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Author(s): Evangelina B. Argüello Caro , Guillermo A. Maroniche , Analía D. Dumón , Mónica B. Sagadín , Mariana Del Vas , and Graciela Truol
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High Viral Load in the Planthopper Vector *Delphacodes kuscheli* (Hemiptera: Delphacidae) is Associated With Successful Transmission of Mal de Río Cuarto Virus

EVANGELINA B. ARGÜELLO CARO,[1,2] GUILLERMO A. MARONICHE,[3] ANALÍA D. DUMÓN,[1] MÓNICA B. SAGADÍN,[1] MARIANA DEL VAS,[3] AND GRACIELA TRUOL[1]

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ABSTRACT *Delphacodes kuscheli* Fennah (Hemiptera: Delphacidae) is the main natural vector of Mal de Río Cuarto virus (family *Reoviridae*, genus *Fijivirus*, MRCV), which infects different gramineae and causes the most important maize (*Zea mays* L.) disease in Argentina. MRCV–vector interactions usually are studied using different winter cereals as hosts. Under experimental conditions, <50% of *D. kuscheli* planthoppers fed on a MRCV-infected plant can transmit the virus to wheat (*Triticum aestivum* L.). This fact is influenced by insect development stage at acquisition and the latency period. This work describes the relation between transmission efficiency and MRCV accumulation in its planthopper vector. First- and third-instar *D. kuscheli* nymphs were allowed to feed on MRCV-infected plants, and 9 or 17 d after the acquisition access period (AAP), viral load of transmitting and nontransmitting planthoppers was quantified by quantitative polymerase chain reaction. The transmitting planthoppers showed significantly higher viral titers than nontransmitting ones, suggesting that successful transmission is positively associated to viral accumulation in the insect. However, planthoppers of the third-instars group did not transmit the virus 9 d after AAP, even when 46% had similar titers to the transmitting insects of the other treatments. These results indicate that additional factors influence MRCV transmission efficiency when acquisition occurs in older planthoppers. This is the first precise quantitative analysis of MRCV in its main vector species and will definitely contribute to better understand planthopper–*Fijivirus* interactions and its epidemiological implications.

KEY WORDS vector–virus interaction, *Fijivirus*, persistent propagative transmission

Planthoppers (Hemiptera: Fulgoroidea: Delphacidae) are severe plant pests that cause phloem sap sucking damage and transmit at least 18 plant viruses (Hogenhout et al. 2008). In particular, *Delphacodes kuscheli* Fennah is the main natural vector of Mal de Río Cuarto virus (family *Reoviridae*, genus *Fijivirus*, MRCV) (Remes Lenicov et al. 1985), the most important viral corn disease in Argentina. MRCV is able to infect maize (*Zea mays* L.) and several other economically important crops and grasses (Rodríguez Pardina et al. 1998) and is only transmitted by planthoppers in a persistent propagative manner (Arneodo et al. 2002, Milne et al. 2005). *D. kuscheli* is the most abundant species in the endemic area and has a demonstrated natural transmission ability (Remes Lenicov et al. 1985, Grilli and Gorla 1999, Remes Lenicov and Virla 1999). Therefore, the study of the interactions between the main vector and MRCV has become an important aspect in the disease epidemiology.

[1] Instituto de Patología Vegetal-Centro de Investigaciones Agropecuarias-Instituto Nacional de Tecnología Agropecuaria (IPAVE-CIAP-INTA). Av. 11 de Septiembre 4755 B° Cárcano. Ciudad de Córdoba CP X5020ICA, Córdoba, Argentina.

[2] Corresponding author, e-mail: earguellocaro@agro.unc.edu.ar.

[3] Instituto de Biotecnología-Centro de Investigación en Ciencias Veterinarias y Agronómicas-Instituto Nacional de Tecnología Agropecuaria (IB-CICVyA-INTA). Las Cabañas y Los Reseros s/n. Hurlingham CP 1686, Buenos Aires, Argentina.

