Journal of Insect Science (*2016*) 16(1): 14; 1–8
doi: 10.1093/jisesa/iev154
Research article

Analysis of Acquisition and Titer of *Maize Mosaic Rhabdovirus* in Its Vector, *Peregrinus maidis* (Hemiptera: Delphacidae)

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Subject Editor: Blake Bextine

Received 30 July 2015; Accepted 9 December 2015

Abstract

The corn planthopper, *Peregrinus maidis* (Ashmead) (Hemiptera: Delphacidae), transmits *Maize mosaic rhabdovirus* (MMV), an important pathogen of maize and sorghum, in a persistent propagative manner. To better understand the vectorial capacity of *P. maidis*, we determined the efficiency of MMV acquisition by nymphal and adult stages, and characterized MMV titer through development. Acquisition efficiency, i.e., proportion of insects that acquired the virus, was determined by reverse transcriptase polymerase chain reaction (RT-PCR) and virus titer of individual insects was estimated by quantitative RT-PCR. Acquisition efficiency of MMV differed significantly between nymphs and adults. MMV titer increased significantly over time and throughout insect development from nymphal to adult stage, indication of virus replication in the vector during development. There was a positive association between the vector developmental stage and virus titer. Also, the average titer in male insects was threefold higher than female titers, and this difference persisted up to 30 d post adult eclosion. Overall, our findings indicate that nymphs are more efficient than adults at acquiring MMV and virus accumulated in the vector over the course of nymphal development. Furthermore, sustained infection over the lifespan of *P. maidis* indicates a potentially high capacity of this vector to transmit MMV.

Key words: vector–virus interaction, propagative transmission, acquisition efficiency, sustained infection

Maize mosaic virus (MMV) is a plant virus (genus *Nucleorhabdovirus)* from the family *Rhabdoviridae*. Like other viruses from this family, MMV has a bacilliform or bullet-shaped virion consisting of a single, negative-sense genomic RNA encapsidated into nucleocapsid (N) protein subunits and surrounded by a lipid bilayer derived from the plant or insect host. It encodes a single viral glycoprotein (G) embedded in the lipid membrane, and G proteins are exposed on the surface of the virion. G is a trimer composed of an ectodomain, a transmembrane domain, and a cytoplasmic tail. The G trimer is essential for virus attachment to cell receptors and entry into host cells by endocytosis (Coll 1995). The matrix (M) protein interacts with both the N and the lipid bilayer of the virion. The phosphoprotein (P) and large (L) protein are required for synthesis of viral RNAs (Jackson et al. 1999). The family *Rhabdoviridae* includes the human and animal pathogen *Rabies virus*; *Vesicular stomatitis virus*, an insect-transmitted livestock pathogen; *Bovine ephemeral fever virus*; and 70 other plant-pathogenic viruses transmitted by arthropods (Jackson et al. 2005). Rabies virus is a progressive neurotropic virus like MMV that is known to affect the behavior of infected humans and animals (Rupprecht et al. 2002). Sigma virus (SIGMAV) is also a rhabdovirus and a naturally occurring disease agent of *Drosophila melanogaster*. SIGMAV replicates in the neural tissues of its insect host and upregulates expression of genes related to innate immunity (Tsai et al. 2008, Teninges 1999). MMV replicates in corn (*Zea mays* L.) and the insect host/vector *Peregrinus maidis* (Ashmead) (Hemiptera: Delphacidae) (Falk and Tsai 1985, McDaniel et al. 1985). MMV multiplication in *P. maidis* was confirmed through enzyme-linked immunosorbent assay over two decades ago (Falk and Tsai 1985). The next report of virus accumulation in the vector came a few decades later when Ammar et al. (2004) used immunofluorescence confocal laser scanning microscopy to study the accumulation and assembly sites of MMV in the tissues of *P. maidis*. The extensive infection of the organs and tissues of *P. maidis* was further investigated by studying the dissemination route of MMV in the insect vector (Ammar and Hogenhout 2008). Collectively, these

studies provided important information on the replication and dissemination of the virus within the vector.

