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Effect of substrate voltage on EPG recordings of ingestion and probing behavior in *Diaphorina citri* (Hemiptera: Liviidae)

Timothy A. Ebert and Michael E. Rogers*

Abstract

Diaphorina citri Kuwayama (Hemiptera: Liviidae) is a major pest of citrus production because it is the vector of *Candidatus* Liberibacter asiaticus (Rhizobiales: Rhizobiaceae), which causes Asiatic huanglongbing, a devastating disease of citrus. Understanding the probing and ingestion behavior of the vector is important in understanding pathogen transmission and possible strategies to reduce disease incidence. We assessed the feeding behavior in *D. citri* by using electropenetrography, wherein a small electric current is passed through the insect. Changes in circuit voltage are recorded, and patterns of voltage changes are subsequently correlated with specific behaviors. However, different laboratories use different equipment with varying applied voltages. It is axiomatic that there will be some voltage at which there will be a change in the behavior of the insect. Current equipment has a range from 0 to 1300 mV, but studies where voltage was reported were in the range from 20 to 600 mV. The purpose of this study was to determine the behavioral response of *D. citri* to voltages in this range. Our results demonstrated that 600 mV DC with an input impedance of 10^9 ohms was below the threshold where *D. citri*'s feeding behaviors were affected. Thus, the outcomes of past studies using different voltages should not have been affected. However, in the present study, we did find that choice of host plant altered *D. citri*'s feeding behavior; thus, it would be beneficial to standardize the host plant if you were studying the effects of non-host factors that may influence feeding, such as insecticides, interspecific competition, or abiotic conditions.

Key Words: experimental design; multivariate analysis; host plant

Resumen

Diaphorina citri Kuwayama (Hemiptera: Liviidae) es una plaga importante de la producción de cítricos, ya que es el vector de *Candidatus* Liberibacter asiaticus (Rhizobiales: Rhizobiaceae), lo que provoca Huanglongbing, una enfermedad devastadora de los cítricos. El entender del comportamiento de prueba y la ingestión del vector es importante en entender la transmisión de patógenos y las posibles estrategias para reducir la incidencia de la enfermedad. Se evaluó el comportamiento de alimentación de *D. citri* mediante el uso de electropenetrografia (EPG), en el que una pequeña corriente eléctrica pasa a través del insecto. Los cambios en la tensión del circuito se registran y los patrones de cambios de voltaje son posteriormente correlacionados con comportamientos específicos. Sin embargo, diferentes laboratorios utilizan diferentes equipos con diferentes voltajes aplicados. Es axiomático que habrá algo de tensión a la que habrá un cambio en el comportamiento del insecto. el equipo actual tiene un rango de 0 a 1300 mV, pero los estudios en los que se informó de tensión estuvo en el intervalo de 20 a 600 mV. El propósito de este estudio fue determinar la respuesta de comportamiento de *D. citri* a voltajes en este rango. Nuestros resultados demuestran que 600 mV CC con una impedancia de entrada de 109 ohmios estuvo por debajo del umbral en el que se vieron afectado el comportamientos de alimentación de *D. citri*. Por lo tanto, los resultados de estudios anteriores que utilizan diferentes voltajes no deben haber sido afectados. Sin embargo, en el presente estudio, encontramos que la elección de la planta hospedera alteró el comportamiento de alimentación de *D. citri*; por lo tanto, sería beneficioso para estandarizar la planta hospedera cuando se estudia los efectos de los factores no-hospederos que pueden influir la alimentación, tales como insecticidas, la competencia entre especies, o condiciones abióticas.

Palabras Clave: diseño experimental; analisis multivariable; planta hospedera

The Asian citrus psyllid, *Diaphorina citri* Kuyawama (Hemiptera: Liviidae), transmits a phloem-limited alpha proteobacterium (*Candidatus* Liberibacter asiaticus) that is the putative causal agent of Asiatic huanglongbing. The psyllid was first reported in Florida in 1998, and the disease was first detected in Florida in 2005 (Halbert & Manjunath 2004; Grafton-Cardwell et al. 2013). The disease kills citrus trees, resulting in extensive economic losses (Farnsworth et al. 2014). In the USA, the Asian citrus psyllid was first reported in Texas in 2001 (French et al. 2001) and California in 2008 (Grafton-Cardwell 2010), and the disease was subsequently detected in both Texas (Kunta et al. 2014) and California in 2012 (Kumagai et al. 2013). Because *Candidatus* Liberibacter asiaticus is phloem limited, it is important to understand the phloem-related feeding behaviors of *D. citri*. However, in this study, we examined how experimental methods might influence results and impact the ability to compare those results among laboratories that have used different methods.