Wheat (*Triticum aestivum* L.) has been proposed as a useful model to study virus–vector interactions, mainly because it is suitable for artificial breeding of the vector, and develops MRCV symptoms earlier than corn (Truol et al. 2001, Arneodo et al. 2002). Under experimental conditions, ≈30–50% of *D. kuscheli* planthoppers that feed on a MRCV-infected plant can transmit the virus to wheat after a maximum latency period of 17 d (Arneodo et al. 2002). Moreover, it has been demonstrated that when *D. kuscheli* acquires the virus as a first-instar nymph, the transmission efficiency is higher than when the acquisition occurs during the third instars (Arneodo et al. 2005). However, little is known about the mechanisms underlying MRCV transmission and the causes of the low and variable transmission efficiency achieved under experimental conditions. The existence of morphological barriers or morphological differences along insect ontogeny were proposed as explanations for a similar observation in the tomato spotted wilt virus (family *Bunyaviridae*, genus *Tospovirus*, TSWV)–*Frankliniella occidentalis* (Pergande) pathosystem (van de Wetering et al. 1996, Moritz et al. 2004). In addition, it was shown that the transmission efficiency of Fiji disease virus by *Perkinsiella saccharicida* Kirkaldy (Hughes et al. 2008) and of TSWV by different genera of thrips is closely related to accumulation of virus particles within the insect vector (Inoue et al. 2004, Rotenberg et al. 2009).

In the current study, we analyzed the relation between transmission efficiency and MRCV load in *D. kuscheli* in conditioning the viral transmission, during different developmental stages of the vector and latency periods.

Materials and Methods

Source and Maintenance of Insects and Virus. The *D. kuscheli* individuals used in this study were obtained from a colony raised in the Vector's Laboratory of Instituto de Patología Vegetal-Centro de Investigaciones Agropecuarias-Instituto Nacional de Tecnología Agropecuaria (IPAVE-CIAP-INTA, Argentina) that originally was isolated from the MRCV disease endemic area (County of Río Cuarto, Province of Córdoba, Argentina) and has been maintained since 2008. The MRCV "RC08" isolate, used as the viral inoculums' source, was isolated from infected oat plants of the Río Cuarto endemic area and maintained in wheat (*T. aestivum* 'ProINTA Federal') since 2008 by serial vector transmissions using *D. kuscheli* as described previously (Truol et al. 2001).

Measurement of MRCV Transmission Efficiency. Transmission trials were carried out using wheat as host to study MRCV–vector interactions (Truol et al. 2001). Groups of male and female *D. kuscheli* planthoppers were allowed to reproduce on healthy wheat plants in plastic containers. Twenty-four hours after oviposition, adults were removed and the plants were grown in breeding chambers under controlled conditions of temperature: 24 ± 1°C, 50% RH, and a photoperiod of 16:8 (L:D) h for egg development. First- (N1) and third-instar nymphs (N3) were obtained 4 and 9 d after eclosion, respectively, and used for subsequent transmission assays. At least 100 nymphs of each life stage were allowed to feed on MRCV-infected wheat for 48 h (acquisition access period [AAP]). The insects then were moved to chambers containing noninfected wheat plants for a latency period depending on each treatment (9 or 17 d after AAP). Next, 1:1 transmission assays were performed by individually transferring one insect to a single noninfected wheat seedling ProINTA Federal (Truol et al. 2001) (inoculation access period [IAP]). After 24 h, each planthopper was removed and stored in absolute ethanol at 4°C until RNA extraction, as described previously by Maroniche et al. (2011). Finally, plants were transplanted to plastic pots and moved to a greenhouse. MRCV infection was analyzed by the scoring of symptom development and DAS-ELISA assays 30 d after IAP (Truol et al. 2001). Each combination of nymph stage and latency period was considered as a treatment: first-instar nymphs, 9 d after AAP (N1L9); first-instar nymphs, 17 d AAP (N1Ll7); third-instar nymphs, 9 d AAP (N3L9); and third-instar nymphs, 9 d AAP (N3L17). Three replicates of 15 insects each were conducted for every treatment ($n = 45$).