P. maidis transmits MMV in a persistent propagative manner (Nault and Ammar 1989, Hogenhout et al. 2008). It is the only known vector of MMV and another plant virus, *Maize stripe virus* (MSpV, genus *Tenuivirus*, not assigned to a family or order) (Ammar et al. 2009, Maramorosch 1955). *P. maidis* is a significant pest in subtropical and tropical environments. The hemipterous insect feeds on the sap of two agronomically important crops, sorghum, and corn, and it develops in three stages: egg, nymph, and adult. The nymphs have five instars and differ from the adults only in size, shape, and their organs of flight and stage duration (Tsai and Wilson 1986). The life cycle of the planthopper is complete in about 1 mo. At 27 ± 1°C, the average length of the nymphal stage is 17.20 ± 1.50 d, and adults are quite long lived with an average lifespan of 36.10 ± 20.0 d (Tsai and Wilson 1986). The adults can have two different wing morphological types: the short-winged and long-winged forms are called brachypters and macropters, respectively. Only the latter have functional wings. Transmission of MMV in the field relies specifically on its vector and it is not known to be transmitted by other insects or by mechanical inoculation (Maramorosch 1955). In the laboratory, MMV can be mechanically passaged using a specialized technique termed vascular puncture inoculation (Louie 1995). Transmission studies showed that *P. maidis* becomes more efficient at transmitting the virus when injected with purified MMV (85%) compared with acquiring the virus from MMV-infected plants (42%) (Falk and Tsai 1985). This suggests that the midgut is a transmission barrier, and that if bypassed, MMV transmission occurs more efficiently.

The amount of virus in the vector is often positively correlated to the efficiency and frequency of transmission. For example, *Tomato spotted wilt virus* (TSWV), a *Tospovirus* from the family *Bunyaviridae*, is transmitted in a persistent propagative manner by *Frankliniella occidentalis*, the western flower thrips. In thrips, the vector acquires TSWV at the larval stage and transmits the virus when in the adult stage (Van de Wetering et al. 1996). Rotenberg et al. (2009) showed that viruliferous thrips transmit TSWV more frequently when virus titers are high in the insect. *Maize chlorotic dwarf virus* (MCDV) and a rhabdovirus, *Maize fine streak virus* (MFSV), are semipersistently and persistently transmitted by *Graminella nigrifrons* (Forbes), respectively (Redinbaugh et al. 2002, Choudhury and Rosenkranz 1983). MCDV-inoculative *G. nigrifrons* exhibit higher transmission frequency when given longer access to infected plants (Choudhury and Rosenkranz 1983). MFSV is a nucleorhabdovirus like MMV, and was shown to infect nymphs earlier and harbor higher titer than adults. With longer acquisition periods on MFSV-infected plants, viral titer and transmission increased in the *G. nigrifrons* (Todd et al. 2010).

The nymphal and adult stages of *P. maidis* can acquire and transmit MMV. This is true also for other propagative viruses such as the *Maize rayado fino virus* (MRFV) and *Oat blue dwarf virus* transmitted by leafhoppers *Dalbulus maidis* and *Macrosteles fascifrons*, respectively. These viruses were shown to be more efficiently transmitted when they were acquired during nymphal stages rather than the adult stage (Banttari and Zeyen 1970, Nault et al. 1980). Ammar et al. (1987) reported that *P. maidis* nymphs were better vectors of MSpV at 2 wk postacquisition than adults, but there were no differences in transmission efficiencies between stages at 4 wk postacquisition.

P. maidis sustains a persistent and propagative accumulation of MMV and allows it to persist without obvious pathogenic effects. Higashi and Bressan (2013) have shown that MMV has inconsistent to minimal direct effects on fecundity, mortality of nymphs, longevity, and time of development. The objectives of this study are to better understand how virus titer changes over time in the developmental stages of the vector by quantifying MMV titer in the nymphal and adult stages of *P. maidis* and to investigate in which of the two stages the insect acquires the virus more efficiently. The data presented here will help further elucidate the cellular and molecular aspects of virus–vector interaction in this pathosystem that may cause potential changes in host behavior and physiology leading to higher efficiency of MMV transmission and spread.

Materials and Methods

Insect and MMV-Infected Plant Maintenance

Laboratory colonies of *P. maidis* were derived from original field collections (1971) by R. Namba in Hawaii (University of Hawaii, Honolulu). Colonies were maintained on sweetcorn cultivar Early Sunglow from Syngenta Seeds (Greensboro, NC) in growth chamber facilities as previously described (Wayadande and Nault 1993). Rearing, and acquisition and transmission experiments were carried out in the growth chambers with a photoperiod of 18:6 (L:D) h at 25 ± 1°C. To have insects of a similar age, more than 400 uninfected adults were allowed to feed and oviposit for 1 wk on healthy corn plants. After 1 wk, the adults were removed and the plants were observed for hatched nymphs. MMV was maintained in corn by serial inoculation using *P. maidis*. Inoculated plants were used as source plants 1 to 2 wk after MMV symptoms became visible as chlorotic striping and streaking with apical bending and stunted growth.