The most rigorous experimental method for studying hemipteran probing and ingesting behavior is through electropenetrography (EPG), a technique first developed over 50 yr ago (McLean & Kinsey 1964). The original equipment design has been improved greatly in terms of both sensitivity and usability (Tjallingii 1978, 1985; Backus & Bennett 2009). The output is a temporal pattern of voltage shifts reminiscent

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of an electrocardiogram or electroencephalogram. Stereotypical repetitive patterns within these recordings are called waveforms. This method has been used to study a number of psyllid species: *D. citri* (Bonani et al. 2010; Zhu et al. 2010; Serikawa et al. 2013; Luo et al. 2015), *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) (Butler et al. 2012; Pearson et al. 2014; Sandanayaka et al. 2014), and *Cacopsylla pyri* L. (Hemiptera: Psyllidae) (Civolani et al. 2011, 2013).

Waveforms in *D. citri* were originally characterized and correlated as non-probing (NP), pathway (C), xylem ingestion (G), phloem contact (D), phloem salivation (E1), and phloem ingestion (E2) (Bonani et al. 2010). Subsequently, the non-probing waveform was subdivided into walking (NP), and standing still (Z) (Youn et al. 2011). Furthermore, pathway has been subdivided into initial stylet penetration and salivation (A), epidermis mesophyll sheath salivation (B), and pathway (C) (Yang et al. 2011; Cen et al. 2012).

The objective of this study was to document behavioral changes in *D. citri* as the applied voltage changed from 20 mV to 150 mV to 600 mV DC. Recently, it was suggested that an upper limit of 150 mV be used for the applied voltage due to distortions in the appearance of the recorded waveforms at higher voltages (Pearson et al. 2014), but this was for another psyllid species with no examinations made of differing voltages on insect feeding behavior. Previous studies in M. E. Roger's laboratory used a substrate voltage of 150 mV DC. However, this voltage is higher than typically reported in the literature (Table 1). In the present study, we examined a range of applied voltages to determine if the applied voltage might influence *D. citri*'s feeding behavior.

Changing the applied voltage in EPG experiments can be used to improve the quality of the recorded signal. Although we know that the applied voltage will affect the insect at some point, we do not know exactly where that critical point occurs. As apparent in Table 1, many researchers do not report the applied voltage. This may be because of a doubt expressed by McLean that the current flowing through an aphid was sufficient to be detectable by the insect (McLean 1977). In turn, this doubt was influenced by earlier work in mosquitoes suggesting that the current flowing through the insect was less than the conjectured static charge generated during flight (Kashin & Wakeley 1965). These assumptions were not tested. Recent data indicate that some insects will alter their behavior in response to the applied voltage (Cervantes & Backus 2015). Because it has been shown that some insects respond to current within the ranges achievable by modern equipment, it is important to determine the effect this might have on interpreting results or comparing results among researchers.

Materials and Methods

PLANTS AND PSYLLIDS

We used *Citrus sinensis* (L. Osbeck.) Valencia orange scion on Kuharske citrange (*C. sinensis* × *Poncirus trifoliate* (L.) Raf.) rootstock (Rutaceae) planted in 3.92 L black plastic pots measuring 18 cm at the rim and 18 cm deep filled with Fafard Professional Custom Mix (Agawam, Massachusetts). Plants were pruned to a height of 51 cm from soil surface. As needed, artificial light was provided by high-pressure sodium lamps (16:8 h L:D photoperiod). Additional data were used in this manuscript (described later), and some of those data come from experiments that used *C. sinensis* Midsweet orange scion on Kuharske rootstock grown under the aforementioned conditions.

Psyllids used for EPG recordings were obtained from a *Candidatus* Liberibacter asiaticus –negative colony maintained in the laboratory at the Citrus Research and Education Center, Lake Alfred, Florida. The colony was tested periodically for *Candidatus* Liberibacter asiaticus by using polymerase chain reaction methods (Li et al. 2006) but has never tested positive (results not shown). We did not sex or age the psyllids, although sex-based behavioral differences had been reported before (Serikawa 2011). The plants used for colony maintenance were the same cultivar and from the same source as the plants used in the experiment.

