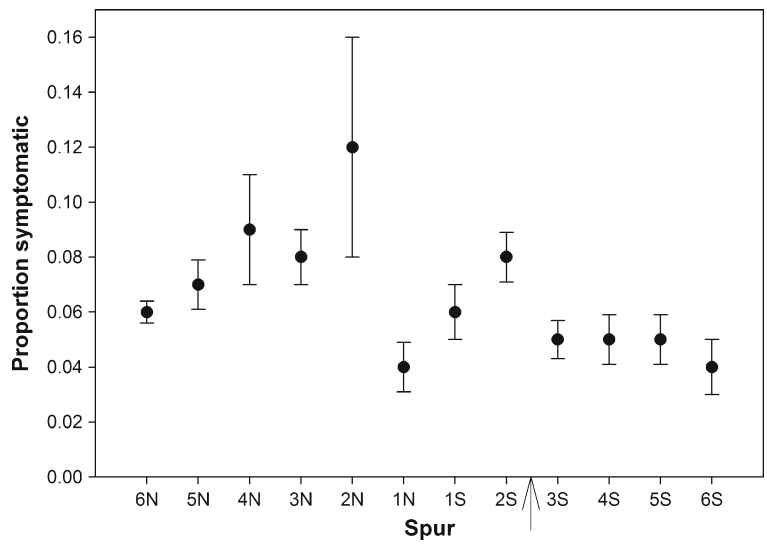

Fig. 3 Proportion of leaves that showed grapevine leafroll disease symptoms in July of Year Two, mean ± standard error. Each point on the x axis represents one vertical spur growing from the horizontal cordon. The vines' trunk is between spurs 1S and 1 N (S = south, N = North). Spurs did not significantly differ in proportion of leaves infected ($P = 0.13$). The *arrow* below the x axis indicates the average spur position of inoculation

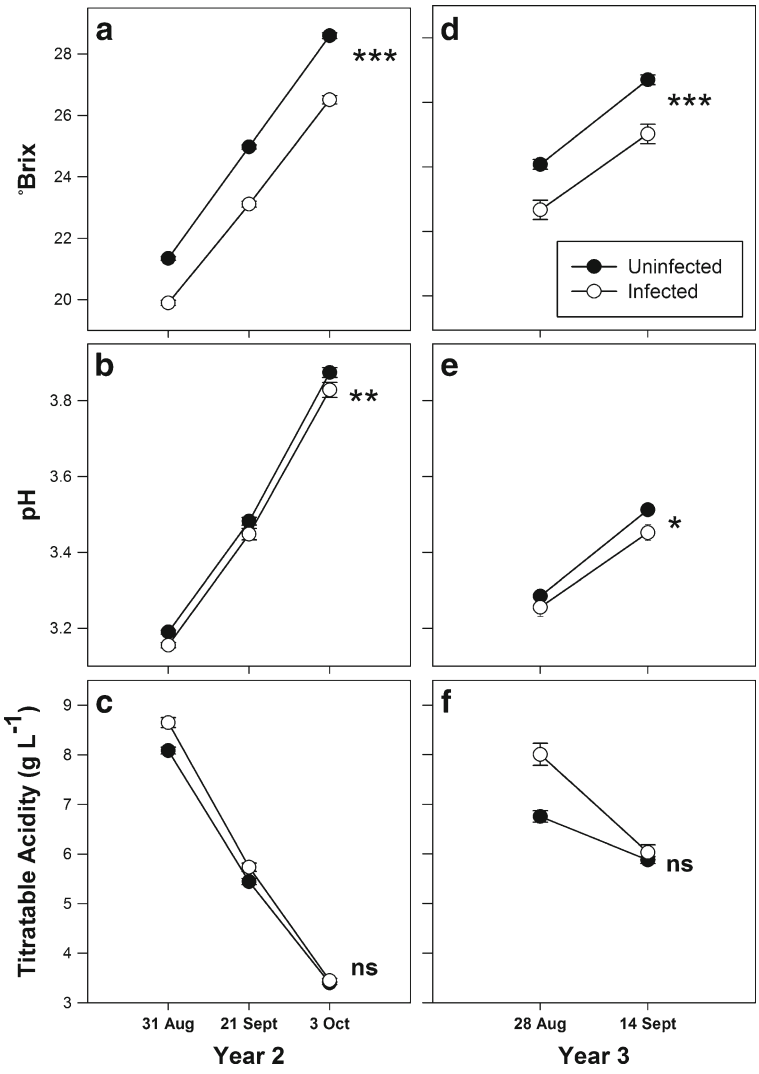

Fig. 4 Degrees Brix (**a** and **d**), pH (**b** and **e**), and titratable acidity (**c** and **f**) of berries in the weeks leading up to harvest in Year Two (**a**, **b**, and **c**) and Year Three (**d**, **e**, and **f**), mean ± standard error. Statistically significant differences are indicated for the final sampling time each year: ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$

was higher in infected vines at the first sampling date (F = 30.931, df = 1/28, $P < 0.0001$), but not at harvest (F = 1.187, df = 1/28, $P = 0.285$) (Fig. 4f).

