

Effects of an optical brightener and an abrasive on iridescent virus infection and development of *Aedes aegypti*

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Accepted: 28 August 2003

Key words: *Aedes aegypti*, Invertebrate iridescent virus 6, optical brightener, peritrophic membrane, Blankophor BBH, silicon carbide, infection, mortality, development rate, Diptera, Culicidae, Iridoviridae

Abstract

The insecticidal properties of certain entomopathogenic viruses can be greatly improved in mixtures with substances that affect the integrity of the insect peritrophic membrane, particularly optical brighteners. We aimed to determine the effect of an optical brightener, Blankophor BBH, and an abrasive compound, silicon carbide, alone and in mixtures, on the prevalence of patent and covert infection of *Aedes aegypti* (L.) (Diptera: Culicidae) by *Invertebrate iridescent virus 6* (IIV-6) (Iridoviridae). The prevalence of patent infection by IIV-6 was < 1.5% in all treatments involving virus. Contrary to predictions, there were significantly fewer patent infections in virus treatments involving Blankophor with or without silicon carbide compared with controls. Covert infection of adults detected by insect bioassay was between 6.7 and 12.2%, although no significant differences were observed between treatments. Exposure to IIV-6 alone or silicon carbide alone did not significantly increase larval mortality compared to the controls, whereas exposure to Blankophor alone, or in any combination with IIV-6 or silicon carbide, clearly increased larval mortality. These effects did not carry-over to the pupal stage. Adult females emerged ~1.5 days later than males. Compared to control insects, female development rate was extended by 11.4 and 12.6% in the treatments involving IIV-6 alone and silicon carbide alone, respectively. The sex ratio at adult emergence did not differ significantly between control insects and those of other treatments. These results support the hypothesis that the gut is unlikely to represent the principal point of infection of mosquito larvae by iridescent viruses.

Introduction

The insecticidal properties of certain entomopathogenic viruses can be greatly improved in mixtures with substances that affect the integrity of the insect peritrophic membrane (Wang et al., 1994; Wang & Granados, 2000). The peritrophic membrane forms a transparent continuous film extending from the anterior midgut to the hindgut (Tellam et al., 1999). This membrane is a semi-permeable tube consisting of chitin microfibrils embedded in a matrix of proteoglycans incorporating proteins and glycoproteins (Lehane, 1997). The membrane is believed to have multiple functions,

including mechanical protection against abrasion, a barrier to pathogens, as well as physiological functions related to digestion and absorption of nutrients (Edwards & Jacobs-Lorena, 2000).

Certain stilbene-derived optical brighteners show a high affinity for β -glucans such as chitin, and thereby affect chitin biosynthesis (Elorza et al., 1983; Roncero et al., 1988; Bartnicki-Garcia et al., 1994). In the midgut of lepidopteran larvae, optical brighteners can solubilize proteins from the peritrophic membrane and inhibit membrane formation (Wang & Granados, 2000). This causes a marked increase in the susceptibility of larvae to infection by baculoviruses (Shapiro & Dougherty, 1994). Optical brighteners have not been tested in mosquitoes, although abrasive

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substances have been reported to result in an increased prevalence of infection in mixtures with iridescent viruses (Undeen & Fukuda, 1994; Marina et al., 1999).

Invertebrate iridescent viruses (IIVs) infect many invertebrate species, especially insects, in damp and aquatic habitats (Williams et al., 2000). IIVs can cause two types of infection: patent and lethal, or covert and non-lethal (Williams, 1995). Patently infected mosquitoes take on an iridescent blue colour and die due to the proliferation of virus particles in host cells, whereas covertly infected mosquitoes appear healthy and may develop to adulthood and reproduce (Marina et al., 1999). Covert infections can be readily detected by PCR and highly sensitive insect bioassay techniques using *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae (Constantino et al., 2001). The route of infection is uncertain for most IIVs, although predation of infected individuals and parasite-mediated transmission has been observed in certain species (Linley & Nielsen, 1968; Mullens et al., 1999; López et al., 2002).

Electron microscope observations indicate that the peritrophic membrane may be an effective barrier to IIV infection of epithelial cells in the gut of mosquito larvae (Stoltz & Summers, 1971). Therefore, the aim of the present study was to determine the effect of the optical brightener Blankophor BBH, and an abrasive compound, silicon carbide, alone and in mixtures, on the prevalence of patent and covert iridescent virus infection of the medically important vector, *Aedes aegypti* (L.) (Diptera: Culicidae). We also determined the effect of these substances on immature mortality, development rate, and adult emergence. Such studies should elucidate the role of the peritrophic membrane as a barrier to iridescent virus infection in larval mosquitoes and the feasibility of formulating these viruses for control of insect vectors with aquatic larval stages (Federici, 1985).