MRCV qPCR Relative Quantification in *D. kuscheli*. Total RNA was extracted individually from each planthopper by using a modified Trizol (Invitrogen, Carlsbad, CA) protocol (Maroniche et al. 2011). RNA concentration and purity were measured with an ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE) and integrity was checked by agarose gel electrophoresis. Synthesis of cDNA was carried out from 500 ng of DNase I-treated total RNA by using Superscript III (Invitrogen) and random primers, according to the manufacturer's protocol. The cDNAs synthesized were used for subsequent quantitative polymerase chain reactions (qPCR) in an ABI7500 Real Time polymerase chain reaction (PCR) System (Applied Biosystems, Foster City, CA), using a QuantiTec SYBR Green PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. MRCV segment S3 (MRCV-S3) was chosen for amplification because it is the most conserved genomic segment (Distéfano et al. 2003) and codes for the major core capsid protein (Distéfano et al. 2009) which in turn is the most abundant viral protein. The MRCV qPCR quantification was performed by amplifying a 121 bp fragment of the MRCV-S3 by using primers S3_F (5′-AGTCATAGATTTGGACGCACTTTG-3′) and S3_R (5′-CAGAATGCATCGTGGGTTAAAG-3′) and 1 μl of nondiluted cDNA as a template. In parallel, the *D. kuscheli* ubiquitin gene (UBI) was amplified as an internal control as described (Maroniche et al. 2011). All the reactions were carried out in triplicate in a 20-μl final volume and using a final primer concentration of 200 nM. A cDNA from noninfected insects of the same development stage was used as a negative control of MRCV-S3 amplification, whereas no template was added to the UBI negative controls. The qPCR cycling conditions were: an initial step of 10 min at 95°C followed by 40 cycles composed of a 15-s denaturalization step at 95°C and 1-min annealing and elongation step at 60°C. A final dissociation step was carried out as a control of the PCR amplification specificity. Output results were processed with the LinReg software (Ruijter et al. 2009) for calculations of threshold cycle values (Ct) and PCR efficiencies.

Absolute Quantification of MRCV-S3 in *D. kuscheli*. An external standard curve was set up to individually estimate the MRCV-S3 copy number in planthoppers of the N3L9 treatment as well as viral titer in all males and females. The standard curve was constructed as follows: a 700-bp PCR product of the MRCV-S3 genomic segment was amplified using primers pS3 up (5′-AATGAATTCGGAGGATAATCGGAAAAAAGAA-3′) and pS3 low (5′- TTAAATCAGAGACGAAACTCTAATGT-3′), purified using a QIAquick PCR purification kit (QIAGEN Science, Germantown, MD) and quantified with a ND-1000 spectrophotometer (NanoDrop Technologies). Ten-fold serial dilutions ranging from 1×10^{11} to 1×10^{5} copies of the MRCV-S3 fragment were used as a template for qPCR amplification as described previously (Maroniche et al. 2011). Two cDNA samples from each treatment were included for normalization between runs. Finally, a standard curve was generated by plotting the mean Ct value against the logarithm of the initial copy number for each dilution, and the copy number of

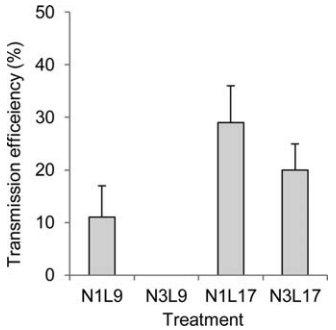

Fig. 1. Transmission efficiency of MRCV by *D. kuscheli* according to different treatments defined by: life stages at acquisition first instars (N1) or third instars (N3), and latency periods (9 or 17 d after AAP [acquisition access period]).

MRCV-S3 was calculated by linear regression of the normalized sample Ct values to the standard curve.