GVB transmission

Four of the field vines that became infected with GLRaV-3 also became infected with GVB. No vines that did not become infected with GLRaV-3 became infected with GVB. Berry quality was compared between the vines with mixed infections and vines with single GLRaV-3 infections using a t test, and there was no significant effect of GVB infection ($P = 0.73$–0.99). The four vines that became infected with GVB were inoculated by insects from four different source vines. Exploratory analyses of other variables, including timing of symptom development, time to GLRaV-3 detection using diagnostics, and severity or within-vine distribution of symptoms, indicated that there was no effect of GVB infection on disease indicators that we measured. Following Swallow (1985), we found that the estimated probability of GVB transmission to vines that became infected with GLRaV-3 by a single insect was $Ps = 0.007 \pm 0.0005$ 95 % CI. No inoculated vines became infected with GFkV.

Laboratory versus field transmission experiment

Similar to the field study, a laboratory study found no significant difference in transmission between the caged

Table 1 Repeated measures ANOVA for berry quality measurements

Response Variable	Source	Year Two			Year Three		
		F	df[c]	P[a]	F	df[c]	P
°Brix	Infection	244	88	<0.001***	30.9	28	<0.001***
	Time	11,463	88	<0.001***	545	28	<0.001***
	I*T[b]	24.2	88	<0.001***	1.54	28	0.23 ns
pH	Infection	7.80	88	0.006**	4.29	28	0.048*
	Time	2409	88	<0.001***	301	28	<0.001***
	I*T	0.263	88	0.769 ns	1.60	28	0.22 ns
Titratable Acidity	Infection	15.9	88	<0.001***	22.7	28	<0.001***
	Time	8072	88	<0.001***	149	28	<0.001***
	I*T	21.8	88	<0.001***	22.1	28	<0.001***

[a] *ns* not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

[b] I*T indicates the interaction between infection and time

[c] The denominator of the degrees of freedom is shown. For both Year Two and Year Three, the numerator of the degrees of freedom is 1

and uncaged inoculation treatments ($\chi^2 = 0.9$, df = 1, $P = 0.34$). In the laboratory experiment, 22 of 30 recipient test vines with insect cages and 19 of 30 recipient test vines with no insect cages became infected with GLRaV-3, for a total of 41 out of 60 vines. In the field experiment, just 29 out of 60 inoculated vines became infected with GLRaV-3. Significantly more vines became infected in the laboratory study than in the field study ($\chi^2 = 8.83$, df = 1, $P = 0.003$). We note that multiple variables differed between field and laboratory, including vine variety, age, and physiological status.

At the conclusion of the 48 h inoculation access period and before insecticide treatment in both studies, we counted the number of mealybugs that we were able to find on each recipient test plant's inoculated leaf. Because the first instar mealybugs are roughly 1 mm in size and pale in color, this provides merely an estimate of how many mealybugs may have successfully settled and fed on each recipient test vine. In the field, we found an average of 0.89 mealybugs per vine (range 0–4 mealybugs per vine). In the laboratory, we found an average of 3.47 mealybugs per vine (range 0–10 mealybugs per vine). A t-test revealed significantly more mealybugs on each recipient vine in the laboratory study ($t = 6.776$, df = 118, $P < 0.0001$). Using the mean number of mealybugs found per recipient vine for each experiment and following Swallow (1985), we found that the estimated probability of transmission by a single insect was $Ps = 0.06 \pm 0.003$ 95 % CI in the field study, and $Ps = 0.10 \pm 0.003$ 95 % CI in the laboratory study, still lower in the field than in the laboratory.

GLRaV-3-infected vines were effective virus sources in Year Two

In October of Year Two, immediately following harvest and 15 months after inoculations, cuttings were taken from experimental vines and brought to the laboratory for testing as sources for transmission of GLRaV-3 by *Pl. ficus*. Transmission occurred from all symptomatic cuttings. No recipient cuttings from asymptomatic source vines became infected with GLRaV-3. Thirty-five of 62 recipient test vines from symptomatic source vines became infected with GLRaV-3. Based on Swallow (1985), the estimated probability of transmission of GLRaV-3 by a single insect was $Ps = 0.08 \pm 0.003$ 95 % CI. Transmission of GVB was not tested.

Discussion

Our vineyard inoculations provide the first mealybug-borne GLRaV-3 transmission study under realistic commercial vineyard conditions, providing corroboration that other laboratory transmission studies of GLRaV-3 are predictive of mealybug-borne transmission in commercial vineyards. In the field study, three months after vector inoculation, GLRaV-3 infections were detected in the petiole of the inoculated leaf of approximately two

thirds of all vines that ultimately became infected, indicating that early localized infections in commercial vineyards can be detected using diagnostics well before the appearance of disease symptoms.