Virus Source and Serial Inoculation

MMV-inoculated seeds of the sweetcorn cultivar Spirit were obtained from M. G. Redinbaugh (USDA-ARS and The Ohio State University). Seeds were inoculated through vascular puncture inoculation (Louie 1995). Fifteen pots were sown with four seeds each. Plants that showed MMV symptoms were used for the initial acquisition of the virus by *P. maidis*. Two hundred uninfected adults were fed with the infected plants for 14 d (acquisition access period, AAP) before transfer to a noninfected corn plant for inoculation. MMV has a latent period of approximately 14 d and a higher proportion of insects acquire MMV with longer acquisition periods (Falk and Tsai 1985, Ammar and Hogenhout 2008). The rearing cages were aluminum-framed (Catalog no. 1450 M) purchased from Bioquip Inc. (Compton, CA). After 14-d AAP, the insects were transferred to a new cage with four pots of healthy corn plants (10 plants per pot) in V2 to V3 vegetative stage for a 7-d IAP. After inoculation, the plants were taken out of the cage and placed in a plastic tub container with Hot Shot insecticide strip (Chemsico, St. Louis, MO) to kill any remaining insects on the plants. After 2 h, the plants were moved to a larger rearing (Catalog no. 1450DC) cage and kept in a growth chamber. Four pots of new healthy corn plants were placed in the cage for a second 7-d IAP. Plants were changed every week for sequential inoculations to maintain virus-infected plants, and this continued for 8 wk or until the number of insects was less than 100.

Acquisition of MMV by P. maidis

First instar nymphs or adults were used to check acquisition efficiency following oral acquisition of the virus from MMV-infected plants. First instar nymphs or adults were collected and moved to MMV-infected corn plants. The insects were allowed a 7-d AAP. Groups of 5 or 20 insects were collected and subjected to a 7-d

holding period on healthy corn plants of V2 to V3 vegetative stage in a 12- by 3-inch butyrate tube cage. Later, the insects were collected and separated by sex and wing phenotype. Individual insects were moved to microfuge tubes and quickly frozen in liquid nitrogen and kept at −80°C until RNA extraction for MMV acquisition analysis by endpoint polymerase chain reaction (PCR). The experiment was done three times. Overall, the total number of insects analyzed was 260 and 131 for nymphs and adults, respectively. The sex of the adults was determined by checking the presence or absence of an ovipositor. The corn plants were maintained in the growth chamber and observed for development of disease symptoms.

RNA Extraction for Groups or Individual Insects and cDNA Preparation

Total RNA from individual and groups of insects was prepared using TRIzol (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation. For individual insects, 50 µl of TRIzol reagent was added to the 1.5 ml microfuge tube containing the individual insect. Homogenization was done at room temperature (RT) using a micropestle and a Kontes cordless pestle motor (Daigger, Vernon Hills, IL). An additional 150 µl TRIzol was added to the homogenized sample to make 200 µl total volume and incubated at RT using a microfuge rotator for 3 min. For groups of insects, 20 third instars (N3), N4, N5, female, or male adults, a total of 1 ml TRIzol was used to grind the insect bodies. For this experiment, there were seven biological replicates. The samples were then centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant was collected and transferred to a new microfuge tube. Twenty microliters (200 µl for groups) of chloroform was added, and the mixture was shaken vigorously for 15 s and incubated at RT for 5 min. After incubation, the mixture was separated by centrifugation at 12,000 × *g* for 5 min at 4°C. The aqueous phase was carefully pipetted and transferred to a new tube. One hundred microliters of isopropanol (500 µl for groups) was added, and the mixture was incubated at RT for 10 min. The mixture was centrifuged at 12,000 × *g* for 5 min at 4°C. The RNA pellet was often invisible until the addition of 1 ml of 75% ethanol. After centrifugation at 7,600 × *g* for 5 min at 4°C, the pellet was air dried and redissolved in 10 µl (50–150 µl for larger pellets) of diethylpyrocarbonate (DEPC)-water. Total RNA (1 µg) was reverse transcribed using a Verso complementary DNA (cDNA) kit (ThermoFisher Scientific, Waltham, MA) in a 10-µl reaction mixture volume. The resulting cDNA was used for the detection of MMV infection by reverse transcriptase PCR (RT-PCR) or quantitative RT-PCR (qRT-PCR).

RT-PCR for Detection of MMV Infection

Detection of MMV infection was done by RT-PCR using GoTaq Flexi DNA Polymerase (Promega, Madison, WI). The M gene encoded by the MMV genome was amplified using the primer set: forward primer (FP), 5′-TCC AGC AAC TCA ATC ATT-3′; reverse primer (RP), 5′-CCT ATC AAT CCT TCC TCT-3′. β-Tubulin was used as internal control with primers: FP, 5′-GCA GTG TGG AAA TCA AAT CG-3′ and RP, 5′-GGC TCC TTC AGT GTA GTG G-3′. In total, 10 µl reaction volume was run using the following thermal cycling conditions: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C with 35 cycles. An insect was considered infected and acquired MMV if it yielded a PCR product for the M gene that was easily visualized in agarose gels (10 µl of PCR product loaded) stained with Ethidium bromide (EtBr). Insects that tested positive for MMV were further analyzed for virus titer by qRT-PCR.

