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BIOLOGICAL CONTROL

Mosquito Iridescent Virus: New Records from Nature and Infections Using *Strelkovimermis spiculatus* (Mermithidae) as a Vector Under Laboratory Conditions

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Abstract
Iridoviridae is a DNA virus family that affects both vertebrates and invertebrates. Immature aquatic stages of many dipteran species infected with iridovirus have been found in different places worldwide. The most represented genera of the Culicidae family are *Aedes* and *Psorophora*. To date, sixteen species of *Aedes* naturally infected with iridoviruses have been reported. Moreover, there are four records for the genus *Psorophora*, one for *Culiseta*, and two for *Culex.* In this paper, we report two new mosquito species as natural hosts of iridoviridae in Argentina: *Aedes albifasciatus* (Macquart) and *Culex dolosus* (Lynch Arribalzaga). We also analyzed the ability of a *Cx. pipiens*-Invertebrate Iridescent Virus to replicate in vivo in the larval stage of two mosquito species, *Culex apicinus* Philippi and *Ae. aegypti* (L.) using *Strelkovimermis spiculatus* as a vector, under laboratory conditions. Although *Ae. aegypti* is the most recognized mosquito vector of important arboviruses responsible for emergent diseases, *Cx. apicinus* and *Ae. albifasciatus* may also be implicated in enzootic or epizootic cycles of virus transmission, such as the St. Louis Encephalitis virus and the Western Equine Encephalomyelitis virus.

Introduction

Iridoviridae is a DNA virus family that affects a wide range of host species, both vertebrates and invertebrates. Among the invertebrate hosts, around 95% are arthropods and the remaining 5% consists of annelids, mollusks, and nematodes. Immature aquatic stages of Diptera infected with iridovirus have been found in different places worldwide. The families with the greatest number of reports are the Simuliidae, Ceratopogonidae, Tipulidae, and Culicidae. The most represented genera of the Culicidae family are *Aedes* and *Psorophora*. To date, sixteen species of *Aedes* naturally infected with iridoviruses have been reported, but *Aedes aegypti* (L), *Aedes albopictus* (L), and *Aedes albifasciatus* (Macquart) are not among them. There are four records for the genus *Psorophora*, one for *Culiseta* and two for *Culex* (Fedorova 1986; Muttis *et al* 2012).

A peculiarity of iridescent invertebrate viruses is their ability to produce two types of infections: patent and sublethal. The first type has obvious symptoms, such as iridescence, and the infected immature always dies. The other type does not produce this symptoms; the individual infected can survive and becomes an adult (Marina *et al* 1999; Williams 1993). Most records from dipterans are patent-infected larvae and little is known about the host range of sublethal infections, and there is only one record in adult *Anopheles* (Huang *et al* 2015).

Different iridoviruses usually differ markedly from each other with regard to their laboratory host range. Some iridoviruses only naturally infect a small number of host species, such as the Invertebrate Iridescent Virus-3 (IIV-3), the type species of the genus *Chloriridovirus*, isolated from the

mosquito *Aedes taeniorhynchus* (Wiedemann) (Clark *et al* 1965). Other viruses, such as IIV-6, originally isolated from *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), have a wider host range, being able to infect numerous species of mosquitoes, including *Ae. aegypti* (Fukuda 1971; Marina *et al.* 2003). Note that *Anopheles*, *Culex*, and *Culiseta* species are susceptible to IIV-6 (Fukuda 1971) but not to IIV-3 (Woodard & Chapman 1968) under laboratory conditions. Studies performed on this issue seem to indicate that certain IIVs are able to infect a number of sympatric host species (Williams *et al* 2005), and the range of species in which the IIVs replicate depends greatly on the route of infection. This appears to be more restricted in nature than in the laboratory, where injected virus can infect species of different taxonomic orders and even different classes (Ohba & Aizawa 1979; Williams *et al* 2005). For example, IIV-22 (isolated from *Simulium* spp.) can infect different species of mosquitoes, sandflies, and at least one triatomine species by intrathoracic inoculation (Tesh & Andreadis 1992).