Statistical Analysis. Transmission trials data were treated as categorical (transmitting: 1, nontransmitting: 0) and analyzed by χ^2 using InfoStat version 2010 software (Di Rienzo et al. 2010). Statistical analysis of Ct values obtained by relative qPCR quantification were carried out using the fgStatics software (Di Rienzo 2010) that uses the Pfaffl method for calculation of the expression ratios (Pfaffl et al. 2002). For the absolute quantification analysis, the number of molecules was $\log_{10}$-converted and statistically analyzed with the Mann–Whitney nonparametric test using GraphPad Prism software (Radushev 2009). The values of N3L9 treatment were further analyzed by the agglomerative clustering method followed by a *t*-test, using InfoStat version 2010 (Di Rienzo et al. 2010).

Results

Transmission Efficiency of MRCV by *D. kuscheli*. To evaluate the influence of developmental stage and latency period on MRCV accumulation and transmission efficiency by *D. kuscheli*, an experimental transmission assay composed of four different treatments was carried out using first (N1) or third (N3) instar nymphs and two alternative latency periods of nine (L9) or 17 (L17) days. Each planthopper was classified as transmitting or nontransmitting based on visual MRCV symptom development in wheat (short, erect, dark green colored leaves with enlarged veins, a large number of tillers, shortening of the internodes with a stunted appearance) and MRCV detection by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in all the plants analyzed (Arneodo et al. 2002). Transmission efficiency of each treatment is presented in Fig. 1. The higher transmission efficiency (28.87%) was achieved in the N1L17 treatment followed by treatments N3L17 (20%) and N1L9 (11.13%). No transmitting insects were detected in the N3L9 treatment. All insects were adults when reaching the IAP, except for N1L9 treatment, in which nine out of 45 insects were at the fifth nymph stage. A χ^2 analysis was run for independence between transmission efficiency of nymphs versus adults, and no relation between transmission and life stage was found ($P = 0.9786$).

A χ^2 test for independence was performed to examine the relation between transmission and treatments. The relation between these variables was significant ($\chi^2 = 16.17$, df = 3, $P = 0.001$), so transmission is related to treatments (i.e., with the life stage at acquisition and latency periods).

Relative Quantification of MRCV Titers in Transmitting and Nontransmitting *D. kuscheli*. To compare the viral titers between transmitting and nontransmitting planthoppers obtained in the transmission assay, the relative accumulation of MRCV was analyzed. After qPCR, the insects were classified as positive or negative according to whether they had detectable levels of MRCV-S3 or not, respectively. On average, 18.3% of the insects were qPCR-negative (Fig. 2). Although all the transmitting insects were qPCR-positive, near 53% were viruliferous (qPCR-positive) but nontransmitters (Fig. 2).

Next, the data obtained by qPCR for treatments N1L9, N1L17, and N3L17 was statistically analyzed by

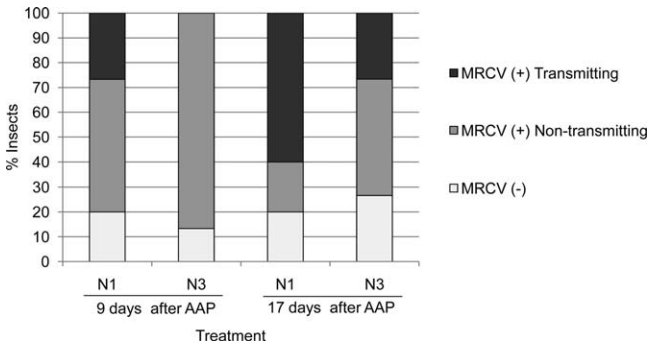

Fig. 2. Transmission efficiency of MRCV by *D. kuscheli*. Different life stages at acquisition (N1: first instars, and N3: third instars) and different latency periods (9 or 17 d after the acquisition access period [AAP]) were measured ($n = 15$). Planthoppers were classified as MRCV positive (+) or negative (-) according to qPCR amplification of a portion of the MRCV-S3 genomic segment. MRCV (+) planthoppers were further classified as transmitting or nontransmitting according to symptom development and ELISA test of the plant tissue.