Quantification of the Virus Titer Using qRT-PCR in Individual or Groups of P. maidis

Individuals or groups of insects were analyzed for virus titer by qRT-PCR. cDNA was prepared as described above, with a total reaction volume of 10 µl. The cDNA was diluted 10 times prior to amplification. From this, 8-µl cDNA was used as template for two replicates in the qRT-PCR experiment. To obtain highly reliable quantification data, we first analyzed different candidate internal reference controls. Specific qRT-PCR primers were designed for the following: actin, β-tubulin, and ribosomal proteins RPI8 and RPL10. Expression stability of these genes was assayed in MMV-infected and healthy *P. maidis*. The best internal reference gene was chosen based on the analysis by the "BestKeeper" excel tool (data not shown). This tool analyzes gene expression variability by calculating the cycle threshold (Ct) set, SD, and coefficient of variance, and then pairwise comparison calculates the correlation between the genes with the "BestKeeper index" (Pfaffl et al. 2004). This application ranked RPL10 as the least variable gene followed by RPI8. Pairwise comparisons used the genes of the BestKeeper index with each of the two best genes, RPL10 and RPI8; RPL10 showed higher correlation. Based on these results, we chose RPL10 as the internal reference control to be used in our qRT-PCR experiments. MMV titer was measured based on the relative expression of the target gene, MMV N, on a nonregulated internal reference gene, RPL10. Beacon Designer software (Premier Biosoft, Palo Alto, CA) was used for the automated design of primers for qRT-PCR: MMV N FP, 5′-GAG CAT CTG GTA GAG GAG-3′, RP, 5′-CAT AGG TTC AGG AGC GTA T-3′ and RPL10 FP, 5′-CGA AGA AGT GGG GTT TCA-3′ and RP, 5′-CTC TGG CCT GTA CTT CAC-3′. Both primer pairs were tested for sensitivity and efficiency. qRT-PCR was performed using CFX 96 Thermal Cycler (Bio-Rad, Hercules, CA) using SYBR green chemistry and RT fluorescence measurements. This technique was carried out using iQ SYBR Green Mix (Bio-Rad). The 10-µl reaction mixture consisted of 2× SYBR Green PCR master mix, 200 nM of each primer, and 40 ng of cDNA. The experiment was run with 40 cycles of 10 s at 95°C and 45 s at 55°C, and each cycle was scanned to quantify the PCR products. Amplification plots in RT were constructed using CFX96 software (Bio-Rad). Quantitative analysis of the MMV N expression was done using the formula of Pfaffl (2001) as average normalized abundance ratios. MMV titer was reported as ratios and log10 values of the normalized abundance ratios.

MMV Titer in Adult P. maidis Through Time

First instar nymphs were allowed a 7-d AAP on MMV-infected plants. After 7 d, the insects were removed and moved to healthy corn plants. Adults were collected at 10, 20, 25, 35, and 40 d post-acquisition to MMV-infected plants. The insects were placed in individual microfuge tubes, quickly frozen in liquid nitrogen, and stored at −80°C until RNA extraction was conducted. RNA was extracted from individual insects following the procedure described above. The insects were checked for MMV acquisition by endpoint PCR. Insects that acquired MMV were further analyzed for virus titer by qRT-PCR. There were three biological replicates. The total number of insects analyzed was 59, 33, 42, 29, and 29 on 10th, 20th, 25th, 35th, and 40th day postacquisition on MMV-infected plants, respectively.

Statistical Analyses

Different statistical procedures were used to analyze the datasets on virus titer. All analyses were done in SAS version 9.3 (SAS Institute

Inc., Cary, NC). Mixed model analysis (PROC MIXED) and the Wilk–Shapiro test (PROC UNIVARIATE) to evaluate the normality of residuals and pairwise comparisons using Tukey adjustments were used to determine if 1) MMV titer differed among the nymphal (N3, N4, N5) and adult developmental stages, 2) titer varied between male and female insects at the different time points post acquisition, and 3) titer change in individual adults over time. The correlation between developmental stages of insect and virus titer was analyzed using Spearman's rank correlation analysis (PROC CORR). The difference in percent acquisition (proportion of individuals testing positive for virus) between nymphs and adults was analyzed using the general model (PROC GENMOD). All tests were done at 5% level of significance.

