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Sublethal iridovirus disease of the mosquito *Aedes aegypti* is due to viral replication not cytotoxicity

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Abstract. Invertebrate iridescent viruses (Iridoviridae) possess a highly cytotoxic protein. In mosquitoes (Diptera: Culicidae), *invertebrate iridescent virus* 6 (IIV-6) usually causes covert (inapparent) infection that reduces fitness. To determine whether sublethal effects of IIV-6 are principally due to cytotoxicity of the viral inoculum (which inhibits macromolecular synthesis in the host), or caused by replication of the virus larvae of the mosquito *Aedes aegypti* (L) were exposed to untreated IIV-6 virus that had previously been deactivated by heat or ultraviolet light. Control larvae were not exposed to virus. Larval development time was shortest in control larvae and extended in larvae exposed to untreated virus. Covertly infected mosquitoes laid significantly fewer eggs, produced between 20 and 35% fewer progeny and had reduced longevity compared to other treatments. Wing length was shortest in mosquitoes exposed to heat-deactivated virus. Multivariate analysis of the same data identified fecundity and progeny production as the most influential variables in defining differences among treatments. Overall, viral infection resulted in a 34% decrease in the net reproductive rate (R_0) of covertly infected mosquitoes, vs. only 5–17% decrease of R_0 following treatments with deactivated virus, compared to controls. Sublethal effects of IIV-6 in *Ae. aegypti* appear to be mainly due to virus replication, rather than cytotoxic effects of the viral inoculum.

Key words. *Aedes aegypti, Iridovirus*, biological control, bionomics, covert infection, cytotoxic protein, deactivated virus, IIV-6, insect pathology, invertebrate iridescent virus, larval development time, mosquito fecundity, mosquito fitness, mosquito longevity, mosquito size, reproductive rate (R_0), sublethal effects, wing length.

Introduction

The debilitating effects of sublethal viral diseases of insects are becoming increasingly recognized as important in the demography and dynamics of insect populations (Sait *et al*., 1994a; Rothman & Myers, 1996; Boots & Norman, 2000). The sublethal effects of such diseases commonly include extended juvenile development rates, reduced adult weight, reduced longevity and a reduction in the net reproductive rate of sublethally infected insects (Sait *et al*., 1994b; Myers *et al*., 2000).

Invertebrate iridescent viruses (IIVs) (*Iridoviridae*) are non-occluded, icosahedral, DNA viruses that principally infect insects, especially mosquitoes, in damp or aquatic habitats (Williams *et al*., 2000). IIVs have been reported to

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produce two kinds of infection: one patent and lethal, the other covert and non-lethal (Williams, 1993, 1995). Patently infected hosts take on a purple or blue iridescence and die in the larval or pupal stage. Covertly infected hosts, however, appear normal, develop to adulthood and may reproduce (Williams, 1993; Marina *et al*., 1999). Patent infections are generally rare and these viruses are not considered useful for biological control purposes. Covert infections, however, have been reported to be orders of magnitude more prevalent than patent infections, making the study of the effects of sublethal IIV disease particularly pertinent (Williams, 1995; Tonka & Weiser, 2000).

Invertebrate iridescent virus 6 (IIV-6), originally isolated from *Chilo suppressalis* (Lepidoptera: Pyralidae), is the type species of the *Iridovirus* genus and represents the standard laboratory model for the study of this group of viruses (Williams, 1998; Jakob *et al*., 2001). Individuals suffering covert IIV-6 infections can be identified using a highly sensitive insect bioassay technique or by polymerase chain reaction (PCR) (Constantino *et al*., 2001). The bioassay involves injection of a semipurified insect homogenate into larvae of *Galleria mellonella* (Lepidoptera: Pyralidae), which develop a patent infection 8–12 days later (Marina *et al*., 2000; Constantino *et al*., 2001). Adult *Aedes aegypti* mosquitoes covertly infected by IIV-6 had reduced longevity, shorter wing length and produced fewer progeny than non-infected conspecifics (Marina *et al*., 1999). This resulted in a 22% reduction in the R_0 of covertly infected insects.

