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Transmission of a pathogenic virus (Iridoviridae) of *Culex pipiens* larvae mediated by the mermithid *Strelkovimermis spiculatus* (Nematoda)

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ABSTRACT

Little progress been made in elucidating the transmission pathway of the invertebrate iridescent virus (MIV). It has been proposed that the MIV has no active means to enter the mosquito larva. We have previously found that the presence of the mermithid nematode *Strelkovimermis spiculatus* is associated with MIV infection in *Culex pipiens* under field conditions. In the present study, we evaluated the transmission of MIV to *C. pipiens* larvae mediated by *S. spiculatus* and several factors involved in this pathway (mosquito instars, nematode:mosquito larva ratio, amount of viral inoculum). Our results indicate that *S. spiculatus* functions as an MIV vector to *C. pipiens* larvae and seems to be an important pathway of virus entry into this system. Moreover, TEM images of *S. spiculatus* exposed to the viral suspension showed no infections inside the nematode but showed that viral particles are carried over the cuticle of this mermithid. This explains the correspondence between MIV infection and the factors that affect the parasitism of *S. spiculatus* in *C. pipiens* larvae.

1. Introduction

This study involved the interaction of three organisms: *Culex pipiens* (L.) (Culicidae), *Strelkovimermis spiculatus* Poinar and Camino (Mermithidae) and the mosquito iridescent virus (Iridoviridae) (MIV). *C. pipiens* is a culicid with worldwide distribution and its importance as a vector of human and animal diseases is well known (Foster and Walker, 2002; Farajollahi et al., 2011). In Argentina, the most common breeding sites for this mosquito species are man-made containers and drainage ditches in suburban areas of the cities. In this natural habitat, several enemies of *C. pipiens*, including an α-proteobacterium (Tranchida et al., 2012), a cyclopoid (Copepoda) (Tranchida et al., 2009), and *Hazardia milleri* (Microporidia) (Campos et al., 1993) have been reported. In addition, *S. spiculatus* has been found parasitizing this mosquito species (Garcia and Camino, 1990). This neotropical mermithid was first isolated from larvae of *Ochlerotatus albifasciatus* (Macquart) in La Plata, Argentina (Poinar and Camino, 1986). The life cycle of *S. spiculatus* consists of free and parasitic stages. Second-instar juveniles (J2 or preparasites) emerge from eggs to aquatic breeding sites and enter the mosquito larva by actively puncturing on its cuticle. Then, the nematode grows inside the mosquito larva until it emerges as a fourth-instar juvenile (J4 or postparasites), resulting in the death of the mosquito immature stage (Camino and Reboredo, 1994).

The family Iridoviridae includes double-stranded linear DNA viruses that affect both vertebrates and invertebrates around the world. Among the five genera that integrate this family, only two are invertebrate iridescent viruses and MIV belongs to the genus *Chloriridovirus*. These are non-occluded viruses and their icosahedral shape allows paracrystalline arrangements in the cytoplasm, which cause the iridescent coloration. MIV was first reported in Florida, affecting *Aedes taeniorhynchus* (Wiedemann) (Clark et al., 1965). After that, the virus has been recorded in many other mosquito species and countries (Williams, 2008). Few studies have been made to elucidate the transmission pathway of this virus. Some authors have proposed that MIV has no active means to enter the mosquito larva and that it therefore crosses the primary barrier through an injury on the external cuticle or peritrophic membrane (Undeen and Fukuda, 1994; Stoltz and Summers, 1971). Other authors have proposed cannibalism, injuries, nematode

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penetration, and hymenopteran endoparasitoid oviposition as possible mechanisms of horizontal transmission. (Williams, 2008). Moreover, Woodard and Chapman (1968) reported transovarial transmission of MIV to larval progeny of *A. taeniorhynchus* adults exposed to MIV as 4th instar larvae.

We have previously reported the presence of MIV infecting *C. pipiens* larvae in the neotropical region (Muttis et al., 2012). We have also found that the presence of the mermithid nematode *S. spiculatus* is strongly associated with MIV infection in *C. pipiens* under field conditions (Muttis et al., 2013).