EPG METHODS

All insects were recorded feeding on the abaxial surface of young leaves. Young leaves were light green, not fully expanded, and of unknown age. The main criterion for defining a young leaf was stickiness. If you rub your finger over a mature leaf, the waxy coating makes the leaf feel slick. You can apply pressure, and your finger will still slide across the surface. Young leaves lacked this waxy coating, and the leaf would get damaged if you forced your finger across the surface. Such leaves are found only within 3 to 5 leaves of the apical meristem.

We used two 4-channel AC-DC monitors (Backus & Bennett 2009) custom-built by William H. Bennett (EPG Equipment Co., Otterville, Missouri) in DC mode with 20, 150, or 600 mV substrate voltage and 160× adjusted amplification at the control box, and 100× fixed amplification in the head-stage amplifier. Data were acquired through a DI710 AD converter (Akron, Ohio) using Windaq software at a sampling rate of 100 Hz per channel. Psyllids were tethered using a 2 cm long and 25.4 µm diameter gold wire (Sigma Cohn Corp., Vernon, New York) attached to thoracic tergites by using silver glue (1:1:1 w/w/w, white glue:water:silver flake [8–10 µm; Inframat Advanced Materials, Manchester, Connecticut]). The other end of the gold wire was attached to a 23 mm long (0.48 mm diameter) copper wire by using the silver glue. This wire was soldered to a 20 mm long and 1.14 mm diameter brass nail that was inserted into the unit's head amp that was set to an impedance of 10^9 ohms. To complete the circuit, a 10 cm long (2 mm diameter) copper wire was inserted into the water-saturated soil of the pot with the plant. There was a 30 min starvation period from the time the insects were removed from the colony until they were placed on the plant. All insects were wired during this period. Psyllids were not chilled or anesthetized with CO_2. Recording

Table 1. Voltages used in EPG studies on members of the Psylloidea.

Voltage in mV	AC or DC	Species	Reference
Unknown	DC	*Diaphorina citri*	(Bonani et al. 2010; Zhu et al. 2010; Yang et al. 2011; Youn et al. 2011; Cen et al. 2012; Luo et al. 2015; Miranda et al. 2016)
	DC	*Bactericera cockerelli*	(Sandanayaka et al. 2014; Mustafa et al. 2015)
	DC	*Cacopsylla pyri*	(Civolani et al. 2013)
15	DC	*Diaphorina citri*	(Serikawa 2011)
30	DC	*Bactericera cockerelli*	(Butler et al. 2012)
25 to 75	AC	*Bactericera cockerelli*	(Pearson et al. 2014)
20 to 600	DC	*Bactericera cockerelli*	(Pearson et al. 2014)

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was started before psyllids were placed on the plant to ensure that all recordings started in the NP behavior. Recordings were 23 h in duration. We chose to measure only the original 6 waveforms (NP, C, D, E1, E2, G) (Bonani et al. 2010).

To reduce electronic noise, the plants and insects were placed in a Faraday cage built as an aluminum frame covered with pure copper screen (16 × 16 mesh = 0.15 mm wire spaced 1.58 mm apart). A ring stand was placed in the Faraday cage to support the electronics and facilitate placement of the insect on a specific leaf. In all cases, the leaf was held with double-sided tape to the outside flat surface of at 35 mm diameter plastic Petri dish. This helped ensure that the insect was feeding at a specific location, and it prevented plant movement from breaking the electrical circuit. Light was provided by overhead fluorescent lights (24:0 h L:D photoperiod). Room temperature was maintained at 26.6 °C.