Results

MMV Acquisition in the Nymph or Adult Stages of P. maidis

In groups of 5 insects per plant, a total of 120 insects overall for nymphs and adults were analyzed and 41 nymphs (68%) and 29 adults (48%) were infected with MMV (Table 1). In groups of 20 per plant, a total of 271 nymphs and adults overall were analyzed. There were 116 nymphs infected with MMV out of 200 (58%), compared with only 23 from a total of 71 adults analyzed (32%) (Table 1). The mean % acquisition for nymphs was 23% higher than the adults ($P = 0.0031$), which indicates that nymphs acquire MMV more efficiently than adults.

MMV Titer in the Different Developmental Stages of P. maidis

To obtain reliable titer quantification data, we used the BestKeeper tool to identify the best internal reference gene for qRT-PCR and this application ranked RPL10 as the least variable gene. Quantification of MMV titer from the third to fifth instar (N3, N4, N5) until the adult stage of *P. maidis* revealed that the virus titer increases postacquisition from MMV-infected plants ($P < 0.0001$) (Fig. 1). MMV titers in N3 and N4 were not significantly different but these two life stages showed significant difference with titer adult insects. MMV titers in N4 and N5 were not significantly different but titer in N4 is significantly different from the adult stage. Correlation analysis showed that the developmental stages and virus titer is positively correlated ($r_s = 0.7080$, $P < 0.0001$) (Fig. 1).

MMV Titer in Individual Brachypterous P. maidis Adults

Analysis of MMV relative abundance in groups of insects showed that titer increased over time and peaked at the adult stage. However, these samples represented a group of exposed insects and consisted of insects infected with MMV and others that were not. We investigated the titer of individual insects to determine the

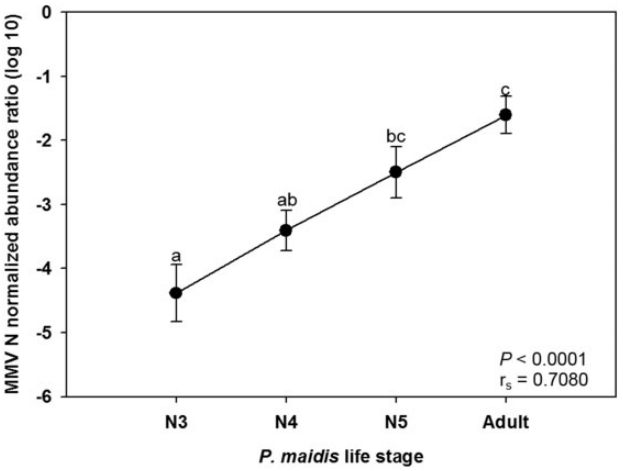

Fig. 1. MMV titer from the developmental stages of *P. maidis* from N3 to adult stage. Groups of 20 insects per developmental stage at day 1 after eclosion were analyzed for MMV titer by qRT-PCR. Titer is reported as log10 values of the normalized abundance ratio of MMV N and RPL10. Mixed model analysis (PROC MIXED) and the Wilk–Shapiro test (PROC UNIVARITE) were used for comparisons of viral titer over time. Tukey's method was used to perform multiple comparisons of means in the different developmental stages. Titer in the developmental stage headed by different letters is significantly different. The correlation between developmental stages and virus titer was analyzed using Spearman's rank correlation analysis (PROC CORR)

Table 1. Percentage acquisition of MMV by *Peregrinus maidis* nymphs or adults

MMV acquisition by *P. maidis* nymphs or adults								
P. maidis held on individual plant	Exp. no.	Nymph			Exp. no	Adult		
		No. of insects analyzed	MMV infected	Mean % acquisition		No. of insects analyzed	MMV infected	Mean % acquisition
Groups of 5	1	20	12	68	1	20	2	48
	2	20	15		2	20	12	
	3	20	14		3	20	15	
Total		60	41			60	29	
Mean total		20	13.7			20	9.7	
Groups of 20	1	70	42	58	1	20	8	32
	2	70	32		2	21	5	
	3	60	42		3	30	10	
Total		200	116			71	23	
Mean total		66.7	38.7			23.7	7.7	
Overall total no. of insects		260	157			131	52	
Overall mean % acquisition				63*				40*

Nymphs or adults were exposed to MMV for 7 d (7-d AAP) and MMV acquisition was determined by RT-PCR. Insects were held on individual healthy plants in groups of 5 or 20 for 7 d.