In this study, we aimed to better understand the behavior of this virus in nature, the virus-nematode relationship and the mechanism of virus entry into *C. pipiens* larvae mediated by *S. spiculatus* as well as the influence of several factors.

2. Materials and methods

2.1. Mosquito larvae

Healthy *C. pipiens* larvae, obtained from the laboratory colony maintained at the insectary of the Centro de Estudios Parasitológicos y Vectores (CEPAVE), La Plata, Argentina, were used for the assays. Larvae were reared in plastic trays for 72 h with finely ground rabbit chow at 25 ± 1 °C and under a 12:12 h light–dark photoperiod.

2.2. Nematodes

Second-instar juveniles (J2 or preparasites) of *S. spiculatus* were obtained 24 h after flooding cages with sand and *S. spiculatus* eggs, as described by Camino and Reboredo (1996).

2.3. Viruses

2.3.1. Purification

Viral particles from a known number of MIV-infected *C. pipiens* larvae were purified to estimate the number of particles per larva. Laboratory MIV-infected third-instar larvae (L3) of *C. pipiens* were homogenized in distilled water with a glass homogenizer. Macerated larvae were filtered through cotton in a syringe. Then, the suspension was subjected to two steps of differential centrifugation (2500 rpm for 15 min and 15,000 rpm for 30 min) to remove cellular debris. The pellet was resuspended in distilled water followed by centrifugation at 12,000 rpm through a 15–60% (*w*/*v*) sucrose gradient. The visible blue band was separated and washed twice in sterile distilled water. To isolate the viral particles, the suspension was centrifuged at 30,000 rpm. This procedure was repeated twice to obtain an average number per larva, using 130 and 204 larvae each time.

2.3.2. Quantification of viral particles per mosquito larva

Viral particles were quantified based on the general methodology described by Constantino et al. (2001), with some modifications. Briefly, the concentration of viral particles from each previous purification was determined by TEM instead of by SEM. Aliquots of viral suspension and polystyrene beads of 460 nm diameter (1.8×10^9 b/ml) (Sigma–Aldrich) were mixed (10 µl of each). To obtain a proportion of viral particles and beads close to 1:1, the stock suspension was diluted 1/100. From this suspension, 2 µl was put on a grid and allowed to dry. Nine squares from the grid were examined under TEM and the viral particles and beads were counted to calculate the virus/beads ratio. The number of viral particles per larva was calculated based on the total number of viral particles in the stock suspension.

2.3.3. Preparation of inocula

To prepare the inocula, one hundred 72-h-old larvae were exposed to three homogenized MIV-infected third-instar larvae and 500 J2 of *S. spiculatus* in 100 ml of water for 96 h and maintained at 25 ± 1 °C. Totally infected third-instar larvae were selected as inoculum and then stored at −20 °C until use. Infected larvae used as inoculum were previously homogenized with a pestle in distilled water.

2.4. Assays

2.4.1. Biotic factors

Twenty healthy *C. pipiens* larvae were exposed to the viral inoculum and *S. spiculatus* preparasites for 24 h in 30-ml plastic containers with 25 ml of distilled water. After that, the larvae were washed in distilled water and transferred to 200-ml plastic containers with 150 ml of distilled water and finely ground rabbit chow. Larvae were evaluated at 72 h post-exposure, the approximate period of time after which infected larvae show an iridescent blue color throughout the body. Larvae were observed under a stereomicroscope on black background and the number of live larvae and infected larvae were recorded. All tests were carried out at 25 ± 1 °C under a 12:12 h light–dark photoperiod. Controls performed for each assay are described in the following sections. In controls, MIV infection, mortality, and the J2:mosquito larva ratio was recorded. Each assay was performed in triplicate *and repeated* three times.

2.4.1.1. Nematode J2:mosquito larva ratio. Four J2:mosquito larva ratios were tested: 1:1, 3:1, 5:1 and 7:1. One MIV-infected larva was used as inoculum.

Three controls were made: larvae exposed to the virus (one infected larva), larvae exposed to each different J2:mosquito larva ratio and larvae reared without any pathogens.

