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MICROBIOLOGY

Pathology of an iridescent virus in immature *Culex pipiens* L. (Diptera, Culicidae)

EVANGELINA MUTTIS, MARÍA VICTORIA MICIELI & JUAN JOSÉ GARCÍA

Abstract: Iridovirus in *Culex pipiens* was reported for the first time in 2012. Later studies of horizontal transmission were performed, in which an interaction with the parasite *Strelkovimermis spiculatus* acting as viral vector was recognized. In the present study, we observed aspects of the pathology produced by an invertebrate iridescent virus in laboratory infected immature *Cx. pipiens* as well as in infected immature *Cx. pipiens* in the field. In the laboratory infected larvae, the infection and mortality were asynchronous. Signs of infection in larvae exposed to the virus were observed between the second and the fourth days post-exposure in 99% of the cases, while the highest daily record of visible infected larvae (52%) was observed on the third day post exposure. Moreover, 79% of confirmed virus infected larvae died in the first 10 days after exposure. The Median Lethal Time was eight days. Several tissues were found to be infected and the common sites of replication were the fat body, epidermis and epithelial derivatives, such as the imaginal discs and the tracheal epithelium. Moreover, infection in the salivary glands, gastric ceca and posterior gut have not been previously documented on other mosquito iridescent viruses.

Key words: *Culex pipiens,* Insect pathology, *Iridoviridae*, Virology.

INTRODUCTION

The *Iridoviridae* is a wide virus family with numerous species and both, invertebrate and vertebrate hosts. The particle has an icosahedral shape, usually 120–200 nm in diameter, and it contains linear double-stranded DNA (King et al. 2012). *Iridoviridae* has many species distributed in six genera, three of them infecting invertebrates and three infecting vertebrates. Among invertebrate hosts, these viruses infect numerous members of the Culicidae family (Williams 2008), but only two of these isolates have been recognized as a species of the genera *Chloriridovirus, Invertebrate iridescent virus-3* (as a type species) from *Aedes taeniorhynchus* and *Anopheles minimus iridovirus* from *Anopheles minimus* (Chinchar et al. 2017). The most representative mosquito genera that have been found harboring iridovirus are *Aedes* and *Psorophora*. However for *Culex*, there have been three isolates from different species: *Culex territans* from Russia and Ukraine (Fedorova 1986), *Culex pipiens* from Argentina (Muttis et al. 2012) and *Culex dolosus* (Muttis et al. 2020) from Argentina.

Viral isolates have been found in many mosquito species belonging to different genera around the world, but studies on the pathology have only been performed in infected *Aedes* species: *Ae. aegypti* (Marina et al. 1999), *Ae. taeniorhynchus* (Matta & Lowe 1970) and *Aedes stimulans* (Anderson 1970). It is known that iridovirus develops in two different ways. One way may result in patent infection, in which large numbers of viral particles in the tissues form a paracrystalline arrangement. This phenomenon produces an optical effect (Bragg diffraction)

(Chinchar et al. 2009) that induces a change in the coloration making the infected tissues iridescent and all the affected individuals die (Clark et al. 1965). On the other hand, the second way of infection is called subletal, it has been demonstrated that larvae infected at the end of their development stage do not show iridescence and emerge as adults likely to transmit the virus to their progeny (Woodard & Chapman 1968, Linley & Nielsen 1968, Marina et al. 1999). The reason why one type of infection occurs instead of the other one is still unknown and subletal infection has not been studied in *Cx. pipiens*.

Here we studied some aspects of *Culex pipiens*-IIV pathology, in particular the patent infection. This virus was isolated in Argentina and that has been the first record in the Neotropical region (Muttis et al. 2012). Afterwards, we performed transmission studies and reported that, in nature, the virus was associated with the juvenile stage of the nematode *Strelkovimermis spiculatus* (Muttis et al. 2013). In the laboratory, horizontal transmission was only possible in the presence of early juvenile nematode. Patent infection of 82 % was recorded when second instar *Cx. pipiens* larvae were exposed to virus and the nematode. Juvenile parasites were observed inside all virus infected larvae (Muttis et al. 2013). The authors concluded that *S. spiculatus* acts as a vector, transporting viral particles into the larval mosquito body (Muttis et al. 2015). The aim of the present study is improve the knowledge of the pathology that *Culex pipiens*-IIV produces in larvae infected in the laboratory, using juvenile nematodes as vectors, and in naturally infected immature stages of *Cx. pipiens*. The infective and lethal times were evaluated. Moreover, the progress of iridescence in the larval body, the tissues and organs affected are being discussed.