All iridoviruses tested to date have cytotoxic properties (Jakob *et al*., 2002). Intrahaemocoelic injection of a solubilized protein extract of IIV-6 into rats or mice causes an acute degenerative hepatitis that is fatal within 24 h (Lorbacher de Ruiz, 1990). Likewise, vertebrate and insect cells lines inoculated with IIV-6 in which the genome has been rendered non-functional by exposure to ultraviolet (UV) light undergo extensive cell fusion and shutdown of macromolecular synthesis within 1–2 h of inoculation (Cerutti & Devauchelle, 1980). By contrast, virus inactivated by heat treatment has denatured proteins and causes no such effects (Ohba *et al*., 1990). The cytotoxic properties of IIV-6 are due to a proteinaceous factor associated with the lipid membrane that lies between the core and the capsid of the virus. Sequencing data suggests this to be a peptide of 46 amino acids that may be distantly related to a spider neurotoxin (Cordeiro *et al*., 1992; Tidona & Darai, 1997; Jakob *et al*., 2001).

The present study aimed to determine whether the sublethal effects of IIV-6 in adult mosquitoes were due to pathological effects of viral infection or the result of exposure to toxic viral proteins during the larval stage. To achieve this, the survival and reproduction of mosquitoes that had been exposed to virus inactivated by heat, or by UV light, were compared with that of mosquitoes exposed to untreated virus, or control insects with no virus exposure. UV-irradiated virus has a non-functional genome but possesses the functional toxic polypeptide, whereas heat-inactivated virus has a denatured toxic protein but possesses a normal genome. It was therefore possible to distinguish between the effect of virus pathology and that of virus toxicity.

Materials and methods

Insects and virus

Eggs of *Aedes aegypti* and larvae of the greater wax moth *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were obtained from laboratory cultures held in the Centro de Investigación de Paludismo and El Colegio de la Frontera Sur (ECOSUR), both in Tapachula, Chiapas, Mexico, respectively. Mosquitoes used in the experiments described below were reared using filtered dechlorinated tap water. All experimental procedures involving mosquitoes were performed at 26 ± 1°C, LD 12 : 12 h light cycle and 75–85% RH. IIV-6 was grown in *G. mellonella* and stored as infected cadavers at -20°C until use.

Purification and quantification of virus

The procedures for purification and quantification of IIV-6 have been described previously (Marina *et al*., 1999, 2000). Briefly, infected *G. mellonella* larvae were thawed and homogenized in sterile distilled water. The homogenate was centrifuged at 490 **g**, 960 **g** and 1250 **g**, each for 10 min to remove insect debris, followed by 15 300 **g** for 10 min to pellet virus. The pellet was resuspended in 300 µl water, layered onto 30% sucrose and subjected to 15 300 **g** for 30 min. The resulting pellet was washed once and resuspended in 1 ml sterile distilled water.

The purified suspension was quantified by direct counting of a mixture of virus and polystyrene beads 460 nm diameter (Aldrich Chemical Co., MO, U.S.A.) using a scanning electron microscope. Five fields of vision from five replicate mixtures were counted for each suspension (>1000 particles in total) (Constantino *et al*., 2001).

Virus inactivation by heat and UV light

Purified virus suspensions containing 2.5×10^{11} virus particles/ml were heated to 70°C for 30 min using a calibrated heating block with a precision of ± 1°C. Thermometers placed in microcentrifuge tubes containing water placed next to the virus samples were used to confirm the temperature for the duration of the treatment.

For UV-inactivation, virus suspension containing 2.4×10^{11} virus particles/ml was sealed inside thin polythene bags and placed for 15 s on the filter of a UV-transilluminator equipped with six 20 W tubes (Hoefer Scientific Instruments UVTM-25, Chicago, IL, U.S.A., peak output at 302 nm).

Serial dilutions of UV- or heat-treated virus samples and of untreated virus suspensions were made and 8.4 µl volumes were injected into third-instar *G. mellonella* larvae.

Control larvae were injected with sterile water. Groups of injected larvae were placed in plastic pots containing semisynthetic diet and reared at 25°C. At 12–14 days post-injection, the proportion of patently infected larvae was determined in each treatment.