Materials and methods

Insects and virus

Eggs of *Ae. aegypti* were obtained from a laboratory colony continuously reared in the Centro de Investigación de Paludismo, Tapachula, Chiapas, Mexico. Larvae were reared from these eggs using filtered unchlorinated water and a diet consisting of a mixture of powdered soya and yeast. Larvae of *G. mellonella* were obtained from a laboratory culture maintained on a semi-synthetic diet in El Colegio de la Frontera Sur (ECOSUR), Tapachula, Chiapas, Mexico. All insects were maintained in a temperature controlled room at 25 ± 1 °C, 75–85% r.h., and L12:D12.

An isolate of *Invertebrate iridescent virus 6* (IIV-6) (genus *Iridovirus*, family Iridoviridae) originally obtained from P. Christian (CSIRO, Canberra, Australia), was produced

by injection in third instar *G. mellonella* as previously described (Constantino et al., 2001). At 7–10 days post-infection, patently infected *G. mellonella* larvae were placed at -20 °C and stored until required. To purify the IIV-6, infected larvae were triturated in 1 ml sterile distilled water and subjected to three steps of centrifugation at 490 g for 10 min, 15 300 g for 10 min, followed by a 30% (wt/vol) sucrose cushion at 15 300 g for 30 min and two washes in sterile water as described by Marina et al. (2003). The purified suspension was quantified by direct counting of a mixture of virus and 460 nm diameter polystyrene beads (Aldrich Chemical Co., MO, USA) using a scanning electron microscope (Constantino et al., 2001).

Treatment of mosquito larvae

Groups of 100 third instar *Ae. aegypti* were randomly selected, placed in plastic dishes with 100 ml of filtered water and subjected to one of the following treatments: (i) water control, (ii) 1 mg silicon carbide powder (Aldrich Chemical Co., MO, USA), (iii) 0.001% (wt/vol) Blankophor BBH (Bayer Corp., Pittsburgh, PA, USA), (iv) 1 mg silicon carbide + 0.001% Blankophor BBH, (v) IIV-6 (vi) IIV-6 + 1 mg silicon carbide, (vii) IIV-6 + 0.001% Blankophor BBH, or (viii) IIV-6 + 1 mg silicon carbide + 0.001% Blankophor BBH. IIV-6 was used at a concentration of 2.4×10^9 particles/ml in all treatments involving virus. Each suspension was gently agitated by compressed air from an aquarium air pump. Following 6 h exposure, the larvae were individually passed through a series of six 1–l washes in clean filtered water. Previous studies had shown this to be an effective means of eliminating residual virus inoculum and other compounds (Marina et al., 1999, 2003).

Each larval treatment group was then carefully placed in a rearing tray (each tray representing one replicate of one treatment) and reared to pupation on the soya + yeast diet. Larvae were inspected twice daily for signs of mortality, pupation, or patent iridescent virus disease characterized by an obvious change of colour to an opalescent blue. Following pupation, pupae were individually placed in 250 ml plastic cups containing 50 ml of filtered water until adult emergence. The duration of the larval stage, larval and pupal mortality, and adult emergence and sex ratio were recorded. Within 24 h of emergence, adults were sexed, individually placed in sterile plastic Eppendorf tubes and stored at -20 °C until required.

A sample of 30 adult mosquitoes was selected randomly from each treatment group and bioassayed to detect the presence of covert infections. The abdomen of each mosquito was removed and individually homogenized in 300 µl of antibiotic solution (0.08% wt/vol aureomycin). Insect debris was sedimented by centrifugation at 190 g for 5 min. The supernatant was placed in a sterile

microcentrifuge tube and 8.4 µl volumes were injected into each of 15 third instar *G. mellonella* using a manual microinjector fitted with a 1 ml syringe (Burkard Co., UK). Injected *G. mellonella* were reared in 50 ml plastic cups with semi-synthetic diet and maintained at 25 ± 1 °C. Control larvae were injected with antibiotic solution alone. At 12–14 days post-injection, larvae were checked for signs of patent iridescent virus infection (whitish-blue iridescence of the ventral epidermis). The complete experiment was performed eight times.