2.4.1.2. Instar of mosquito larva. Larvae of each instar were exposed to one MIV-infected larva and a 5:1 J2:mosquito larva ratio. This ratio was selected to obtain a high rate of parasitism with the lowest number of parasites. Three controls were made for each instar: larvae exposed to the virus, larvae exposed to a 5:1 J2:mosquito larva ratio and larvae reared without any pathogens.

2.4.1.3. Amount of inoculum. Increasing amounts of inoculum were tested. A preliminary test with more than six MIV-infected larvae was carried out to decide the maximum amount of inoculum to be used in the experiment. We thus selected half, one, three and six infected larvae. To use half larva, one infected larva was homogenized in 500 µl of distilled water and 250 µl of this stock was used as inoculum. Three controls were made: larvae exposed to each amount of inoculum, mosquito larvae exposed to a 5:1 J2:mosquito larva ratio and larvae reared without any pathogens.

2.4.2. Abiotic factor

For this assay, the infection was performed with an 80-fold higher volume of water. Twenty healthy second-instar mosquito larvae were exposed to the viral inoculum (one homogenized MIV-infected larva) and juvenile nematodes (5:1) in plastic pails (23.5 cm diameter) with 2 l of dechlorinated water for 72 h. Two controls were made: healthy larvae rinsed in the container without any pathogens and mosquito larvae exposed to juvenile nematodes (5:1). This assay was performed in triplicate and repeated two times.

2.4.3. Multiple parasitism and MIV infection

To understand why MIV-infected larvae parasitized by late juvenile nematodes occurred at field but not at experimental

conditions, we exposed second-instar mosquito larvae to a 5:1 J2:mosquito larva ratio in 300 ml of dechlorinated water. When larvae were at the late third or early fourth instar, 30 of these were isolated in 30-ml plastic containers with 25 ml of distilled water and exposed again to preparasites but at this time MIV was added. After that, the larvae were transferred to 200-ml plastic containers with 150 ml of distilled water and finely ground rabbit chow. After 72 h of virus exposure, larvae with symptoms of MIV infection were separated and observed under optical microscopy to detect the presence of nematodes within their bodies.

2.5. Electron microscopy

Preparasites of *S. spiculatus* were exposed to the viral suspension for 24 h and processed to be observed under an electron microscope. The J2 suspension was concentrated at 1500 rpm and fixed in 2% glutaraldehyde for 2 h at 4 °C and washed with phosphate buffer. After that, the sample was embedded in agar and fractionated in small pieces and fixed again in 2% glutaraldehyde for 2 h at 4 °C and washed with phosphate buffer. The pieces were postfixed in 1% osmium tetroxide for 2 h at 4 °C and dehydrated using increasing concentrations of ethanol into acetone and embedded in Epon-Araldite resin. Ultrathin sections (60 nm) stained with 2% uranyl acetate and lead citrate were examined with an electron microscope (JEOL JEM 1200 – EX II).

2.6. Statistical analysis

The prevalence of MIV infection in all assays was calculated from the total number of live larvae. The number of MIV-infected larvae and the number of live larvae for each biotic factor was subjected to analysis of variance (ANOVA). When the results were significant ($p < 0.05$), Tukey's HSD test (method: 95.0%) was used for multiple comparisons between treatments.

3. Results

3.1. Virus quantification and larval equivalent

A concentration of virus per larva in the order of 10^8 was obtained from both quantifications. The mean number of viral particles per larva (larval equivalent) was 5.85×10^8.

3.2. Assays

3.2.1. Instars of exposed larvae

When the larvae of the different instars were exposed to MIV and the nematode, all were susceptible to infection by the virus. The mean prevalence of MIV infection varied between 82.5% ($n = 180$) for first-instar larvae and 5% ($n = 180$) for fourth-instar larvae. The differences in the number of infected larvae among all larval instars were highly significant ($F = 19.91$, $df = 3$, $p < 0.01$). The post hoc test showed significant differences in the number of infected larvae between instars except between first- and second-instar larvae (Table 1). No significant differences were recorded in the number of live larvae at 72 h among larval instars ($F = 2.13$, $df = 3$, $p > 0.05$) (Table 1). No infection by MIV was recorded in any of the controls and the mortality of healthy larvae did not exceed 13% ($n = 240$).