We previously reported the presence of IIV infecting *Cx. pipiens* larvae in the Neotropical region (Muttis *et al* 2012), and we also found that the presence of the nematode *Strelkovimermis spiculatus* Poinar and Camino is associated with IIV infections in *Cx. pipiens* under field conditions (Muttis *et al* 2013). This neotropical mermithid was first isolated from larvae of *Ae. albifasciatus* in La Plata, Argentina (Poinar & Camino 1986). Additionally, in horizontal transmission studies, we only achieved the successful transmission of the virus when the nematode was present. Furthermore, viral particles were observed stuck to the cuticle in ultrathin sections of juvenile parasites previously exposed to the virus, indicating that *S. spiculatus* functions as a vector of *Cx. pipiens*-IIV to *Cx. pipiens* larvae (Muttis *et al* 2015).

In this paper, we report two new mosquito species as natural hosts of iridoviridae: *Ae. albifasciatus* and *Culex dolosus* (Lynch Arribalzaga). Moreover, we analyzed the ability of a *Cx. pipiens*-Invertebrate Iridescent Virus to replicate in vivo in the larval stage of two mosquito species, *Culex apicinus* Philippi and *Ae. aegypti* using *S. spiculatus* as a vector under laboratory conditions. Although *Ae. aegypti* is the most recognized mosquito vector of important arboviruses responsible for emergent diseases, *Cx. apicinus* and *Ae. albifasciatus* may also be implicated in enzootic or epizootic cycles of virus transmission, such as the St. Louis Encephalitis virus and the Western Equine Encephalomyelitis virus (Avilés *et al* 1992; Díaz *et al* 2012).

Materials and Methods

Field survey

Mosquito sampling was performed sporadically during 2 years at three different sampling sites in Buenos Aires province, Argentina (Fig 1). The sites were composed of drainage ditches outside houses in suburban areas of Berisso. These drains collected polluted water from the houses as well as rainwater, being a normal breeding site for immature mosquitoes of the *Cx .pipiens* complex. The rain ditches in Los Hornos (La Plata) were in rural areas characterized by grassland and rice fields. The water accumulated in these ditches came from rain and was not polluted. Lastly, the flooded puddles in Ensenada were in natural forest areas where large puddles often form; their temporary existence depends almost exclusively on exceptional floods in the Río de La Plata. At all the sites, larvae were collected using a dipper and transported to the laboratory in plastic containers with water from the sites.

Taxonomic identification

The third and fourth instar larvae were counted and identified using taxonomic keys for immature dipterans (Darsie & Mitchell 1985). Some healthy larvae from each sample were mounted on slides with mounting resin to visualize the specific taxonomic characters following the technique proposed by Gaffigan & Pecor (1997). Infected larvae were observed under a stereomicroscope and grouped by morphological similarity. One infected larva of each species was mounted to confirm the taxonomic identification.

Nematodes

Nematode prevalence was calculated for each sample. The percentage of parasitism was determined using 20–150 fourth instar larvae per sample. Larvae were placed individually in multiwell plates for nematode emergence. The emerging parasites were identified according to their original descriptions (Poinar & Camino 1986).

Viruses

Mosquito larvae collected at the different sites were observed using a stereomicroscope on a black background to detect the turquoise iridescence characteristic of iridovirus infections. Virus prevalence was calculated in the total number of larvae collected in the corresponding sample. The presence of viruses belonging to the *Iridoviridae* family in larvae with symptoms was confirmed by PCR assays using MCP primers that amplified a conserved region of the major capsid protein, as described by Muttis *et al* (2012). Viral particles in the infected larvae were partially purified before DNA extraction to remove cellular debris. The DNA was purified by standard phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.* 1989). PCR amplification was carried out under standard conditions (1X standard buffer, 0.25 U Taq DNA polymerase, 1.5 mM MgCl2, 1 mg/ml BSA, 0.2 mM each dNTP, and 1 μM each primer). The amplification profile used was as