Statistical analysis

Patent and covert infections in larvae and adults, respectively, and mortality in the larval and pupal stages were subjected to analysis of variance (ANOVA) of arcsine \sqrt{x} transformed percentage values. When the ANOVA resulted in significant F-values, mean separation was performed by Tukey test ($P = 0.05$). The duration of the larval and pupal stages were subjected to analysis of covariance with treatment as a factor and sample size as a covariate. Mean separation was performed by Bonferroni test (Zar, 1999). In all cases, the supposition of normality, homoscedasticity, and the distribution of residual values were examined for each data set. All statistical procedures were performed using the SPSS package (SPSS, 1999).

Results

The prevalence of patent infection by IIV-6 was very low (< 1.5%) in all treatments involving virus (Table 1). However, contrary to predictions, compared to the results with virus alone, there were significantly fewer patent infections in virus treatments involving Blankophor with or without silicon carbide ($F_{3,21} = 5.83$, $P = 0.005$). The mixture of IIV-6 with silicon carbide did not result in a significant increase in patent infections compared to IIV-6 alone.

The prevalence of covert infection of adults (6.7–12.2%) was approximately 10-fold greater than that observed for patent infections, although no significant differences were observed among treatments ($F_{3,21} = 1.18$, $P = 0.35$) (Table 1). No patent or covert infections were detected in treatments not involving IIV-6.

Exposure to IIV-6 alone or silicon carbide alone did not result in a significantly increased probability of mortality in the larval stage compared to the control treatment. In contrast, exposure to Blankophor alone, or in any combination with virus or with silicon carbide, clearly increased larval mortality compared to the controls ($F_{7,49} = 12.7$, $P < 0.001$) (Table 2). However, these effects did not carry-over to the pupal stage, in which no significant differences

Table 2 Mean (± SE) mortality of larvae and pupae of *Aedes aegypti* following exposure to *Invertebrate iridescent virus 6* (IIV-6) alone or in mixtures with silicon carbide and Blankophor BBH (optical brightener)

Treatment ^a	Mortality (%) ^b	
	Larvae	Pupae
Control	1.4 ± 0.6 a	3.0 ± 0.6 a
Blankophor	28.6 ± 7.4 e	9.6 ± 4.3 a
Silicon carbide	2.5 ± 0.9 ab	3.4 ± 0.6 a
Blankophor + silicon carbide	24.8 ± 6.6 de	3.8 ± 0.7 a
VII-6	4.8 ± 1.2 abc	7.7 ± 3.8 a
VII-6 + Blankophor	15.3 ± 3.7 cde	7.4 ± 3.9 a
VII-6 + silicon carbide	7.0 ± 1.1 abc	5.6 ± 1.7 a
VII-6 + Blankophor + silicon carbide	10.4 ± 2.8 bcd	10.2 ± 6.2 a

^aFor all treatments there were eight replicates each comprising 100 larvae.

^bValues followed by the same letter are not significantly different for comparisons within each column (ANOVA, Tukey test $P > 0.05$). Percentages were arcsine transformed for analysis but are shown here as the original values.

Table 1 Mean (± SE) prevalence of patent and covert infection of larvae and adults of *Aedes aegypti*, respectively, following exposure to *Invertebrate iridescent virus 6* (IIV-6) alone or in mixtures with silicon carbide and optical brightener (Blankophor BBH)

Treatment ^a	Patent infection of larvae		Covert infection of adults	
	n ^b	Percentage ^c	n ^b	Percentage ^c
IIV-6	800	1.3 ± 0.3 a	240	12.2 ± 2.7 a
IIV-6 + Blankophor	800	0.4 ± 0.2 b	240	6.7 ± 3.0 a
IIV-6 + silicon carbide	800	1.4 ± 0.4 a	240	10.0 ± 3.8 a
IIV-6 + Blankophor + silicon carbide	800	0.5 ± 0.3 b	240	8.9 ± 2.2 a

^aNo patent or covert infections were observed in insects from the four treatments not involving virus (IIV-6).

^bNumber of insects tested. For all treatments there were eight replicates each involving 100 larvae and 30 adults (females).

^cValues followed by the same letter are not significantly different for comparisons within each column (ANOVA, Tukey test $P > 0.05$). Percentages were arcsine transformed for analysis but are shown here as the original values.