MATERIALS AND METHODS

Pathology in laboratory infected *Culex pipiens* larvae

Infection assay

Cx. pipiens larvae were exposed to the virus in the laboratory for pathology studies.

Second instar larvae used for assays were obtained from a colony kept at the insectary in CEPAVE. For horizontal transmission, we used the strain *Cx. pipiens*-IIV previously isolated by Muttis et al. (2013, 2015) and infective juveniles of *S. spiculatus* (Nematoda, Mermithidae) were used as vectors in a ratio of five nematodes per mosquito larva. The juvenile nematodes were obtained from a colony kept at CEPAVE, as described by Camino & Reboredo (1996). Briefly, adults nematodes were kept in plastic cages (15 x 20 cm) filled with wet sand, in which they laid the eggs. The infective juveniles hatched after we filled the cages with water. The inoculum was Cx. *pipiens* larvae with iridescence all over the body corresponding to ≈5.85 x10^8 viral particles that were obtained following the protocol by Muttis et al. (2015). Infected larvae were homogenized in distilled water before be used as inoculum. Five replicates each consisting of 48 second instar larvae (total= 240) were arranged in 200 ml plastic containers with distilled water. For each trial, larvae, viral inoculum and juvenile parasites were added. After 24 h, larvae from each replicate were transferred to their corresponding container with clean distilled water and finely ground rabbit chow. The assay was maintained at 25°C in an incubator. Then, 48 h after exposure, the larvae were observed daily under a stereoscopic microscope with a black background in order to report the time at which iridescence began to be detected and the day when the infected larvae died. The numbers of iridescent larvae and dead larvae were recorded every 24 h within a period of ten days.

The median lethal time values were calculated, with the respective 95% confidence limit, by using the statistical software for correlated data developed by Throne et al. (1995).

Pathological studies were performed on 180 *Cx. pipiens* larvae from the infection assay.

A total of 10 larvae were dissected or fixed for microscopic studies. The virus pathology was followed on the remaining 170 larvae until they naturally died. Larvae not showing signs of infection were not studied.

Optical microscopy and ultrastructural studies

After 72 h post viral exposure, seven larvae that presented visible signs of infection were dissected using fine forceps and were observed under an optical microscope to evaluate the infection in the internal organs. On the other hand, three larvae, one of each 24, 48 and 72 h post viral exposures was fixed in glutaraldehyde (2.5 %) for electron microscopy studies. Larvae of 48 and 72 h post exposure presented visible signs of infection. As the 24 h exposure larvae had not shown signs of infection, one larva was selected since the juvenile nematode was observed inside its body, therefore, the probability of being infected was high. Larvae were cut to separate the thorax and abdomen, and 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. Three ultrathin sections (60 nm) from each piece were stained with 2% uranyl acetate and lead citrate and were examined with an electron microscope (JEOL JEM 1200 – EX II).

Pathology in field collected immature stages

Samples of immature mosquitoes were taken from several drainage ditches located in suburban areas of La Plata, Buenos Aires province, Argentina, from September 2010 to January 2013 (sampling previously reported in Muttis et al. 2013). The immature specimens were collected using a dipper and were transported to the laboratory in plastic containers with water from the same sites and then kept at room temperature. They were observed under a stereoscopic microscope with a black background in order to detect the iridescence in larvae previously infected and were daily examined until they died. The infected larvae were identified using taxonomic keys (Darsie & Mitchell 1985).

RESULTS

Pathology in laboratory infected *Cx. pipiens* larvae

Infection assay

Iridescence appeared in 99% of the 170 larvae that were examined until they died between the second and fourth day after exposure. The highest daily record of visible infected larvae (52%) was recorded on the third day (Fig. 1). Iridescent larvae molted to the third instar and died. There were no records of fourth instar larvae or pupae with signs of patent disease.

Mortality began on the fourth day and 78.7% (n=170) of patent infected larvae died between four and 10 days (Fig. 1). The remaining larvae (21.3 %) died after this time. The Median Lethal Time was 8 days (7-9 days). On the fourth and fifth days, the appearance of iridescence and mortality overlapped (Fig. 1). The larvae which never developed any sign of infection (n=60) might not have been infected or could have been infected asymptomatically.