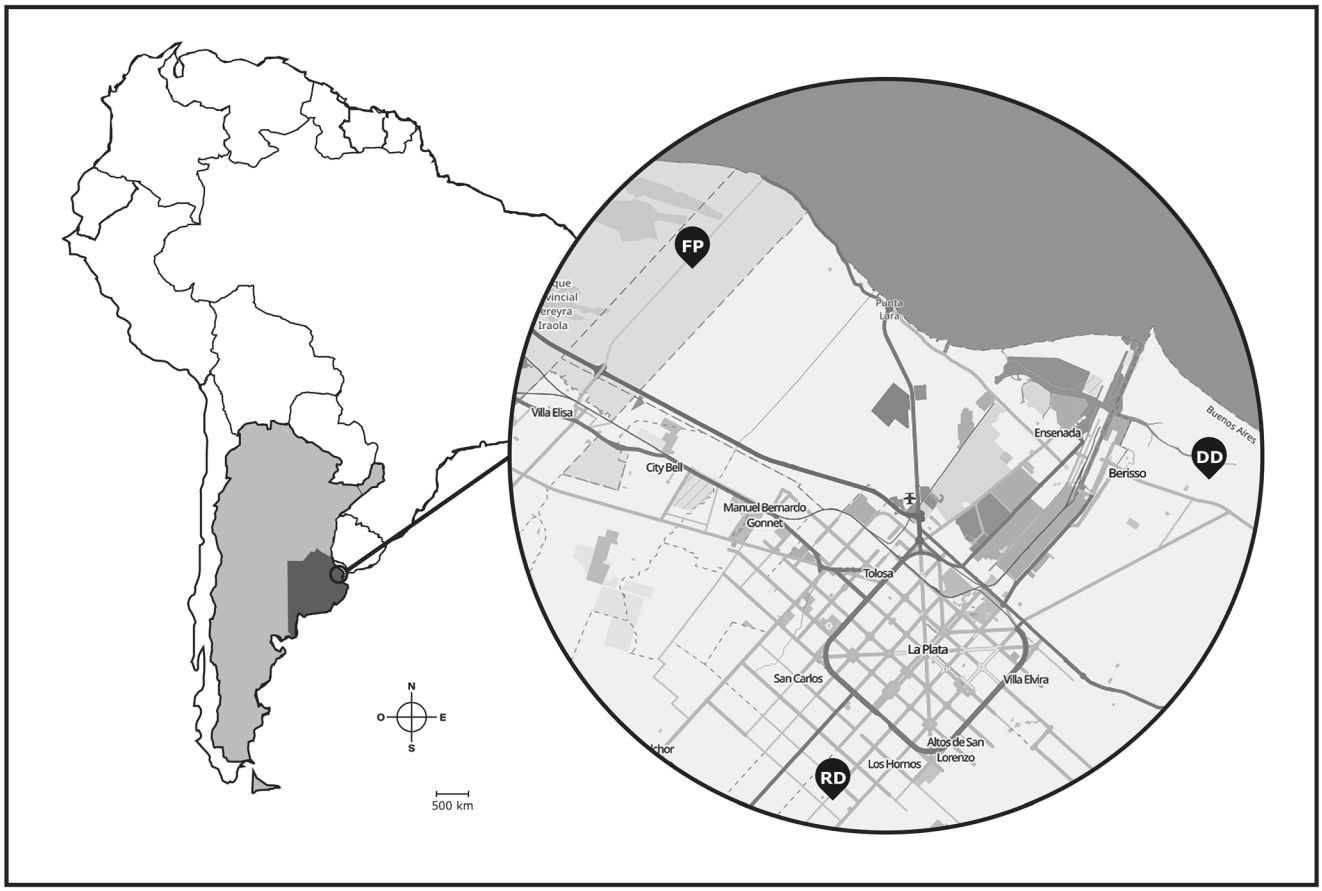

Fig 1 Map showing the location of the three sampling sites. Drainage ditches (DD) in Berisso City, rain ditches (RD) in Los Hornos (La Plata City), and flooded puddles (FP) in Ensenada.

follows: one initial denaturing step of 1 min at 94°C, 35 cycles of 10 s at 94°C, 10 s at 56°C, and 20 s at 72°C, followed by a final extension step of 3 min at 72°C.

In addition, to compare the iridoviruses from different mosquito species, we designed two pairs of primers based on *Cx. pipiens*-IIV DNA. This genome was digested with the restriction endonuclease HindIII, and a library was obtained by cloning into pZeRO plasmid. Eight clones of this library were sequenced by the standard Sanger method (Macrogen). Based on these sequences, a set of two pairs of PCR primers were designed using standard criteria, IC28 primer (F 5′-ATTT TATGTGAAAGGATACCGAAT-3′, R 5′ TAAGCTTTTGACAT TAATCTGTCT-3′) and IC35 primer (F 5′-TTTGCGGTGCATGT CTCACT-3′, R 5′-AGTATATGTTGAAATACAACGCTAT-3′). The corresponding amplicon sizes were respectively 994 bp and 654 bp. The performance of each pair of primers was tested by PCR using the original plasmid clone as template. The amplification profile was adapted to each primer.