3.2.2. J2:mosquito larva ratio

MIV prevalence increased from 38% ($n = 180$) with the lower ratio (1:1) to 88.3% ($n = 180$) with the highest ratio (7:1) when 72 h-old larvae were exposed to different proportions of nematodes. The difference in the number of MIV-infected larvae obtained between ratios was highly significant ($F = 12.78$, $df = 3$, $p < 0.01$), except for the pairs 3:1–5:1 and 5:1–7:1 (Table 2). There was no significant difference in the number of live larvae among all ratios except between ratios 1:1 and 7:1 ($F = 3.17$, $df = 3$, $p > 0.05$) (Table 2). In controls with larvae exposed only to the virus, one MIV-infected larva was recorded ($n = 180$). On the other hand, none of the larvae were infected in the other controls. In the control with larvae exposed only to the nematode, the parasitism prevalence was higher than the prevalence of MIV infection in assays with both pathogens, except for the ratio 7:1 (Fig. 1). The mortality of healthy larvae did not exceed 5.5% ($n = 180$).

3.2.3. Amount of inoculum

Next, 72 h-old *C. pipiens* larvae were exposed to different amounts of MIV inoculum (infected larvae, IL): 1/2 IL ($\approx 2.92 \times 10^8$ viral particles), 1 IL ($\approx 5.85 \times 10^8$), 3 IL ($\approx 1.75 \times 10^9$) and 6 IL ($\approx 3.51 \times 10^9$), assuming that each larva had around 5.85×10^8 viral particles. The prevalence of MIV infection decreased from the first inoculum (86.1% ($n = 180$)) to the fourth inoculum (43.3% ($n = 180$)). We found no significant differences in the number of infected larvae for the different amounts of inoculum ($F = 7$, $df = 3$, $p < 0.01$), specifically between the pairs 1/2 IL–3 IL and 1/2 IL–6 IL (Table 3). The mortality of healthy larvae did not exceed 5% ($n = 60$).

3.3. Abiotic factor

The prevalence of MIV infection was 42% ($n = 120$). The nematode prevalence in the control was 41.5% ($n = 40$). The mortality in the control of healthy larvae was 10% ($n = 40$).

3.4. Multiple parasitism

At 72 h post-exposure, 10 larvae ($n = 30$) were detected with MIV infection, five of which had the nematode inside, being three of them at the late juvenile stage. In subsequent days of observation, all larvae died between the fifth and ninth day and only one postparasite emerged before the host died.

3.5. Electron microscopy of S. spiculatus preparasites exposed to MIV

Several cross-sections of juvenile nematodes exposed to MIV were observed under TEM. Many viral particles were located

Table 1
Variation of MIV infection on different instar of *Culex pipiens* larvae exposed to 5:1 nematode (J2): mosquito larvae ratio.

Instar of larvae	N° larvae exposed	N° larvae live[a]	N° larvae infected[a]
1st	180	14.9 ± 2.7 a	12.3 ± 2.8 a
2nd	180	17.0 ± 1.9 a	11.2 ± 4.4 a
3rd	180	17.1 ± 2.2 a	6.4 ± 4.5 b
4th	180	17.7 ± 2.6 a	0.9 ± 1.3 c

[a] Mean ± standard deviation. Values followed by a common letter are not significantly different by Tukey's HSD test ($p = 0.05$).

Table 2
Variation of MIV infection of *Culex pipiens* larvae exposed to an increasing nematode (J2): mosquito larva ratio.

J2: larva ratio	N° larvae exposed	N° larvae live[a]	N° larvae infected[a]
1:1	180	19.2 ± 0.9 a	7.3 ± 2 a
3:1	180	18.5 ± 1.1 ab	11.2 ± 3.2 b
5:1	180	18.3 ± 1.4 ab	13.9 ± 3.7 bc
7:1	180	17.1 ± 2.1 b	15.1 ± 2.2 c

[a] Mean ± standard deviation. Values followed by a common letter are not significantly different by Tukey's HSD test ($p = 0.05$).