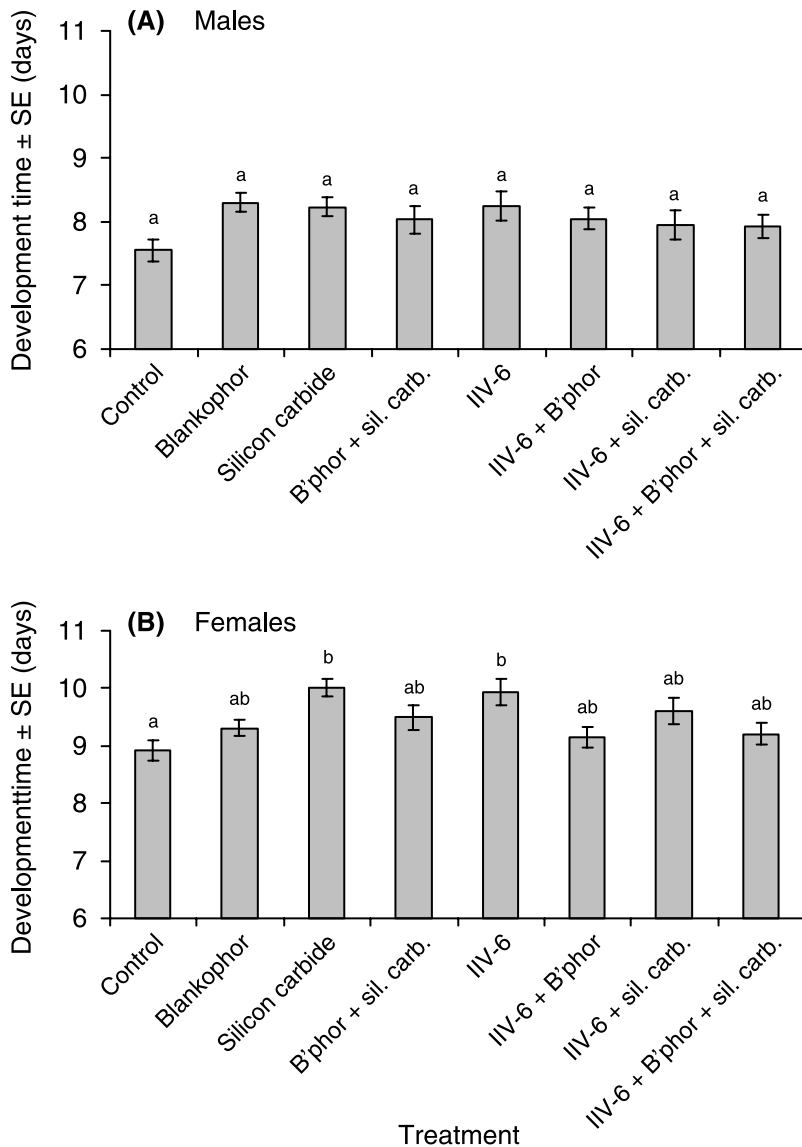


Figure 1 Mean (\pm SE) development time expressed as the interval between treatment of *Aedes aegypti* larvae and emergence of adults: (A) males and (B) females. Insects were treated with optical brightener (Blankophor BBH; B'phor) and silicon carbide (sil. carb.) alone or in mixtures with *Invertebrate iridescent virus 6* (IIV-6). Columns headed by the same letter are not significantly different for comparisons within each sex (ANCOVA, Bonferroni test $P > 0.05$).

occurred among treatments, although mortality ranged from 3.0% in the controls to a maximum of 10.2% in the treatment involving IIV-6 + Blankophor + silicon carbide ($F_{7,49} = 1.22$, $P = 0.31$) (Table 2).

Females emerged, on average, 1.5 days later than males ($F_{1,118} = 136$, $P < 0.001$) (Figures 1A, B). The interval from treatment to adult emergence differed significantly among treatments for female ($F_{7,48} = 3.12$, $P = 0.008$) (Figure 1B), but not for male insects (Figure 1A), although the calculated F-value was borderline significant ($F_{7,48} = 2.06$, $P = 0.06$). Compared to control insects, female development rate was extended by 11.4 and 12.6% in the treatments involving IIV-6 alone and silicon carbide alone, respectively. The sex ratio (proportion male) in control insects at

adult emergence was 0.520 (range of 95% C.L.: 0.477–0.563) and was similar to that of other treatments (range 0.485–0.539) ($F_{7,56} = 0.77$, $P = 0.61$). This indicates that *Ae. aegypti* females were more sensitive than males to certain treatments in terms of development times, but not in terms of a significant differential mortality of the sexes.