PROCESSING RAW DATA

Ebert 2.0 (http://www.crec.ifas.ufl.edu/extension/epg/sas.shtml) was used to analyze the data. Ebert 2.0 is a version of Ebert 1.0 (Ebert et al. 2015), modified specifically for analysis of data from psyllids. Ebert 2.0 calculated all variables in the Sarria workbook (Sarria et al. 2009) except those involving pd, F, and E1e. While some variables were removed, others were added for a more detailed analysis of the D and G waveforms. Variables measured included: number of probes to first D, number of probes to first G, number of D, total duration of D, duration of Np before first D, duration of Np before first G, mean duration of D, average number of D per probe, average number of G per probe, time from first probe to first D, time from first probe to first G, time from start of probe with first D to first D, time from start of probe with first G to first G, number of probes after first D, number of probes after first G, number of probes <3 min after first D, number of probes <3 min after first G, number of sustained G (>10 min), time to first sustained G (>10 min), duration of longest D, duration of longest G, and percentage of probing in D. We also added standard deviation of the duration of waveforms Np, C, G, D, E1, and E2. There were in total 85 variables available in the following analyses (for a list, see supplementary file online at http://purl.fcla.edu/fcla/entomologist/browse).

DATA ANALYSES

Synopsis

All variables were transformed to improve model fit based on a quantile plot of the residuals. Treatment differences were assessed using the Tukey–Kramer multiple-comparison procedure as implemented in Proc Glimmix in SAS (SAS Institute 1988). Use of mixed-model ANOVA results in improved power of statistical tests compared with non-parametric ANOVA (Gbur et al. 2012). Given the results from this analysis, additional analyses were performed using data from previous experiments as a means to provide further context (Table 2). The equipment and setup were the same for all of these past experiments as for this experiment, except that the voltage used in these other trials was always 150 mV. These data provide small differences that can be compared with the observed effects of voltage.

These data were analyzed using Proc StepDisc to reduce the number of variables. The variables identified in this step were subsequently used in the Proc Discrim procedure in SAS to get Mahalanobis distances and to get a classification error rate. The Mahalanobis distance is a unitless measure of the difference between groups or treatments. It is useful for comparing multivariate treatment effects when the units of the independent variables are different. The classification error rate is where SAS builds a statistical model but leaves one observation out of the data set. It then uses the statistical model to try and predict where that observation comes from. SAS repeats the procedure for every insect. The goal is to have more correct classifications than would be predicted by chance alone.

Details of Data Analysis

There are two options when using Proc Glimmix for dealing with heteroscedastic variables, and models with residuals that are not normally distributed. Historically, these problems were handled by transforming the data, but in Proc Glimmix one can change the underlying distribution. Both options were tried, with counts either Poisson distributed or square root transformed, and time was either Gamma distributed or log transformed. Percentages were either Beta distributed or logit transformed. If logit transformation did not help, then arcsine square root transformation was attempted. The other continu-

Table 2. A list of supplemental sources of data used in this research. All plants used the Kuharske rootstock with either Valencia or Midsweet scion. The number of insects in each trial is *n*.

Trial	*n*	Date	Plant	Voltage	Insecticide
A[a]	8	17 Mar 2015	Valencia	150 mV	Dibrom[b]
B	37	10 Jul 2013	Midsweet	150 mV	
C	27	11 Jul 2013	Midsweet	150 mV	
D	22	10 Sep 2014	Midsweet	150 mV	
E	22	10 Jul 2014	Valencia	20 mV	
F	21	10 Jul 2014	Valencia	150 mV	
G	20	10 Jul 2014	Valencia	600 mV	
H	21	14 Nov 2014	Valencia	150 mV	
I	18	16 May 2013	Midsweet	150 mV	
K	21	16 Nov 2012	Midsweet	150 mV	
L	14	3 Jul 2013	Midsweet	150 mV	
M	20	4 Sep 2013	Midsweet	150 mV	
N	18	24 Jan 2014	Valencia	150 mV	
O[c]	20	2 Apr 2015	Valencia	150 mV	Admire[d]

[a]Trial A had 8 insects feeding on plants treated with dibrom 21 d earlier. Plants were held in the greenhouse and subject to standard growing practices.
[b]1,2-dibromo-2,2-dichloroethyl dimethyl phosphate, CAS 300-76-5.
[c]Trial O used plants previously treated with imidacloprid with residues at 30 ppb including analytes. This value was from a combined sample of many flushes, but lacks replication.
[d]N-(1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl) nitramide, CAS 105827-78-9.

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ous variables were either log transformed or distributed lognormal, exponential, or tcentral (*t*-distribution). A quantile plot was examined to determine the best approach. In general, counts were square root transformed, percentages were logit transformed, and everything else was log(variable + 0.1) transformed (for specific cases, see supplemental material online at http://purl.fcla.edu/fcla/entomologist/browse). These transformations were done with the sole purpose of improving the statistical models. We also attempted transforming all variables to a standard mean and variance using Proc Standardize, but no qualitative difference in outcome from multivariate procedures was observed. Therefore, the results we present are from non-standardized values.