Laboratory transmission assays

Horizontal transmission tests were conducted using the mermitid *S. spiculatus* to transmit the *Cx. pipiens*-IIV to two mosquito species. The species evaluated were *Cx. apicinus* and *Ae. aegypti*, species that naturally breed in man-made containers.

Healthy *Ae. aegypti* larvae for the assays were obtained from the laboratory colony kept at the insectarium of the Centro de Estudios Parasitológicos y Vectores (CEPAVE), La Plata, Argentina. Healthy *Cx. apicinus* larvae were collected from a swimming pool located in La Plata. Infective juveniles (second instar or J2) of *S. spiculatus* were obtained from the nematode colony kept at CEPAVE following the procedures described by Camino & Reboredo (1994).

To prepare the inoculum, we followed the protocol used by Muttis *et al* (2015). Briefly, second instar *Cx. Pipiens* larvae from a colony kept at CEPAVE were infected with *Cx. pipiens*-IIV. After 72 h, the third instar larvae that showed symptoms of infection in the whole body were stored at − 20°C until use. For the assay and control, we used one infected larva (≈ 5.85×10^8 particles) per plastic container (150 ml), previously homogenized with a pestle in distilled water. Viral particles were counted as previously described by Muttis *et al* (2015); that is, viral particles from a known number of third instar larvae were purified, and the concentration of the suspension was estimated using transmission electronic microscopy

(TEM). Aliquots of the viral suspension and polystyrene beads of 460 nm diameter (1.8×10^9 beads/ml; Sigma-Aldrich) were mixed (10 µl of each). The stock suspension was diluted to obtain a proportion of viral particles and beads close to1:1. Nine fields were examined under TEM, and the viral particles and beads were counted to calculate the virus/beads ratio.

For each species, twenty healthy second instar mosquito larvae were exposed to the viral inoculum, being the proportion of five J2 per mosquito larva (5:1), and then kept for 24 h in plastic containers (30 ml) with 25 ml of distilled water. Then, larvae were washed in distilled water and transferred to plastic containers (200 ml) with 150 ml of distilled water and finely ground rabbit chow. Virus infection in larvae was evaluated at 72 h post exposure, the approximate period after which infected larvae show an iridescent blue color throughout the body in *Cx. pipiens* larvae (Muttis *et al* 2015). Larvae were observed under a stereomicroscope on a black background, and the numbers of live and infected larvae were recorded. All tests were carried out at 25 ± 1°C under a 12:12 h light–dark photoperiod. Three controls were performed: one of them with healthy larvae without any viruses or nematodes, another one with larvae exposed to nematodes (5:1), and the third with larvae exposed to the virus without nematodes. Larvae of each mosquito species exposed to the nematode (20 larvae per assay) were individualized until parasite emergence. The number of live and infected larvae was recorded, as well as the number of parasitized larvae. Each assay was performed three times on different dates, each one with three replicates.

Data analysis

The prevalence of virus infection was calculated considering the total number of live larvae. The Test of Proportion was used to compare the percentages of infected larvae of *Ae. aegypti* and *Cx. pipiens*. The test was performed using R software, version 3.3.2.

Results

Field survey

A considerable number of samples collected from drainage ditches in Berisso were positive for mosquito larvae, commonly identified as belonging to the *Cx. pipiens* complex. On one single occasion we found larvae of *Cx. dolosus*, and 28% of them ($n = 25$) were infected with an iridovirus. The *S. spiculatus* prevalence in the same sample was 76%, whereas one larva had both the parasite and the viral infection.

In rain ditches, mosquito larvae of *Ae. albifasciatus* ($n = 4600$) were detected in 48 samples, whereas the nematode was found in 24 of them, with prevalence varying from 11 to 100%. Larvae infected with iridovirus were only found in one sample. The prevalence of virus and nematode in this particular sample ($n = 1800$) was 0.17% and 40%, respectively.

In the flooded puddles, mosquito larvae were detected in 16 samples, with 960 specimens corresponding to *Aedes crinifer* (Theobald) and 246 to *Psorophora ferox* (Von Humboldt). There were no larvae infected with virus or parasitized with nematodes.