Discussion

We hypothesized that both abrasive particles and an optical brightener (chitin synthesis inhibitor) would increase the prevalence of IIV infection in *Ae. aegypti* larvae compared to conspecific larvae inoculated with virus alone. Instead, we found that these compounds had

minimal effects on the prevalence of patent and covert IIV infection and observed unexpected effects on the survival and development of this mosquito.

Mosquito larvae possess a type II peritrophic membrane, which is produced by specialized zones of epithelial cells in the cardia (proventriculus) and moves as a continuous sleeve down the length of the midgut (Jacobs-Lorena & Oo, 1996). The semi-permeable characteristics of the membrane are determined in large part by the presence of membrane pores. These pores are estimated to have a maximum diameter of between 2 and 10 nm for most insect species (Lehane, 1997) or a maximum of 21–36 nm for certain Lepidoptera and Orthoptera (Barbehenn & Martin, 1995). An earlier permeability study using sugars suggested the presence of much larger pore sizes (25–4000 nm) in *Ae. aegypti* membranes (Zhuzhikov, 1970), although the accuracy of these measurements appears to be in question (Lehane, 1997). In the hydrated state, IIV particles of the *Iridovirus* genus, such as IIV-6, have a diameter of approximately 170 nm whereas IIVs of the *Chloriridovirus* genus are larger, around 220 nm in diameter (Wagner et al., 1973; DeBlois et al., 1978; Hemsley et al., 1994).

Stoltz & Summers (1971) reported that chloriridovirus (IIV-3) particles failed to penetrate the peritrophic membrane of *Ochlerotatus (Aedes) taeniorhynchus* (Wiedemann) larvae and were degraded by digestive enzymes shortly after entering the midgut. Similar results were observed following the inoculation of mosquito larvae with a smaller iridovirus (IIV-1) from the dipteran *Tipula paludosa* Meigen. They hypothesized that IIV particles would only gain access to mosquito gut epithelial cells via occasional fractures in the peritrophic membrane.

Stilbene-derived optical brighteners are recognized as effective synergists for a number of insect viruses, particularly baculoviruses (Shapiro & Dougherty, 1994; Hamm, 1999). The mode of action of these brighteners involves, among other things, degrading the integrity of the peritrophic membrane, thus making it more porous to pathogenic virus particles (Wang & Granados, 2000). Contrary to our expectations, treatments involving Blankophor caused a small decrease in the prevalence of patent infection, with the same tendency observed for covert infections, although this was not significant. This could be related to the anti-feedant properties of certain optical brighteners, especially when ingested in mixtures with virus (Sheppard & Shapiro, 1994; Martínez et al., 2003). This would have resulted in a reduced consumption of inoculum and therefore a lower probability of infection compared to insects treated with virus alone.

Interestingly, the optical brightener Calcofluor white, which is identical to Blankophor BBH at a higher pH, did not affect the permeability of the peritrophic membrane to

the dye dextran blue (molecular weight: 2×10^6 Da) in larvae of *Anopheles gambiae* Giles (Edwards & Jacobs-Lorena, 2000). In contrast, the permeability of the multilayered peritrophic membrane of *Calliphora erythrocephala* (Meigen) (Diptera: Calliphoridae) larvae was greatly increased in the presence of Calcofluor white (Zimmermann & Peters, 1987). The sensitivity of peritrophic membranes to this brightener appears to differ markedly across dipteran species.

Using silicon carbide 'whiskers' in mixtures with IIV-3 Undeen & Fukuda et al. (1994) reported an increase in the prevalence of patent infection of from 4.8% in *O. taeniorhynchus* larvae treated with IIV-3 alone to 17.5% in larvae treated with IIV-3 + silicon carbide whiskers. These whiskers are needle shaped fibres (0.6 μm wide and 5–80 μm long) used to punch holes in insect or plant cells for genetic transformation. As such, they are likely to have caused more severe damage to the peritrophic membrane than the silicon carbide grains that we used.

In a previous study on the sublethal effects of IIV infection of mosquitoes, we observed that suspensions of virus with finely ground sand increased the prevalence of patent infections (Marina et al., 1999), which is what originally stimulated this study. Scanning electron microscope examination of ground sand particles revealed a heterogeneous mixture of particle sizes, the majority between approximately 5–200 μm and of various shapes (Figure 2A). In contrast, the commercially produced silicon carbide powder had a much more uniform distribution of sizes of around 30 μm , almost all of which had the form of polyhedral grains (Figure 2B).