We used a stepwise discriminant analysis (Proc StepDisc) to select a limited number of variables from the list of 85. Because these 85 variables were all correlated to some degree, it was possible that StepDisc could find a solution that was dependent on a specific variable entering the model and could find a better model if that variable was made unavailable. After the first run, one or more of the variables that entered this first model were removed and Proc StepDisc was run again. Stepwise discriminant analysis is similar in concept to stepwise regression analysis. There is a dependent variable and many independent variables. The method searches through the independent variables to find the one that is most significant (*P*-value to enter). It adds that variable to the model, and then recalculates all the *P*-values for all variables in the model. It searches these values and removes variables that have become non-significant (*P*-value to remove). Initially we ran the analysis using a *P*-value to enter of 0.08 and to remove of 0.06, but values of 0.3 and 0.2, respectively, were also tried. The best model was selected based on having the fewest number of independent variables and the ability of the model to distinguish between treatments. This model is not presented, but the variables from this model were used in Proc Discrim. By using a smaller number of variables, we eliminated most of the problems with correlation amongst the variables, and we were able to use nearly all the insects (287 out of 290 in the data set). We used Mahalanobis distances to understand how far apart the treatments were from each other. We used misclassifications to understand how accurate the model was in classifying the data.

We next focus on the problem of running an experiment with a large number of variables. It is seldom clear in the EPG literature how many variables are subjected to a statistical analysis. It is possible that researchers have reported all the variables that they tried, or that they tried all the variables and only reported the ones that showed a significant difference. In the latter case, this probably represents examination of 30 or more variables. Given this number, what is the probability of finding a few significant variables by chance alone?

In our case, we had 85 variables. Given that the null hypothesis was true and assuming that the outcome for each variable was independent of the outcome for the others, the probability of finding exactly 2 significant variables is $(0.05)^2*(0.95)^{85-2}$ (the general formula is $p^r q^{n-r}$ where $q = 1 - p$) multiplied by the number of ways that one can withdraw 2 objects from a list of 85. The general formula for doing this is where r is the number of items to be selected from a list of n items. In our specific case, this is to get a probability of about 0.13. Do this for all outcomes from 0 to 85, as in . Take the sum of the outcomes for the first three values in this series to find the probability of observing two or fewer significant variables. The probability of finding more than three significant variables is 1 minus this value or about 0.8 (Ross 1984). This does not mean that one should dismiss these significant outcomes. However, with so few significant differences, it does mean one should view these outcomes with some suspicion as the significant outcomes could have happened by chance alone. The exact number of significant variables necessary to rise above blind chance is related to the α used to declare significance, and the number of variables examined. As stated earlier, the assumption of independence needed in these calculations is not valid. It is unclear if these equations overestimate or underestimate the problem because that would be determined by whether the correlations between variables with significant differences was greater or less than the correlations between variables without significant differences.

All variables discussed in this manuscript are by insect. We used the name of this variable even after statistical analysis. However, the value displayed no longer matches the name. Thus, the total duration of E1 for each insect becomes a mean when these totals are averaged across insects. An elegant naming system was proposed to solve this issue for non-sequential variables (Backus et al. 2007), but the naming conventions become somewhat less user friendly when applied to the already long names for the sequential variables.

Results

The Tukey–Kramer multiple-comparison procedure resulted in 2 variables that showed significant treatment differences: number of probes after the first G, and number of short probes after the first G. If significance is determined by a *P*-value less than 0.05, then there is a 0.2 chance of finding 2 or fewer significant results and a 0.8 chance of finding 3 or more significant results. So there were fewer significant differences than we would expect by chance alone.

We could assume symmetry where if 2 variables were withdrawn from a group of 85 variables, the probability that they have significantly different means (significance is declared if $\alpha < 0.05$) equals the probability that the 2 samples have significantly similar means (by symmetry, the null-hypothesis is true if $\alpha > 0.95$) (Ebert et al. 1998). This should be true if the null-hypothesis is true. In the current study, there were 6 variables with *P*-values above 0.95: DurScndZ, NumD, NumE2, TtlDurD, PrcntPrbD, and TmFrstSusDFrstPrb (see supplemental data). Thus, there were more variables showing similarity than we should expect given that there were only 2 variables with *P*-values less than 0.05.