Grapevine leafroll disease symptoms first appeared early in the year of the growing season following mealybug-mediated inoculations, and were present in all infected vines within a two week time frame. Appearance of disease symptoms was more consistent and narrow in timing than was diagnostic detection, which increased for two years following inoculations. Symptoms first appeared without localization to the point of inoculation, indicating that systemic infection had established before the first expression of symptoms. Furthermore, newly infected field vines were effective sources for mealybug-borne transmission one year after inoculation, providing additional evidence of rapid establishment of systemic infection. Berry quality was also affected one year after inoculations, indicating that infection had an effect on vine physiology as early as one growing season following inoculations. Only vines that were infected with GLRaV-3 also tested positive for GVB, indicating that GVB may have some dependence on GLRaV-3 during transmission or establishment in a new host. There were much fewer infections with GVB than with GLRaV-3. There was no evidence that GVB affected disease symptoms or progression compared with vines that were infected only with GLRaV-3.

Results of laboratory-based transmission studies can differ from realistic field conditions (e.g. Hooks et al. 2009), and there is considerable variation in estimates of transmission efficiency of GLRaV-3 among laboratory studies (Almeida et al. 2013). The laboratory and field studies were consistent with each other in that there was no effect of caging the insect vectors on the recipient test vines on virus transmission. There was higher transmission efficiency based on our laboratory experiment compared with our field study. This may have been due in part to the controlled conditions indoors compared with outdoors, and the improved ability of first instar mealybugs to settle and feed on recipient test vines in the laboratory. We note that different grape varieties were used as recipient test vines in the field and laboratory studies, which limits the direct comparison of the two studies. In addition, plants used in the laboratory study were only tested for GLRaV-3 (not GVB and GFkV); it is possible that interactions among virus species could have influenced vector transmission and pathogen establishment. The physiological status of mature field vines compared to greenhouse cuttings that were several weeks old at the time of inoculation may also influence virus transmission. Despite these relevant caveats, the overall finding is suggestive that laboratory studies may overestimate vector transmission efficiency. Other studies that have compared laboratory and field results have had mixed findings. For example, Hooks et al. (2009) also found a higher transmission efficiency of *Banana bunchy top virus* by aphids based on laboratory experiments compared to commercial agricultural conditions. Another study that included transmission of *Cucumber mosaic virus* and *Zucchini yellow mosaic virus* by multiple aphid species found that the relative transmission rates between field and laboratory conditions depended both on aphid and virus species (Castle et al. 1992). We propose that this question should be addressed in more detail in future studies due to its epidemiological relevance.

While our findings are informative, similar studies should be performed with other mealybug species, genetically distinct variants of GLRaV-3, grape cultivars, and in different climatic conditions to evaluate the general applicability of the results (Bahder et al. 2013; Blaisdell et al. 2015; Salem-Fnayou et al. 2006). For example, berry quality of white wine varieties is affected by GLRaV-3 infection, yet resulting disease symptoms are not readily identifiable (Kovacs et al. 2001). Another open question with respect to disease progression is the amount of time that passes after graft-mediated infections before symptoms can be identified or diagnostic tools can detect new GLRaV-3 infections in the recipient vine. Even though spread of grapevine leafroll disease was documented 25 years ago (Engelbrecht and Kasdorf 1990), many parameters that contribute to spread and progression remain poorly understood.

In Napa Valley, *Ps. maritimus* produces two synchronous generations per year that do not overlap, and our inoculation study coincided with the emergence of the second generation in July (Daane et al. 2012). Differing climatic conditions during the first generation, which usually emerges in March, could potentially lead to different transmission efficiency and different timing of disease development. Varied climatic conditions could lead to variation in host traits and resulting host susceptibility, variation in the virus life cycle in response to varying environmental conditions within host and vector, and variation in mealybug activity that could lead to higher or lower transmission efficiencies. There is a need for understanding how the interactions

between host, vector, and pathogen are affected by climate and seasonality.

Other mealybug species in California produce higher numbers of overlapping generations; therefore vines may be vulnerable to new infections throughout the year (Daane et al. 2012). For example, *Pl. ficus* produces four to seven overlapping generations per year, and is not yet present in most areas of Napa Valley, CA, where our field study was conducted (Daane et al. 2012). If the invasive *Pl. ficus* establishes more widespread populations in Napa Valley and other areas of Northern California at the higher densities typically associated with this species, spread of GLRaV-3 could dramatically increase.

In summary, the information provided by our study regarding key biological traits of GLRaV-3 can inform sound management practices. For example, when certified virus-free material has been used for planting, newly symptomatic vines can be used to infer that the infection resulted from insect-borne inoculations made during the previous growing season, and that the newly symptomatic vines can be an efficient source for further disease spread. Decline in crop quality can be expected during the same growing season in which symptoms first appear, which may influence roguing strategies based on economic models (e.g. Atallah et al. 2012; Ricketts et al. 2015).

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