All treatments tended to increase larval development time compared to control insects. We believe that this is a result of physiological stress or a reduction in feeding rate when exposed to virus, silicon carbide, or optical brightener. Recently, we reported an increase of approximately 0.5–1.0 days in the development times of *Ae. aegypti* larvae exposed to IIV-6 or virus inactivated by heat or ultraviolet (UV) light (Marina et al., 2003). We assumed that exposure to infectious or inactivated IIV particles could elicit apoptosis (programmed cell suicide) of gut epithelial cells, resulting in a reduction in feeding. This concept has now received some support from the finding that a vertebrate iridovirus, *Frog virus 3* (genus *Ranavirus*) elicits apoptosis in vertebrate cells in vitro, even when the virus has been inactivated by heat or UV light (Chinchar et al., 2003). These authors conclude that some component of the iridovirus particle can trigger apoptosis in host cells in the absence of expression of viral genes, i.e., without causing an infection.

Optical brighteners can cause physiological perturbations in the gut of lepidopteran larvae fed virus (Sheppard & Shapiro, 1994; Sheppard et al., 1994) and increased

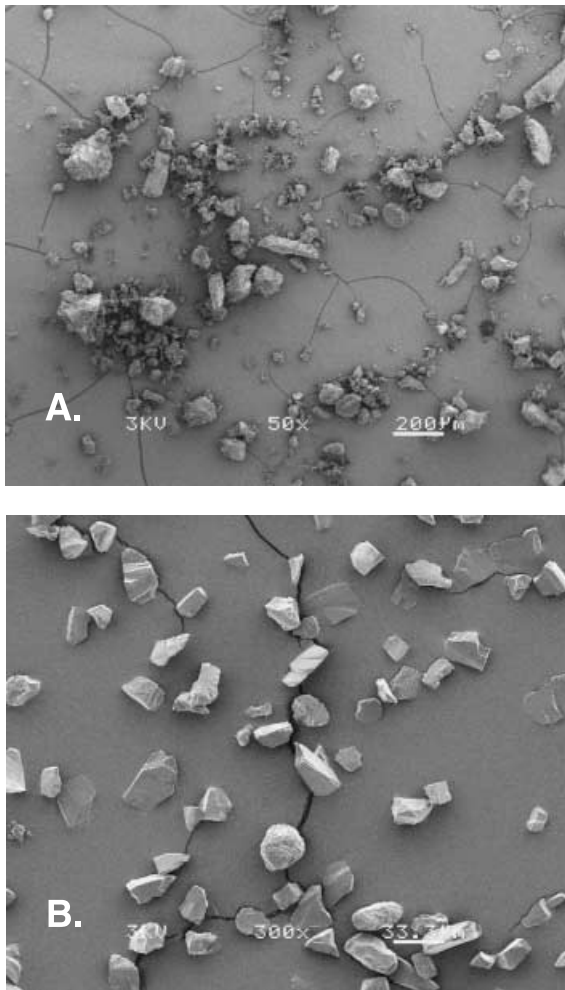


Figure 2 Photomicrographs indicating size, shape, and variation in (A) ground sand particles used in previous studies on IIV-6 infection in this species (Marina et al., 1999) (bar = 200 μm), (B) silicon carbide particles used to inoculate *Aedes aegypti* larvae (bar = 33.3 μm). Both images were obtained using a Topcon SM-510 scanning electron microscope at 30 kV.

mortality (Wang & Granados, 2000). In our study, the mortality of mosquito larvae increased significantly in every treatment involving Blankophor, compared to that of control insects (Table 2). However, the different treatments did not affect the prevalence of mortality of *Ae. aegypti* pupae and consequently mosquito survival to the adult stage largely reflected patterns of mortality observed in the larval stage.

These results lend support to the hypothesis that the gut may not be the principal point of infection of mosquito larvae by iridescent viruses. In contrast, recent studies with nematode parasites (Mullens et al., 1999) and a

hymenopteran endoparasitoid (López et al., 2002) have demonstrated a very efficient vectoring of IIV particles into insect host tissues during the process of parasite penetration or stinging with the ovipositor. As cannibalism and aggressive interactions between IIV-infected and healthy invertebrates are believed to be possible mechanisms of transmission (Williams, 1998), we are now testing the effect of larval mosquito density on the probability of infection by this virus.

Acknowledgements

We thank Alvaro Hernández, Américo Rodríguez, Alma Delia López, and Francisco Solís for technical assistance, Guadalupe Nieto for electron microscopy, and Javier Valle for statistical advice. This work was supported by a CONA-CyT doctoral scholarship to C.F. Marina and by SIBEF project 990502009.

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