Virus identification

Viral DNA was extracted from field-infected larvae (two of *Ae. albifasciatus* and three of *Cx. dolosus*) and one laboratory-reared *Cx. pipiens* larva infected with the iridovirus *Cx. pipiens*-IIV.

The presence of iridovirus in positive samples was confirmed in one larva for each species by PCR using the MCP primers. An expected fragment of approximately 300 bp was obtained. Furthermore, the total number of DNA samples

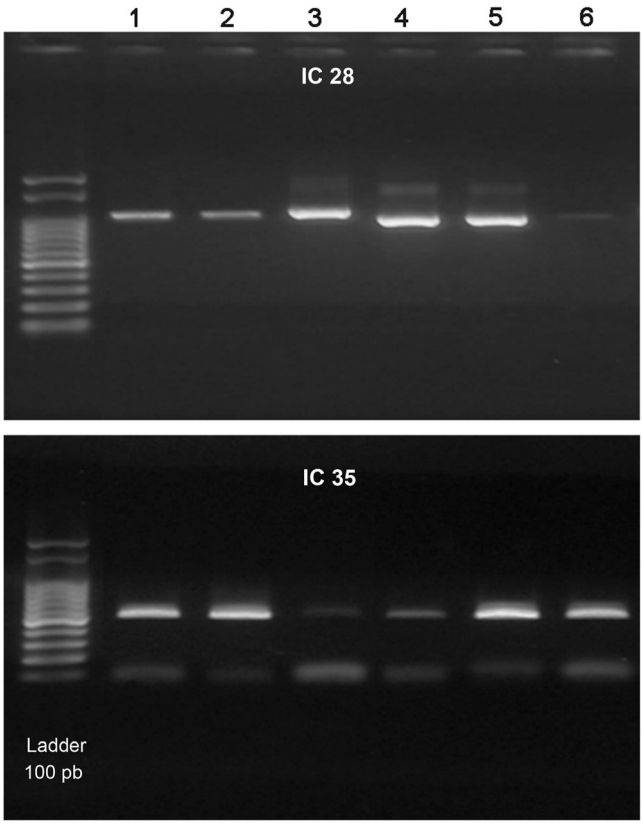

Fig 2 Agarose gel image showing IC28 and IC35 amplification products and ladder 100 pb in the first column. For IC28, the DNA samples belong to individual iridovirus infected larvae: *Aedes albifasciatus* (1, 2), *Culex dolosus* (3–5), and *Culex pipiens* (6) as positive control. For IC35, DNA samples belong to individual iridovirus infected larvae: *Aedes albifasciatus* (1, 2), *Culex dolosus* (4–6), and *Culex pipiens* (3) as positive control.

Fig 3 Second instar *Aedes aegypti* larva infected with the *Culex pipiens*-Invertebrate Iridescent Virus from Argentina. An iridescent blue coloration is observed in the whole body of the larva 72 h post-infection.

was typified using the IC28 and IC35 primers. The expected fragment was amplified in each case, confirming that the viral DNA purified from different mosquito species belonged to viral isolates similar to *Cx. pipiens*-IIV (Fig 2).

Transmission assays

The relatively high mortalities in the control treatments with *Cx. apicinus* can be explained by the fact that, as opposed to *Ae. aegypti*, larvae were collected in the field. Nevertheless, larval stages of both *Cx. apicinus* and *Ae. aegypti* (Fig 3) were susceptible to infection with *Cx. pipiens*-IIV using *S. spiculatus* as a vector. The results of the Test of Proportion applied to compare the percentages of infected larvae of *Cx. apicinus* and *Ae. aegypti* were not significant (chi-square = 3.54, df = 1, *p* value = 0.06). Parasitism by *S. spiculatus* in the controls was 13% ($n = 4$) for *Cx. apicinus* and 77% ($n = 44$) for *Ae. Aegypti*, whereas larval survival was 50% ($n = 60$) and 95% ($n = 60$) respectively. The numbers of live and infected larvae as well as the percentages are shown in Table 1.