Table 1. Relative abundance of MRCV-S3 in transmitting versus nontransmitting *D. kuscheli* planthoppers, within different experimental treatments: insect development stage at acquisition (first instars: N1, or third instars: N3), and latency period (9 and 17 d) using Ubiquitin as reference gene

Treatment	Mean relative abundance of MRCV-S3	SE	*P* value[a]
N1 L9	16,832.07	15,126.34	0.0011
N1 L17	1,711.34	6,355.58	0.0037
N3 L17	516.7	9,972.27	0.0127

SE: standard error.
[a] For transmitting versus nontransmitting insects within each treatment.

the Pfaffl method (Pfaffl et al. 2002) within each treatment, considering the nontransmitting insects as the control group and the transmitting insects as the test group. This analysis revealed that transmitting insects had significantly higher MRCV titers ($P < 0.0127$) than nontransmitting insects (Table 1), indicating a strong association between viral titers and the MRCV transmission capacity of the planthopper vector *D. kuscheli.* This calculation could not be performed for treatment N3L9 because transmitting insects were not present in this group (Fig. 1).

Absolute Quantification of MRCV in Transmitting and Nontransmitting *D. kuscheli.* Even though planthoppers belonging to the N3L9 group were not able to transmit the virus, 86.67% were qPCR-positive (Fig. 2) and clearly differed in their viral load values (Fig. 3). So, absolute quantification was performed to compare viral load in individual insects of this treatment, as relative quantification could not be used because of the nonexistence of a control group. To estimate the MRCV-S3 copy number in these insects, Ct values were extrapolated to a calibration curve obtained by amplifying serial dilutions of a MRCV-S3 dsDNA fragment by qPCR. Further statistical analysis of this group by the agglomerative clustering method established the existence of two significantly different subgroups based on their viral titer ($P < 0.0001$): one with lower viral concentration (cluster 1) and a second one with higher viral titer (cluster 2) (Table 2). In turn, the viral concentration values of cluster 2 were statistically similar ($P > 0.1$) to the values observed in transmitting insects of the other treatments. These results indicate that insects that acquired the virus as third instars were not capable of transmitting MRCV after a latency period of 9 d, even though nearly 46% had viral titers equivalent to those of transmitting insects of the other treatments.

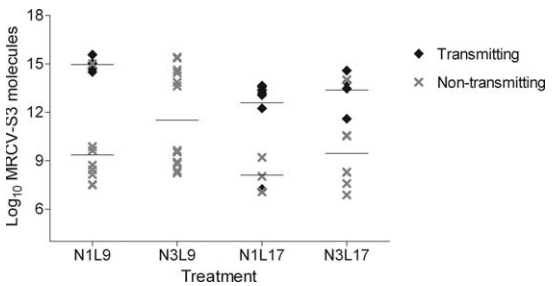

Fig. 3. Estimation of MRCV load in transmitting and nontransmitting *D. kuscheli* by absolute qPCR quantification of MRCV-S3 genome segment. Planthoppers were grouped according to developmental life stage at viral acquisition and different latency periods (AAP). N1L9: first-instar nymphs, 9 d after AAP ($n = 14$); N3L9: third-instar nymphs, 9 d after AAP ($n = 13$); N1L17: first-instar nymphs, 17 d after AAP ($n = 13$); N3L17: third-instar nymphs, 17 d after AAP ($n = 11$). Black horizontal lines show the mean value for each group.

Table 2. Individual analysis of MRCV-S3 titer in nontransmitting *D. kuscheli* planthoppers that acquired the virus as third instars after 9 d after acquisition access (N3L9 treatment) obtained by qPCR and analyzed by agglomerative clusters method

Insect	Log_{10} no. MRCV-S3 molecules	Cluster[a]
1	8.37	1
2	8.86	1
3	8.80	1
4	8.24	1
5	9.64	1
6	9.53	1
7	13.62	2
8	14.62	2
9	14.44	2
10	13.86	2
11	15.36	2
12	15.42	2
13	8.91	1

Cluster 1: low viral titer, cluster 2: high viral titer.
[a] T-Student $P < 0.0001$, Mann–Whitney: $P = 0.0012$.