In the next step, we took the control treatments from experiments that we had done over the last 4 yr using the same methods as reported herein, but always at 150 mV applied voltage. We then compared the results from this experiment against the control treatments from those other experiments. The goal was to understand how the differences between the voltage treatments compared to the differences among other controls (Table 2). We noted that the distances between the voltage treatments (square box) were small relative to the other differences in the table, and within the voltage treatments there were no significant differences at $\alpha = 0.05$ (Table 3).

The distance between the different voltage treatments was less than the distance between any of the other Valencia trials. The average Mahalanobis distance between the voltage treatments was 0.66 (Table 4). The average within Valencia trial distance excluding the voltage treatments was greater than this distance (1.97 versus 0.66) and greater than the distance between any of the voltage treatments and the other Valencia trials (1.56). The same pattern holds if one examines the number of significant differences as a fraction of the total number of differences. None of the distances within the voltage treatment were significant, but many of the other distances were significant (Table 4). To eliminate the possibility that the results are an artifact of having a treatment that had pesticide residues, we also present the results having eliminated trial A from the analysis. While this changed the numbers, it had no effect on the overall conclusion that the voltage treatments were more similar to each other than the controls from other experiments conducted over the last 4 yr.

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Table 3. Mahalanobis distances between trials (lower triangular matrix) and the associated *P*-value (upper triangular matrix). The box highlights the voltage experiment results. The letters are for the trials (Trl) as described in Table 2.

Trl	A	B	C	D	E	F	G	H	I	K	L	M	N	O
A		0.00	0.02	0.00	0.01	0.01	0.03	0.08	0.02	0.00	0.01	0.00	0.00	0.01
B	3.55		0.00	0.38	0.16	0.11	0.00	0.01	0.01	0.01	0.60	0.30	0.01	0.00
C	2.19	2.77		0.00	0.00	0.00	0.00	0.02	0.23	0.08	0.01	0.00	0.00	0.21
D	3.00	0.40	2.29		0.75	0.88	0.08	0.49	0.10	0.05	0.10	0.16	0.42	0.00
E	2.90	0.60	2.88	0.25		0.96	0.14	0.22	0.00	0.00	0.03	0.02	0.12	0.00
F	2.57	0.71	2.84	0.17	0.10		0.06	0.42	0.01	0.01	0.02	0.04	0.28	0.00
G	2.31	1.51	1.61	0.96	0.81	1.06		0.19	0.01	0.00	0.01	0.00	0.00	0.01
H	1.75	1.25	1.15	0.42	0.67	0.48	0.74		0.34	0.16	0.03	0.00	0.46	0.00
I	2.56	1.27	0.63	0.94	1.74	1.52	1.65	0.58		0.93	0.20	0.01	0.16	0.02
K	3.15	1.32	0.89	1.09	1.80	1.62	2.12	0.79	0.14		0.15	0.00	0.26	0.03
L	3.01	0.38	1.85	1.10	1.48	1.60	1.81	1.56	0.92	1.01		0.20	0.01	0.04
M	3.54	0.49	3.56	0.77	1.38	1.16	2.30	1.72	1.62	2.04	0.91		0.00	0.00
N	3.71	1.35	2.21	0.51	0.91	0.66	1.91	0.49	0.89	0.71	2.04	2.06		0.00
O	2.97	2.28	0.60	2.41	2.46	2.84	1.47	1.70	1.31	1.26	1.44	3.90	2.60	

We looked at the ability of this model to predict the placement of a new insect. If the model was good, then all new insects would be correctly placed into their respective treatment/trial. Trial L was the most successful, with half of the insects from this trial being correctly placed (Table 5). Trial B was next at 29%. In general, the classification success rate was very low (median 10.5% correct). Although there may be some significant differences in these data, the best model that we could develop was ineffective at distinguishing differences between the voltage treatments. The misclassified insects for E and G would often be placed in trial A. Misclassified insects for F were frequently misclassified as coming from trial H. So the effect of voltage on the psyllid was generally less than the difference between two controls run a few months apart.