Optical microscopy and ultrastructural studies

Iridescence first appeared in the thorax of infected larvae, in all cases in the fat body. Moreover, in 80% of them (n=170) it appeared in the fat body and the imaginal discs at the

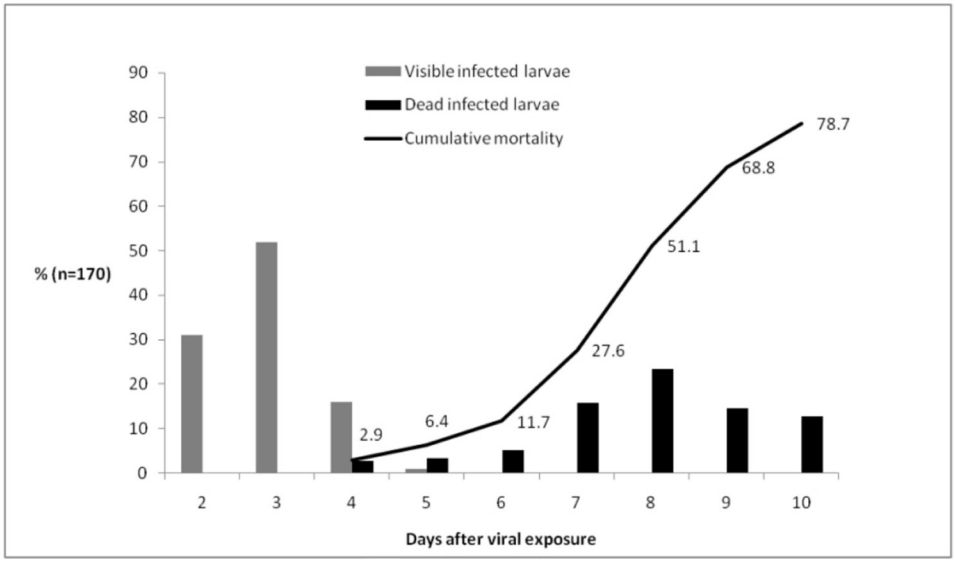

Figure 1. Iridescence and mortality on 170 visible infected larvae. Percentage of iridescent larvae that appeared inthe respective day after viral exposure (n=170) (gray). Percentage of dead infected larvae recorded daily (black). Cumulative percentage of infected larvae that died on each day after exposure (black line).

same time. The viral infection progressed to the abdomen (Figs. 2a, 2b), while the iridescent coloration gradually intensified. Before death, the whole larval body showed iridescence, including the head, anal papillae and siphon (Fig. 2c). Iridescence was observed in the internal organs, clearly in both the anterior digestive tube, including the esophagus, gastric ceca (Fig. 2c), salivary glands (Fig. 2d), and posterior digestive tube (Fig. 2e). Moreover, iridescence was observed in Malpighian tubules (Fig. 2e). Images of uninfected larvae from *Cx. pipiens* colony at CEPAVE are shown for comparative purposes (Figs. 2f, 2g).

As ultrathin sections from larva fixed at 24 h after exposure did not show any viral particles in the thorax and abdomen in any of the sections observed, it is probable that this larva was not infected. However, numerous viral particles were observed in the thorax of larva fixed at 48 h (Fig. 3a), while a few viral particles were found in the abdomen, specifically in the tracheal epithelium (Fig. 3b), but no particles were found in tissues around the tracheas (Fig. 3b). In contrast, ultrathin sections of larva fixed at 72 h showed an accumulation of a high number of viral particles in both the thorax and abdomen.

Pathology of immature stages collected in the field

Of 33 field-collected visibly infected immature specimens, those that were collected in the second larval instar died after 2 to 6 days (Average= 4.33 ± 1.8, n= 12), in the third instar they died after 2 to 7 days (5.25 ± 2.2, n= 4) and in the fourth instar after 1 to 9 days (5.23 ± 3.2, n= 17). Moreover, one infected pupa was found and it died 3 days later (Fig. 4a). Infected larvae in the first instar were occasionally recorded but they were not followed up in laboratory. The infected larvae generally died in the same larval instar in which they were found, although they occasionally molted once before dying. No iridescent infected larva or pupa was observed to complete its development and emerge as an adult.

In addition, the iridescence of naturally infected larvae was observed more intensely than the laboratory infected ones and the color was mostly turquoise (Fig. 4b), although larvae with green iridescence or mixed coloration were also collected (Fig. 4c).