The transmitting insects that had acquired the virus as first instars showed significantly higher viral titers at nine (N1L9) than at 17 d of latency (N9L17) ($P = 0.0028$) (Fig. 3). No significant differences in MRCV accumulation were found between nontransmitting insects of N1L9, N1L17, and N3L17 treatments.

Two insects belonging to treatments N1L9 and N3L17 had high viral titers but did not transmit the virus, and a single insect from treatment N1L17 was able to transmit despite having a low viral titer (Fig. 3).

Finally, no significant differences in the levels of viral accumulation between males and females were found, for either transmitting ($P = 0.0629$) or nontransmitting insects ($P = 0.4752$) (Table 3).

Table 3. Estimation of MRCV titer in transmitting and nontransmitting females and males of *D. kuscheli* planthoppers by qPCR of segment S3 (MRCV-S3)

	Sex	Mean (log_{10} MRCV-S3 copies)	SD	*P* value[a]
Transmitting	Females	13.51	1.42	0.6209
	Males	12.40	2.96	
Nontransmitting	Females	10.12	2.92	0.5588
	Males	8.80	1.38	

SD: standard deviation.
[a] Mann–Whitney test.

Discussion

MRCV is only transmitted by planthoppers in a persistent propagative manner (Arneodo et al. 2002). In this type of transmission, viruses replicate and move throughout the insect host to different organs and finally reach the salivary glands. Therefore, successful transmission implies the overcome of diverse barriers within the vector. Currently, many molecular studies are focused on clarifying the mechanisms related to transmission efficiency to develop more effective strategies to control viral plant diseases (Hogenhout et al. 2008). In the case of plant reoviruses such as MRCV, the underlying mechanisms that determine that only a few of the infected planthoppers are able to transmit the virus to uninfected plants (Boccardo and Milne 1988, Arneodo et al. 2002, Dhileepan et al. 2006) are still unknown.

In the current study, we measured if the effective transmission of MRCV by the planthopper vector *D. kuscheli* is associated to the viral titer in the insect at different developmental stages or after different latency periods. For this purpose, an experimental transmission assay of MRCV was conducted using first or third instars of *D. kuscheli*, with minimum and maximum latencies of 9 and 17 d, respectively. The greatest transmission efficiency (28.88%) was obtained when the acquisition occurred at a more juvenile stage (first-instar nymphs) combined with a long latency period (17 d) (Fig. 1). In turn, after the minimum latency period of 9 d, 11.13% of first instars transmitted the virus, whereas none of the third instars were transmitters (Fig. 1). These results are in accordance to previously published work on MRCV experimental transmission by *D. kuscheli* (Arneodo et al. 2005).

After the transmission trial, the viral titers of each transmitting and nontransmitting planthoppers were analyzed indirectly by quantifying MRCV-S3 concentration by using a previously developed qPCR protocol (Maroniche et al. 2011). The results showed that the planthoppers capable of transmitting MRCV have significantly higher viral titers than the nontransmitting insects (Table 1), suggesting that the level of MRCV infection must exceed a certain threshold for viral transmission to be successful. In turn, the viral load might be directly linked to the insect susceptibility to viral infection. In relation to this, Contamaine et al. (1989) detected the existence of refractory genes that present polymorphic alleles in natural populations of *Drosophila melanogaster* Meigen and prevent the replication of sigma virus (family *Rhabdoviridae*, genus *Sigmavirus*, SIGMAV). Ziegler and Morales (1990) suggested that susceptibility of *Tagosodes orizicolus* (Muir) to infection by rice hoja blanca virus (genus *Tenuivirus*, RHBV) was associated with a recessive gene that, in homozygous condition, determined the insect's ability to tolerate viral infection. A similar hypothesis could explain the different levels of MRCV infection in planthoppers of the same population but would not explain the variation between first and third instars observed in this study (Fig. 3). In this case, the planthopper's susceptibility to MRCV infection might also be associated to a permissive physiological state, because of a less developed antiviral defense status. The participation of innate immune responses (Toll, immune deficiency [Imd], Janus kinase–signal transducers and activators of transcription [Jak-STAT] pathways) (Tsai et al. 2008) and RNA mediated interference (RNAi) (Zambon et al. 2006) in controlling virus replication in insects is well known. Recently, Zhang et al. (2010) reported an increase in the expression of genes associated with anti-viral response such as RNAi, JAK-STAT, and Imd partial cascades in *Laodelphax striatellus* Fallen planthoppers infected with rice stripe virus (family Unassigned, genus *Tenuivirus*, RSV). Moreover, Xu et al. (2012) found that an RNAi pathway is stimulated in *Sogatella furcifera* (Horvàth) when infected with southern rice black-streaked dwarf virus (family *Reoviridae*, genus *Fijivirus*, RBSDV), indicating that this pathway might play an important role in insect defenses to viral infection. Interestingly, it has been reported that activation of Toll and Imd pathways is dependent on insects' age because age influences the maturation of the fat body, the main site of synthesis of innate immune factors (Lemaitre and Hoffman 2007). Therefore, the greater MRCV transmission efficiency of first instars compared with third instars (Fig. 1) may be explained by a more permissive immune system in the first group, resulting in an increased viral accumulation and thus a higher proportion of transmitters.