*Significant difference ($P = 0.0031$) in mean % acquisition between nymph and adult *P. maidis* was analyzed using the general model PROC GENMOD of SAS v. 9.3.

variability and difference between males and females. For analysis, MMV titer was converted to log10 values of the normalized abundance ratios (Fig. 2). In individual females, the mean average MMV titer was 1.15 with a minimum average titer of 1.4×10^{-4} and a maximum average titer of 9.49 (Supp Table 1). In males, the mean average titer was 4.54 with a minimum of 2.16×10^{-2} and a maximum of 20.3 (Supp Table 1). The mean MMV titer showed that the MMV titer in individual males and females is significantly different ($P = 0.0004$), with the mean MMV titer of males higher than in females by fourfold.

MMV Titer in Adult P. maidis Through Time.

Our experiments revealed that the mean MMV titer of adults from day 10 to 40 postacquisition to MMV-infected plants was significantly different (Fig. 3A) ($P = 0.0054$), in the range of 0.341–1.51 in females and 1.06–5.17 in males (Supp Table 2). The mean titer at day 10 was significantly different from the mean titer at day 25, 35, and 40 ($P = 0.0300$, 0.0035, and 0.0464, respectively) (Fig. 3A), but the mean titer at days 20–40 postacquisition to MMV-infected plants was not significantly different ($P = 0.2068$–0.9998) (Fig. 3A). The mean MMV titer in females and males from days 10 to 40 from the three biological replicates revealed a significant difference ($P = 0.0382$) (Fig. 3B, Supp Table 2), and the MMV titer in males (2.98) was higher than in females (0.853) over the time course experiment. The mean titer of males at day 10 were significantly higher than females ($P = 0.049$) and there was an overall trend for males to have higher MMV titers than females, although not always statistically different (Fig. 3B). Only at the 35th day did the female planthoppers have a higher virus titer ($P = 0.0198$). Overall, the titer of males was 3.5-fold higher than females, which is consistent with the titer observed previously with the individual adult brachypters (Fig. 2).

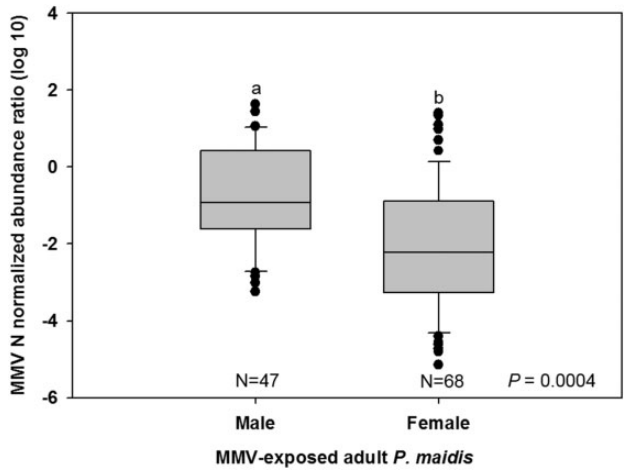

Fig. 2. A box-and-whisker plot of MMV titer data distribution from individual adult male or female *P. maidis*. MMV titer is reported as log10 values of the normalized abundance ratio of MMV N and RPL10. The percentile values for distribution of the titer data were computed using SigmaPlot v.10.0. The boxes show the 25th and 75th percentiles of MMV titer in male or female *P. maidis*. The median value is the line inside the box while the whiskers on top and bottom of the box represent the largest and smallest titer, respectively. Solid points on top or bottom of the whiskers represent outliers. *N* is the number of adult *P. maidis* analyzed. Letters over the box plots indicates significant difference ($P = 0.0004$) analyzed using mixed model analysis (PROC MIXED).

Discussion

Our work presents data quantifying a plant rhabdovirus in its insect vector using a qRT-PCR platform for virus titer analysis. In some plant viruses transmitted by insects, the life stage of the vector during the time of acquisition is important for transmission efficiency. In those cases, the nymphal or larval stages are more susceptible to infection than the adults (Todd et al. 2010) and accumulation and replication of the virus in the vector as it ages increases the frequency of transmission (Nagata et al. 1999, Anhalt and Almeida 2008, Inoue et al. 2009, Todd et al. 2010). In *P. maidis*, MMV can be acquired in the nymphal and adult stages of the vector and the insect can remain viruliferous during its lifetime. We found that 20% more insects were positive for virus acquisition when they were exposed to infected plants at the nymphal stage than when exposed at the adult stage. This result showed that the chance of *P. maidis* acquiring the virus is higher when it is exposed to MMV-infected plants at the nymphal stage than when it is exposed at the adult stage. As MMV can be acquired early in the lifespan of *P. maidis*, this could mean longer persistence and higher titer in the