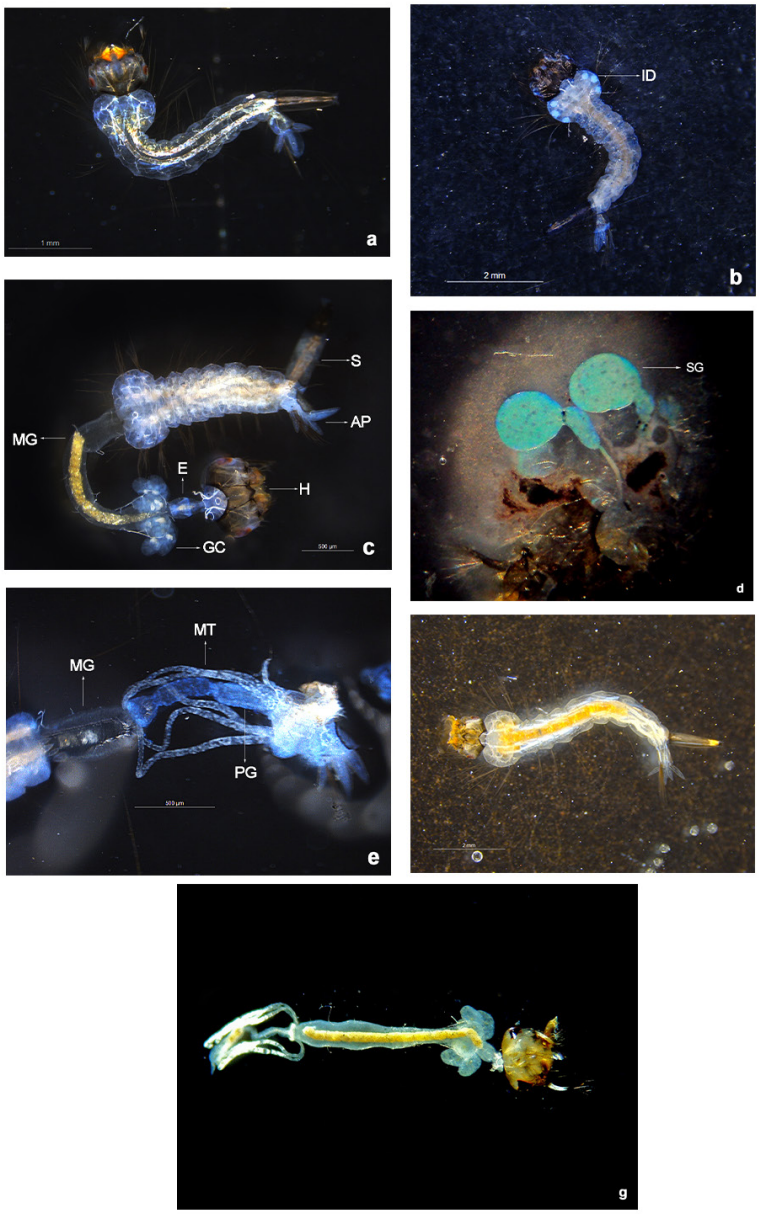

Figure 2. Infected *Culex pipiens* larvae exposed to the iridovirus during 72 h. (a) larva with iridescence in thorax and abdomen. (b) Larva with iridescence in thorax, abdomen and imaginal disk (ID) notoriously infected. (c) Dissected larva with the anterior gut and midgut (MG) outside the body, the esophagus (E) and gastric caecum (GC) are iridescent. The infection is evident in the head (H), siphon (S) and anal papillae (AP). (d) Infected salivary glands (SG). (e) Dissected larva showing the midgut (MG), the iridescent posterior gut (PG) and Malpighian tubules (MT). (f) Uninfected larva. (g) Gut extracted from uninfected larva.

DISCUSSION

The pathology produced by iridovirus in the laboratory infected *Cx. pipiens* larvae had similarities with that produced in other mosquito species, but it also showed certain differences. In our study, the iridescent color was first evident in the thorax and then in the abdomen, while it became more intense, as was observed in *Ae. taeniorhynchus* by Linley & Nielsen (1968). Studies in the same mosquito species showed that iridescence appears just before dying, always in the last larval instar, no matter in which instar the larvae were infected (Clark et al. 1965, Linley & Nielsen 1968). Also, it was reported that laboratory or field infected larvae only occasionally molted to pupa, but none were observed emerging as adults (Chapman et al. 1966, Matta & Lowe 1970). We observed the opposite situation in the laboratory, iridescence appeared a short time after viral exposure and the larvae could