We next tested for differences between the psyllid feeding on 2 suitable hosts: Valencia and Midsweet. There were 23 variables that showed significant differences (Table 6). There are several features that are worth noting. Firstly, there were no significant variables associated with E2. Secondly, psyllids on Midsweet spent more time ingesting xylem than did psyllids on Valencia. Furthermore, the psyllid spent less time trying to find the xylem, and they made fewer repeated probes after finding the xylem. Thirdly, psyllids on Midsweet spent less time in C, thereby indicating that Midsweet had some feature that made it easier for the psyllid to reach phloem or xylem (Table 6). The Mahalanobis distance in psyllid ingesting behavior between Valencia and Midsweet was 5.4 with a *P*-value of 0.0004. It is therefore likely that there was a biologically significant difference in the behavior of the psyllid on these hosts.

Table 4. Average Mahalanobis distances and the mean number of significant distances for differences in probing and ingesting behavior of *D. citri*. "All" includes all the data; "Not A" includes all treatments except Trial A. EFG is restricted to values from treatments E (20 mV), F (150 mV), and G (600 mV). "Val-EFG" are all the trials on Valencia but not E, F, or G. "EFG*Val" are only values between EFG and all other Valencia. Val+EFG consists of trials A, E, F, G, H, N, and O. Midsweet consists of trials B, C, D, I, K, L, and M. "Val*Mid" is the distances between Valencia and Midsweet.

	Mahalanobis distance		Mean number of significant distances	
	All	Not A	All	Not A
EFG	0.66	0.66	0	0
Val-EFG	1.97	1.45	0.67	0.67
EFG*Val	1.56	1.33	0.58	0.44
Val+EFG	1.69	1.26	0.54	0.42
Midsweet	1.28	1.28	0.47	0.47
Val*Mid	1.68	1.45	0.74	0.69

Discussion

The original goal of this experiment was to document the effect of voltage on *D. citri*'s probing and ingesting behavior. We looked at 85 variables representing accepted measures for assessing significant differences in EPG data (Sarria et al. 2009, http://www.epgsystems.eu/systems.htm) or simple extensions of variables therein (e.g., Sarria has "time to first E2" so we added "time to first D"). Only 2 variables showed a significant treatment difference, and we showed that by chance alone one should expect more than 2 significant variables. We also showed that there were an unusually high number of variables with *P*-values of 0.95 or higher. We showed through a discriminant analysis that all the voltage treatments were clustered closer together than one should expect from running a series of controls. Furthermore, the classification model was unable to correctly identify the treatment from which the voltage data originated. For these reasons, we conclude that *D. citri* is unable to detect voltages of 600 mV or less under these experimental conditions. It would strengthen this conclusion if we had been able to identify a voltage that was detectable by *D. citri*, but that value lies outside the range of methods currently reported in the literature. That said, it is possible that waveforms may change shape somewhat at higher voltages as was observed for *B. cockerelli* (Pearson et al. 2014). This should be expected because the increased voltage improves the signal-to-noise ratio from the resistance component while making it worse for the emf component. It is therefore unclear if a change in waveform in *B. cockerelli* was associated with a concomitant change in behavior, though this may be the case.

To better understand the effect of voltage, we included some metadata. As an unexpected outcome, we were able to show that there was a detectable shift in the behavior of *D. citri* feeding on Midsweet versus Valencia oranges. The difference was associated with xylem ingestion being more attractive for psyllids on Midsweet and for psyllids on Midsweet to have fewer probes. There are a few possibilities for how this occurs. It is possible that host plant volatiles are different, thereby influencing a psyllid's perception of host quality. It is possible that small changes in morphology provide some mechanical protection in Valencia, or that Valencia has more chemical defenses in terms of the number of oil glands per leaf. Finally, it is possible that Mid-

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Table 5. Classification results for a "new" insect. Trial is the letter designation for the trials (Table 1). Correct was the number of insects from each trial correctly placed into that trial. Error was the number of times a mistake was made. "Most common" was the trial where most of the individuals from each treatment were placed. "Number misclassified" was the number of insects placed into each trial that did not belong there. Misclassified percentage is the number misclassified divided by the sum of all misclassified for that treatment. Misclassified source is the treatment most likely to be misclassified into the listed trial.