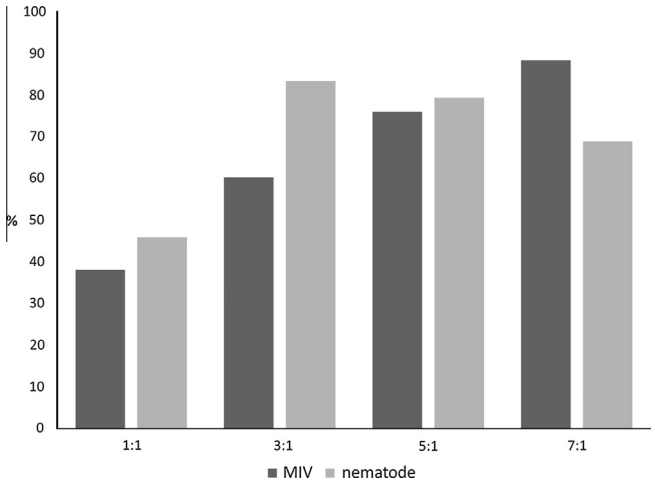

Fig. 1. Comparison between MIV prevalence (%) in *Culex pipiens* larvae in assays with different of *Strelkovimermis spiculatus* (J2): mosquito larva ratio and nematode prevalence (%) in controls without virus.

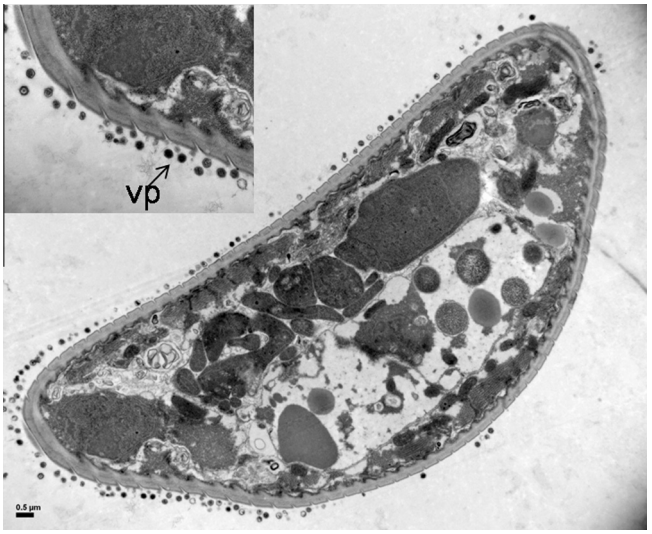

Fig. 2. Ultra-thin sections of *Strelkovimermis spiculatus* (J2) after exposure to MIV for 24 h. Viral particles (vp) are surrounding the sections close to the cuticle.

around sections and many of them looked as if they were stuck to the cuticle. No particles were observed inside the bodies of the nematodes (Fig. 2).

4. Discussion

We studied the *C. pipiens*-MIV transmission mediated by the mermithid *S. spiculatus*. Mosquito larvae of all instar were susceptible to the virus infection mediated by the mermithid. However, there were significant differences between the prevalence of infection between early and late instars. A similar decrease in the parasitism by *S. spiculatus* in mosquito larvae was observed by Achinelly and Camino (2005). When we tested the effects of different J2:mosquito larva ratios, the MIV prevalence increased by 50% from 1:1 to 7:1. Similar results were obtained by Camino and García (1992) working with factors that affect the parasitism by *S. spiculatus* in mosquito larvae. Higher number of preparasites could increase the probability of larvae to become infected by the virus, which is expected if the virus entry is due to the nematode. When we exposed mosquito larvae to different amounts of viral inoculum in the presence of the nematode, we obtained an unexpected result: MIV prevalence decreased significantly from the lowest amount of inoculum to the highest, instead of increasing, as shown in other studies without the nematode (Linley and Nielsen, 1968b). In this study, we expected that the MIV prevalence remained uniform because the infection in this case appears to be related to the presence of preparasites. Looking for an explanation of these results, we observed that some J2 became hooked to tissue debris which was higher with the inoculum and thus would not be able to look for a host. Another possibility is that the viral particles affect the nematodes in some way, preventing them from entering the hemocele of larvae. In several TEM observations, there were no viral particles inside the nematode, suggesting that J2 did not become infected by MIV. Further studies should be carried out to understand this result. As abiotic factor, we evaluated the effect of a higher volume of water on the prevalence of MIV in mosquito larvae and concluded that a 80-fold higher volume reduces the percentage of infection from 75.9% (25 ml) to 42% (2000 ml) ($n = 60$) using the same J2:mosquito larvae ratio. This situation is closer to the natural habitat in which the preparasites and viral particles should meet each other before finding the mosquito larva.