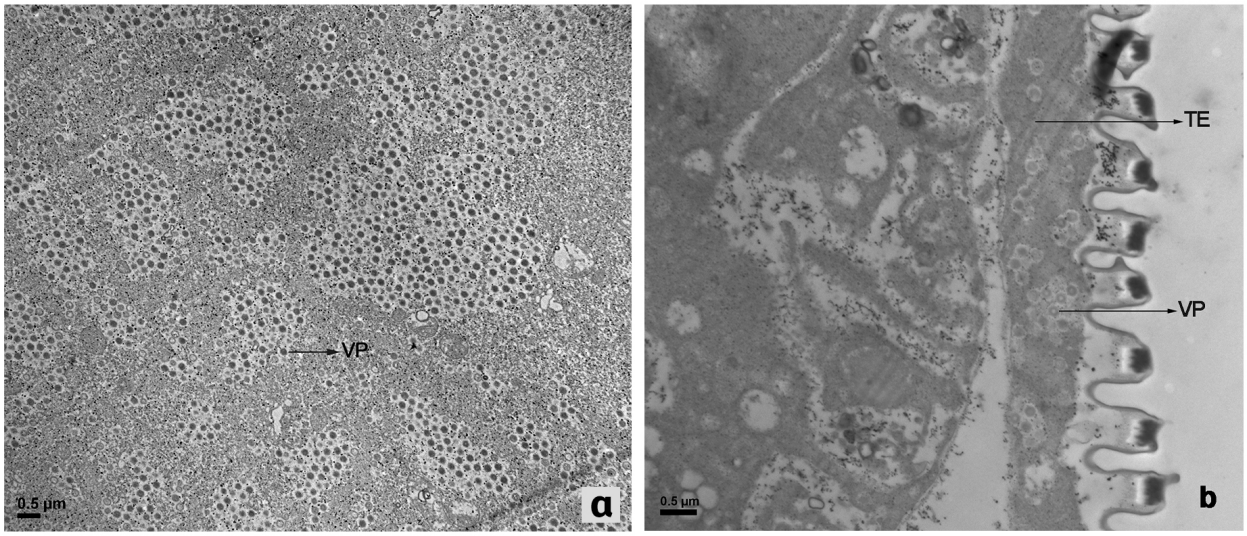

Figure 3. Ultrathin sections from a larva fixed at 48 h post viral exposure showing viral particles (VP) in the thorax (a) and tracheal epithelium (TE) of the abdomen (b).

survive several days before dying. Moreover, larvae never molted more than once and never reached the last larval instar. Whereas in the field, infected fourth instar larvae and a pupa were observed. At temperatures lower than 25 ° C, the progress of infection could be retarded, as well as the mosquito developmental period. Therefore at cooler temperatures, larvae infected in the first instar may be able to develop to the fourth instar or pupa before they die. On the other hand, larvae that remain in late instar for a long time could be infected and they could develop signs of infection in the same stage. The difference in the progress of pathology, faster in our study compared to studies on *Ae. taeniorhynchus*, could be explained by the infection pathway, since we used a direct way of viral inoculation into the insect hemocele by using the nematode, whereas in the other studies with mosquito iridescent virus, larvae were infected by ingestion of viral particles. Otherwise, the contrast in the duration of the infection process between mosquito species could be related to species specific differences in susceptibility to the virus.

In ultramicroscopic studies, viral particles were not detected in ultrathin sections of larva at 24 h after exposure, therefore this larva could maybe not have been infected, or virus particles may not have been present in these sections. In 48h postexposure larva a large number of viral particles were observed in the thorax, whereas in the abdomen, viral particles were only detected in the tracheal epithelium, with no virus being observed in the surrounding tissues.

Infections in the tracheal epithelium were reported and mentioned as very important by other authors (Hall & Anthony 1971). Moreover, the role of the tracheal system as a dispersion route inside the insect was reported for a baculovirus from *Autographa californica* in the lepidopteran *Trichoplusia* (Engelhard et al. 1994). Although *S. spiculatus* prefers to enter the larva's hemocele through the thorax, it is also capable of entry from any point in the body (Sanad et al. 2013). For this reason, it is still uncertain why the iridescence always began in the thorax in our study.