Discussion

We reported two mosquito species as new natural iridovirus hosts: *Cx. dolosus* and *Ae albifasciatus*. These records are significant because only three mosquito hosts have been reported so far in the Neotropical region. Infected *Cx. dolosus* larvae were found in drainage ditches, at the same site where we had previously reported *Cx. pipiens* larvae infected with *Cx. pipiens*-IIV (Muttis *et al* 2012). This suggests that the same viral strain could be involved in both infections. Conversely, infected *Ae. albifasciatus* larvae were collected from a very different sort of site, namely rain ditches. Although both isolates were similar in the studied genomic fragments, they might be different strains or even different virus species. In order to elucidate this assumption, further genetic studies will be conducted to compare these isolates. Records of iridovirus in mosquitoes from other places worldwide have not reported any association with nematodes (Clark *et al* 1965, Chapman *et al* 1966, Anderson 1970). Nevertheless, the virus infections presented here and those previously reported by Muttis *et al* (2012, 2013) occurred in places where nematode populations were well established. This situation was reported at the *Ae. albifasciatus* breeding site around Los Hornos, La Plata, where the immature stages were associated in nature with the nematode *Strelkovimermis spiculatus* (Micieli & García 1999; Campos & Sy 2003). Furthermore, both *Cx. dolosus* and *Cx. pipiens* were found to be infected with established nematode populations in the drainage ditches (Muttis *et al* 2012, 2013). Moreover, there are no records of iridovirus in *Psorophora ferox* (Van Humboldt) and *Aedes crinifer* (Theobald) larvae breeding in flooded puddles, which are sites without any *S. spiculatus* populations. Although there is a record of parasitism by *S. spiculatus* in *Ae. crinifer*, the reported prevalence is very low (Maciá *et al* 1995). This virus was not detected in natural populations of *Aedes aegypti*, one of the most studied mosquito species that breed in artificial containers in the absence of nematode parasites.

Table 1 Infection assays with the iridovirus *Culex pipiens*-IIV in two mosquito species. Evaluations performed at 72 h post exposition treatment.

		Total larvae	Live larvae	Infected larvae	Prevalence (%)
Culex apicinus	Trial[a]	180	122	63	52
	Control1[b]	60	38	0	0
	Control2[b]	60	34	0	0
Aedes aegypti	Trial[a]	180	173	69	40
	Control1[b]	60	55	0	0
	Control2[b]	60	54	0	0

[a] Second instar larvae were exposed to one homogenized infected larvae + *Strelkovimermis spiculatus* (five nematodes per mosquito larva). Three replicates of 20 l were performed and repeated three times

[b] Healthy larvae reared without the virus (Control1); larvae exposed only to *Culex pipiens*-IIV (Control2)

On the other hand, *Cx. pipiens*-IIV was able to infect two new mosquito species at the laboratory, using *S. spiculatus* as a vector: *Cx. apicinus*, which belongs to the same genus from which the virus was isolated, and *Ae. aegypti*. The fact that the virus could infect a mosquito species of another genus suggests that *Cx. pipiens*-IIV could have a broad host range. Although the prevalence of the virus was apparently higher for *Cx. apicinus* (52%) than for *Ae. aegypti* (40%), there were no significant differences between the two species ($p = 0.06$). Moreover, Muttis *et al.* (2015) reported a prevalence of 70.1% in *Cx. pipiens* larvae in similar assays with *Cx. pipiens*-IIV. These differences may be interpreted due to the different levels of parasitism between the mosquito species. However, the prevalence of the nematode does not follow the same pattern of the virus infection, being lower (13%) for *Cx. apicinus* and almost equal (≈ 80%) for the other two species (Muttis *et al.* 2015). The similarity in the parasitism prevalence between *Cx. pipiens* and *Ae. aegypti* is in agreement with a study conducted by Achinelly & Camino (2005) in which no significant differences were observed in the parasitism prevalence between these species. It seems that the difference in the virus prevalence in our studies is not related to the pathway of transmission. Even though *Cx. pipiens*-IIV seems to have a broad host range in the laboratory, higher virus prevalence was observed in their natural host species *Cx. pipiens* for which it seems to be more virulent, followed by *Cx. apicinus* and *Ae. aegypti*. These differences may be linked to issues related to the immunological response of the insect or virus replication. Further assays should be performed to analyze this hypothesis.

In this study, we report the presence of a virus belonging to the *Iridoviridae* family in *Cx. dolosus* and *Ae. albifasciatus* for the first time, adding these two species to the short list of naturally infected mosquitoes in the Neotropical region. Moreover, we have demonstrated that *Ae. aegypti* and *Cx. apicinus* are susceptible to our field-collected iridovirus (*Cx. pipiens*-IIV) and that its transmission is possible using the nematode *S. spiculatus* as a vector.

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