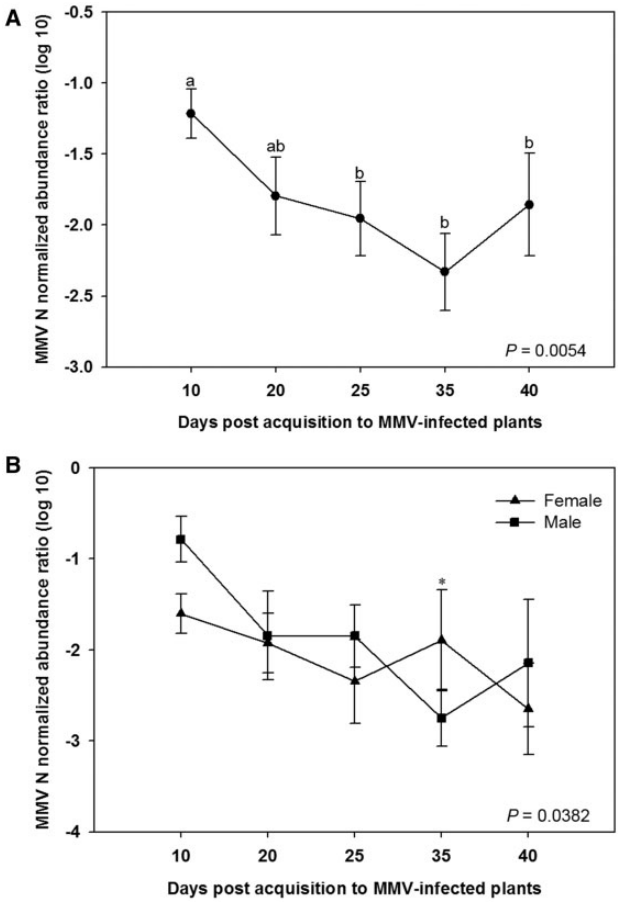

Fig. 3. MMV titer from individual adult *P. maidis* from day 10 to 40 postacquisition on MMV-infected plants. (A) The days postacquisition to MMV was analyzed for its effect on MMV titer in mixture of female and male adults. (B) The difference between female and male titers at days 10, 20, 25, 35, and 40 postacquisition to MMV-infected plants was analyzed. Statistical analysis used was a Wilk–Shapiro test for testing normality of residuals and a mixed model analysis (PROC MIXED). MMV titer is reported as log10 values of the normalized abundance ratio of MMV N and RPL10. Asterisk at day 25 postacquisition to MMV is the time point where female mean titer is significantly different than male.

vector because the virus replicates in the vector (Ammar and Nault 1985, Hurd 2003, Ammar et al. 2004, Ingwell et al. 2012). Thus, the developmental stage of the vector was important for acquisition efficiency.

We sought to characterize the changes in MMV titer as planthoppers develop because this could impact transmission efficiency. Quantitative measurement of MMV titer in the developmental stages of *P. maidis* using qRT-PCR was performed and virus titer was expressed as the normalized abundance of MMV N RNA to an internal reference standard, a ribosomal protein, RPL10. A 7-d AAP was allowed for the first instar (N1) and the insects were transferred to healthy plants afterward. A positive correlation between the titer and developmental stage of the vector was found when we analyzed the titer in groups of 20 insects per developmental stage starting at N3, and the titer continuously increased from the nymphal to adult stage.

In the early adult stage (1–3 d after eclosion from N5), we analyzed virus titer in individual female and male brachypters. For other propagative viruses, the ability of an insect to transmit is associated with accumulation of the virus in the salivary glands (Hogenhout et al. 2008). The results of the quantitative measurement of MMV in *P. maidis* showed that the two sexes harbor different amounts of virus, with males having mean titer fourfold higher than females. To explain this result, we observed that the amount of RPL10 RNA in females is more abundant than in males. RPL10 has been shown to have constant expression among the developmental stages of *P. maidis*, so we used the same gene as an internal reference in the analysis of viral titer for individual insects. The difference in the actual RNA amount can be due to the size or body mass of the insect and the severity of the infection in the tissues of the individual insects being analyzed. Female brachypters are bigger than male brachypters, and although we know that MMV had already replicated in almost all of the tissues and organs of the insects at the time of the assay, the virus in the individual insects may have different rates of replication on the different parts of their body. In TSWV research, males have been found to have higher TSWV N RNA relative to actin RNA, but expressing the virus titer as copies of viral N RNA (cDNA) per insect, the females have more than twice the amount of N RNA molecules (Rotenberg et al. 2009).