The affected tissues reported in this study were generally similar to those reported by 238 other authors (Anthony & Comps 1991). The

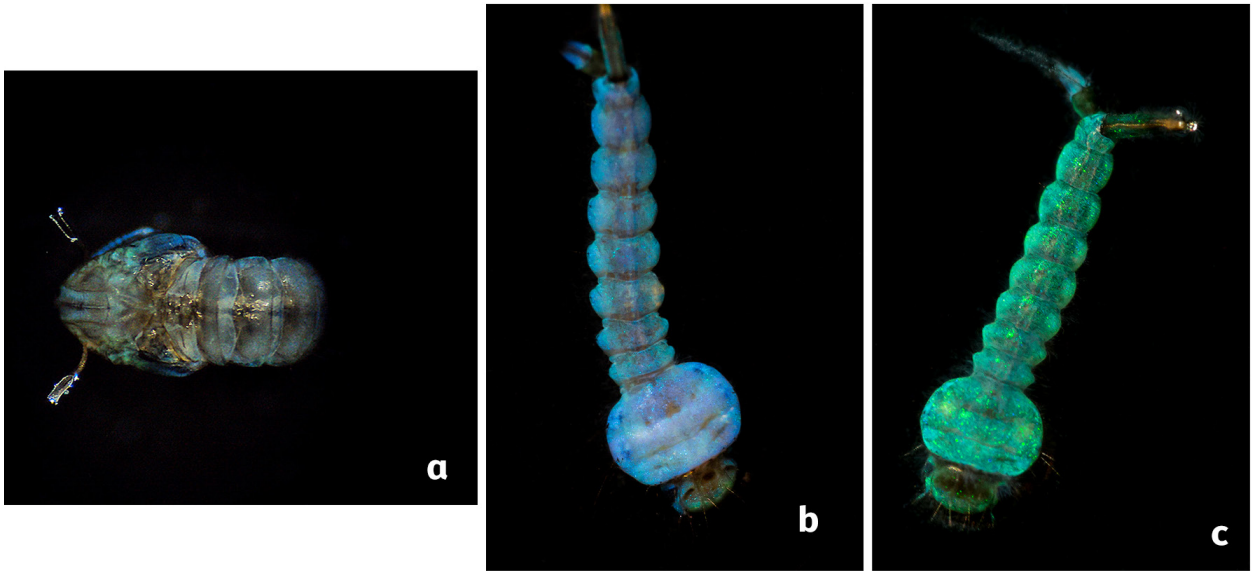

Figure 4. Field infected immatures. (a) Infected pupa. (b) Fourth instar larvae with turquoise iridescence. (c) Fourth instar larvae with green-turquoise mixed iridescence.

fat body, epidermis and epithelial derivatives, such as the imaginal discs and the tracheal epithelium, are common sites of replication. However, infection in salivary glands, gastric caecum and posterior gut have not been documented previously (Williams 2008). It would be interesting to know if the virus has the same affinity for the salivary gland in sub-lethally infected adults.

Iridescence of yellow, orange, red and green color has been previously reported, in addition to turquoise (Chapman et al. 1966, Clark et al. 1965, Anderson 1970, Williams 2008), but we have only seen iridescence in the turquoise and green range. The color has been related to the size of the virus, where particles between 110 and 130 nm in diameter have shown a turquoise coloration pattern, whereas green, yellow or orange were related to the 160-200 nm range (Williams 2008). Unlike previous information, this iridovirus of *Cx. pipiens*, which has a viral particle size of 158 nm (Muttis et al. 2012), produces turquoise iridescence in most of the infected specimens with some recorded larvae showing a green color pattern. The information presented reveals that the mosquito iridoviruses from *Cx. pipiens* do not exhibit a homogenous behavior pattern as IIV-3, but rather they present particularities. For instance, in this host-virus system, the developmental period of infection varies greatly between individuals, causing progressive mortality from 4 days to more than 10 days. This behavior could be important in the mosquito iridescent virus cycle occurring in drainage ditches. In this environment with permanent water, overlapping cohorts of *Cx. pipiens* and *S. spiculatus* are breeding, asynchronous mortality of infected larvae over an extended period, increases the period during which inoculum is present in the aquatic environment and may be available for transmission to susceptible conspecific larvae. To improve the understanding of the viral cycle in these environments, it would be important to carry out studies on the persistence of the infective virus in the soil.

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Author contributions

The author contributions were as follow. J.J.G., M.V.M and E.M. planned and designed the study. E.M. executed experimental work and wrote the manuscript.