Trial	Correct	Error	Most common	Number misclassified	Misclassified percentage	Misclassified source
A	1	7	E	5	2.0	B,C,E,G,H
B	10	24	B	30	12.0	D
C	4	23	O,C,D,M	22	8.8	O
D	1	21	B,M	27	10.8	B
E	2	20	B	20	8.0	B
F	2	19	B,C,H,I	11	4.4	H
G	0	20	I	12	4.8	B,C
H	2	19	O,F,I	17	6.8	F,I
I	2	17	H	24	9.6	G
K	4	16	K	13	5.2	O
L	7	7	L	11	4.4	I
M	0	20	B,D,N	20	8.0	C,D
N	2	16	E	19	7.6	B,M
O	2	20	C	18	7.2	C

sweet has a xylem composition that is more favored by the psyllid or that Valencia has more chemical defenses in the xylem that discourage psyllid ingestion. It has already been shown that small morphological differences can influence psyllid behavior (Ammar et al. 2013, 2014). If future research can identify specific causes, it might be possible to breed citrus to be a less favorable host for the psyllid.

We end with a few words about the data analysis. We cannot say that past research using only univariate methods is inappropriate. Partly this is because it is unclear how past analyses were performed. Do all researchers using the Sarria workbook (Sarria et al. 2009) perform a statistical analysis of all the variables calculated therein? If so, do they report all the variables that were significant or only a subset of the significant variables that made the most sense given their understanding of the biology of the organisms studied? How many variables were calculated in manuscripts that did not list the software used in the analysis? Our perception of the results should be modified by the number of significant variables compared to the total number of variables examined. One approach to get around the problems in having a large number of

Table 6. ANOVA for differences between probing behavior of *D. citri* on Midsweet (M) versus Valencia (V). M > V are the variables where the mean from Midsweet exceeded that for Valencia. Numerator and denominator degrees of freedom (num df and den df, respectively), and probability of a greater difference using *F* test (*P* > *F*). Untransformed means and standard error of mean are also provided.

					Midsweet		Valencia	
M > V	Variable	Num df	Den df	*P* > *F*	Mean	SE	Mean	SE
Probes	MnPrbs	1	288	0.0206	4,153.9	282.50	3,511.1	309.07
	sdPrbs	1	285	0.0499	5,442.2	344.44	4,594.2	375.87
Np	MnDurNP	1	288	0.0004	3,977.0	450.73	2,532.1	493.12
	sdNP	1	286	0.0007	4,806.6	437.15	3,523.8	475.23
G	DurG	1	288	0.0411	5,118.8	318.55	4,216.8	348.51
	MeanG	1	288	0.0492	1,764.5	109.05	1,478.5	119.31
	meanNGPrb	1	267	0.0187	0.34	0.031	0.25	0.034
	NumLngG	1	267	0.0186	2.68	0.124	2.25	0.136
	PrcntPrbG	1	288	0.0086	14.00	0.981	10.17	1.073
D	DurNnprbBfrFrstD	1	288	0.0006	16,789	867.5	10,261	867.5
	meanNDPrb	1	288	0.0355	0.22	0.023	0.16	0.025
	PrcntPrbD	1	288	0.0264	0.38	0.035	0.27	0.039
E1	MnDurE1	1	288	0.0045	70.77	6.333	42.89	6.929
	PrcntPrbE1	1	288	0.0291	0.76	0.093	0.46	0.102
V > M								
Probes	NumPrbs	1	288	0.0007	16.89	1.167	22.52	1.276
NP	NumNP	1	288	0.0004	17.20	1.165	23.00	1.275
C	NmbrC	1	288	0.0020	22.29	1.249	27.92	1.366
	TtlDurC	1	288	0.0155	25,948	942.3	31,092	942.3
	NmbrShrtC	1	288	0.0085	2.86	0.475	4.56	0.520
	PrcntPrbC	1	288	0.0261	57.00	1.915	62.97	2.095
G	CtoFrstG	1	267	0.0002	3.56	0.380	5.70	0.418
	TmFrmFrstPrbFrstG	1	267	0.0007	8,736.6	826.26	12,822	826.3
	NumPrbsAftrFrstG	1	288	0.0537	12.24	1.032	15.92	1.129

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univariate tests is to use multivariate methods. These would include approaches like discriminant analysis (as we described here), factor analysis (which would include principle component analysis [Serrano et al. 2000]), and cluster analysis. They cannot replace more simple methods; rather, they provide a more holistic perspective as part of a comprehensive and balanced analysis of the data from experiments such as the one presented herein.

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