Analysis of the mean titer of adults through time indicated that mean titer at day 10 postacquisition on MMV-infected plants was significantly different from days 20 to 40 postacquisition; however, the mean titer at day 20 is not significantly different from the titers at days 25 to 40. These results suggest that MMV titer in adults, although it persists inside the vector, is relatively consistent for the duration of the adult life span. When we analyze the mean titers of males and females, male mean titer is higher, which is consistent with results from the previous experiment on acquisition when we looked at individual adults collected at the early adult stage. We expected variability in the titer per insect, but the mean titer of males was consistently 3.5 - to 4-fold higher than that of females (Suppl Tables 1 and 2). Aside from body size, the difference in the virus titer between the sexes may be explained by the difference in female and male cellular or molecular activities that would impact the virus lifecycle. For example, the physiological needs as they develop into adults likely differ between males and females and this may affect virus replication. Males and females also have different nutritional requirements as they age (Auclair 1963, Dadd 1973). In adult tsetse flies *Glossina palpalis*, a vector of the flagellate protozoan *Trypanosoma brucei gambiense*, which causes sleeping sickness, the amino acid components in adult males occur at higher concentrations than in females (Balogun 1971). In *D. melanogaster*, the insulin-signaling pathway (ISP) controls the body and organ size by controlling the nutritional conditions to the growing organs (Shingleton et al. 2005). ISP is also involved in the allocation of energy to specific ongoing processes related to reproductive physiology. In female *D. melanogaster*, insulin signaling seems to have a stimulatory effect on ecdysteroid production. Insulin receptor mutant flies displayed impaired ovarian ecdysteroid synthesis (Tu et al. 2002). In male fruitfly testes, ISP was found to regulate spermatogenesis and affected spermatocyte growth (Ueishi et al. 2009). Physiological and related behavioral differences between male and female insects may play a role in virus titer and transmission efficiency.

In both humans and animals, females have been shown to have enhanced levels of immunoreactivity compared with males (Cannon and St. Pierre 1997, Taskiran et al. 1997, Rolf 2001, Fedorka et al. 2004, Ziebell et al. 2011). Females must provide an appropriate environment for egg fertilization to succeed. In males, maintaining the integrity of seminal fluid proteins that are transferred to the female is important because this influences female reproductive physiology (Kubli 2003). Research efforts in our laboratory to characterize the effects of MMV infection in behavior and physiology of the vector are underway. Based on previous work, we hypothesize that *P. maidis* females may increase immunocompetence for successful fertilization of eggs. On the other hand, males with a higher virus titer could represent a reduction in immunocompetence or redistribution of immune cells (hemocytes) within the different components of the innate immune system. The males could put more energy into other physiological systems such as reproduction to favor good-quality seminal proteins, which could influence female fertilization. In the adult male cricket, *Gryllus texensis*, Adamo et al. (2000) found that the phenoloxidase activity and resistance to *Serratia marcescens* declined at the onset of sexual behavior compared with levels in females and younger males. Whether this could be true in adult *P. maidis* remains to be tested. On the other hand, MMV might be able to counteract the antiviral mechanisms of the vector by targeting the pathways of the immune defense response, including RNA interference (RNAi). Several viruses evade immunity controlled by the RNAi pathway by encoding viral suppressors of RNA silencing (VSRs). Infection of *Drosophila* with *Cricket paralysis virus* (CrPV) induces antiviral silencing and CrPV encodes a VSR (Wang et al. 2006). There are several mammalian VSRs identified as suppressors of host RNAi including NS1 of *Influenza A, B, and C* viruses (Li et al. 2004), NSs of *LaCrosse virus* (Soldan et al. 2005), and Tat of *Human immunodeficiency virus* (Bennasser et al. 2005).

The new findings on *P. maidis* virus acquisition efficiency and virus accumulation over the lifetime of the insect provide important insights relevant to understanding the role of virus titer on the physiology and behavior of the vector which can affect the transmission process. The ability of *P. maidis* to support a lifelong MMV infection indicates that these insects have a high capacity for transmitting the virus. The life span for *P. maidis* can exceed 100 d which means that even a single generation of viruliferous planthoppers could transmit virus for a good portion of the corn or sorghum production cycle (Tsai and Wilson 1986). Further studies characterizing the function of vector genes involved in interaction with MMV G or proteins that are differentially expressed during viral infection may help us to identify steps in the transmission process to disrupt vector–virus interaction. The combination of molecular biology resources for *P. maidis* and knowledge of the virus–vector interaction will enable the identification of interacting molecules (Whitfield et al. 2011, Yao et al. 2013, Barandoc-Alviar et al. 2014).

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

We would like to thank M.G. Redinbaugh, USDA-ARS for providing the *Peregrinus maidis* culture and Maize mosaic virus. The project was supported by the National Science Foundation CAREER grant IOS-0953786. This is contribution number 13-293-J from the Kansas Agricultural Experiment Station.

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