The difference in transmission efficiency and virus accumulation observed between first and third instars might also be because of morphological barriers. It is known that the first hurdle facing persistent virus entry into the hemiptera body is a multilayer of laminae, probably analogous to the peritrophic membrane, a semipermeable layer of chitin and proteins that protects the microvilli of the midgut from food particles and pathogen entry (Chapman 2003). This membrane is not fully developed in younger individuals, which could lead to an increased inflow of virus particles in the body of less developed insects (Ammar et al. 2009). Consequently, the increased susceptibility to MRCV infection of younger insects might result in a greater likelihood of transmitting the virus.

Noticeably, the planthoppers that acquired the virus as third-instar nymphs were not able to transmit MRCV after 9 d of latency, even though nearly 46% of the insects of this treatment had viral loads equivalent to transmitting insects of other treatments (Fig. 3). This lack of transmission might be a consequence of a greater physical distance between two key organs in persistent propagative viral transmissions: the midgut and salivary glands. If this was the case, infective particles would fail to reach the salivary glands at shorter times even when the virus is actively replicating in other tissues. In agreement with this hypothesis, Moritz et al. (2004) showed that in *F. occidentalis*, the main vector of TSWV, the three tissues involved in the virus transmission (midgut, visceral muscles, and salivary glands) are spatially associated during larval stages but not in the adult stage, which would limit

viral acquisition only to the larval stage. The tracking of MRCV throughout infected *D. kuscheli* body by immunofluorescence, as well as the qPCR quantification of viral titers in the insect salivary glands, certainly will improve our understanding of the vector ontogenic factors influencing viral transmission.

There were no significant differences in viral titer between males and females of *D. kuscheli*, in both transmitting and nontransmitting insects (Table 3). For *Tospovirus*-thrips pathosystem, Rotenberg et al. (2009) showed that male thrips were more likely than females to transmit TSWV multiple times, even when they showed significantly fewer virus copies. The authors associated this difference in transmission ability to the feeding behavior of each sex. Even so, the result obtained in this work supports the observations of Arneodo et al. (2002), who reported no differences in transmission efficiency of MRCV between *D. kuscheli* males and females. In accordance, Ornaghi et al. (1999) reported no differences in transmission efficiency in natural populations of *D. kuscheli.* The fact that there is no difference in transmission efficiency between males and females could be because of the nondifferential viral accumulation between sexes.

In summary, the experimental evidence presented here shows that the efficiency of MRCV transmission by *D. kuscheli* is strongly linked to the number of individuals that reach a minimum threshold of viral concentration. However, there might be other barriers for transmission associated to the age of the vector. This work contributes to better understand the mechanisms underlying *Fijivirus* transmission and its epidemiological implications.

Acknowledgments

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