In several field MIV-infected mosquito larvae, late juvenile stages of *S. spiculatus* have been observed emerging as postparasites from the larvae (Muttis et al., 2013). In contrast, in the present study, the nematodes were not able to develop to late stages and emerge from MIV-infected larvae. We carried out experimental assays where we found that the multiple parasitism by this mermithid (observed previously by Campos and Sy (2003) in mosquito larvae at field) could be the cause of this situation in nature.

Although in related *A. taeniorhynchus*-MIV studies virus infection was obtained when merely placing cadavers together with healthy larvae under experimental conditions (60% as a maximum) (Linley and Nielsen, 1968a; Undeen and Fukuda, 1994), we have been almost unable to obtain infection in this way (only two infected larvae in 3500 exposed larvae, 0.06%) or when using several injury mechanisms (diatomaceous earth, gross sand on agitation, crowding) at different intensities (unpublished data). Instead, we were able to obtain high levels of infection (100% as a maximum) using the preparasite juvenile of the nematode as a virus vector in laboratory conditions. This parasite punches the cuticle of mosquito larvae to complete part of its life cycle and this would be the time in which the virus enters the larval hemocele. TEM observations of preparasites exposed to MIV showed that viral particles are carried over the cuticle of this mermithid but no infection was detected inside the nematode as previously observed in a mermithid parasite of Isopods (Poinar et al., 1980).

The evolutionary relationship of the major capsid protein (MCP) sequence of *C. pipiens*-MIV and the sequences from the six different viruses of the family Iridoviridae shows that *C. pipiens*-MIV MCP clusters most closely with IIV-22 from *Simulium sp* related to MIV-3 from mosquitoes (Muttis et al., 2012). In this sense, the *C. pipiens*-MIV system appears to be very different from *A. taeniorhynchus*-MIV, in the horizontal transmission mechanism,

Table 3
Variation of MIV infection of *Culex pipiens* larvae exposed to an increasing of viral inoculum and a 5:1 nematode (J2): mosquito larva ratio.

Inoculum	N° larvae exposed	N° larvae live[a]	N° larvae infected[a]
1/2 infected larva	180	15.8 ± 3.5 a	13.5 ± 3.7 a
1 infected larva	180	15.5 ± 2.4 a	11.5 ± 3.0 ab
3 infected larva	180	15.3 ± 3.3 a	8.1 ± 3.3 b
6 infected larva	180	17.3 ± 2.9 a	7.5 ± 2.8 b

[a] Mean ± standard deviation. Values followed by a common letter are not significantly different by Tukey's HSD test ($p = 0.05$).

and also in the timing required for the detection of signs of infection. Many authors refer that *A. taeniorhynchus*-MIV signs begin in the late third instar or fourth instar and that death ensues in the fourth instar regardless of whether the infection initiated early (Linley and Nielsen, 1968a; Undeen and Fukuda, 1994). In contrast, *C. pipiens*-MIV signs appear and death ensues in the same or one-instar later to which the larva was infected (unpublished data). This system appears to be more similar to that found by Mullens et al. (1999) on the biting midge *Culicoides variipennis sonorensis* parasitized by the mermithid *Heleidomermis magnapapula*, which has also shown strong association between the presence of the mermithid and Iridovirus infection both at field and laboratory conditions, where the transmission without nematode was very low (≈0.27%).

Our results indicate that *S. spiculatus* functions as an MIV vector to *C. pipiens* larvae and seems to be an important pathway of virus entry into this system. Although progress has been made in the study of the MIV in natural populations of *C. pipiens*, the knowledge of its behavior in nature needs to be improved. Vertical transmission and covert infections which are known in black flies and mosquitoes (Williams, 2008) could play a role in dispersion of this natural pathogen-mosquito system.

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