Toxic effects of IIV-6

Groups of 200 third-instar *A. aegypti* larvae were placed in plastic containers containing 100 ml dechlorinated water and 100 mg of finely ground sand, shown in previous studies to enhance the prevalence of infection, probably by damaging the peritrophic membrane lining the insect gut (Marina *et al.*, 1999). Larvae were then inoculated with 4.3×10^9 particles/ml of IIV-6 that had been treated as follows: (i) heat-deactivated at 70°C for 30 min, (ii) UV-deactivated by 15 s exposure and (iii) untreated virus. Control mosquito larvae were not inoculated with virus. Both virus inactivation treatments caused eight orders of magnitude decrease in virus activity. The water in each container was gently agitated by an air tube placed in the water for a period of 6 h, after which larvae were washed five times to eliminate virus inoculum. Larvae were finally divided among plastic rearing trays, fed a powdered yeast and soya mixture daily until pupation. The duration of the larval stage, the incidence of larval mortality and of patent IIV infection were noted.

Pupae were transferred to 250 ml plastic cups containing 50 ml dechlorinated water and held individually until adult emergence. Following emergence, mosquitoes were sexed and 50 male–female pairs of adult mosquitoes were randomly selected from each treatment, placed together in plastic cups and fed 10% sugar solution on a cotton wool pad. After 48 h, males were removed, placed together in groups of eight in plastic cups and offered sugar solution daily until death.

Each female mosquito was numbered, given a bloodmeal and individually placed in a plastic cup containing 40 ml water and a wooden spatula as an oviposition substrate. Following oviposition, females were individually transferred to a new plastic cup and offered bloodmeals at 4-day intervals. This process was repeated until death.

Eggs laid at each oviposition bout were counted and placed in water at 24 ± 1°C. The number of larvae emerging from each oviposition was recorded.

After death, the wing length of the female mosquito was measured from the axial incision to the outer margin of the R1 vein (Xue & Ali, 1994). The fimbrial scales were not included in this measurement and the wing was selected from the left or right side of the body at random to avoid possible bias arising from insect asymmetry. Wing length was used as an indicator of body size/mass (Siegel *et al.*, 1992). The abdomen of each female was then removed and homogenized in 500 µl of antibiotic solution (0.08% aureomycin). Insect debris was removed by centrifugation at 190 **g** for 5 min. Volumes of 8.4 µl of the remaining suspension were injected into 10 third-instar *G. mellonella* larvae, which were placed in 50 ml plastic pots containing semisynthetic diet and held at 25°C. The presence of patent IIV infections in these larvae 10–12 days post-injection was used as an indicator of covert infection of the mosquito. The experiment was performed twice.

Statistical analysis

The effect of exposure to heat or UV light on the activity of virus suspensions was subjected to χ^2 tests on the prevalence of patently infected *G. mellonella* larvae at the dilutions tested. To analyse treatment effects on larval development time, development times were categorized into six intervals from 8 to 13 days and were subjected to correspondence analysis for each replicate separately.

The effects of exposure to untreated and inactivated virus on mosquito fitness correlates were compared using a one-way ANOVA and mean separation was achieved by Tukey's test. Fecundity, progeny production and longevity data were $\sqrt{x}$ transformed whereas wing length data were $\log_e(x)$ transformed prior to analysis. A multivariate ANOVA was also conducted and the significance of treatment effects was determined by examination of *F*-values generated by Pillai's trace. The contribution of each variable to the overall *F*-value was determined by examining the magnitude of the standardized canonical coefficients (Winer, 1971). For both analyses, adult mosquitoes that proved to be covertly infected in the *G. mellonella* bioassay were analysed as a separate group.

Results

Virus inactivation by heat and UV light

Heat exposure to 70°C for 30 min reduced the activity of virus by a factor of 2.5×10^8 compared to untreated control virus ($\chi^2 = 4.36$, d.f. $= 1$, $P < 0.04$). Similarly, exposure to UV light for 15 s reduced the activity of virus by a factor of 2.1×10^8 ($\chi^2 = 4.40$, d.f. $= 1$, $P < 0.04$).

Toxic effects of IIV-6

No mortality was observed in control larvae (0/398) whereas mortality of larvae in the other treatments was 1.5% (6/401) in the UV-inactivated virus treatment, 1.3% (5/392) in the heat-inactivated virus treatment and 3.4% (13/391) in larvae exposed to untreated virus, the latter treatment accounted for 57% of the χ^2 value ($\chi^2 = 14.6$, d.f. $= 3$, $P = 0.002$). Only three larvae developed patent infections during the larval stage. All three were removed from the rearing area to avoid cross-contamination and all died prior to pupation.

The pattern of larval development times differed between replicates and therefore correspondence analysis was applied to each replicate separately (Fig. 1). The duration

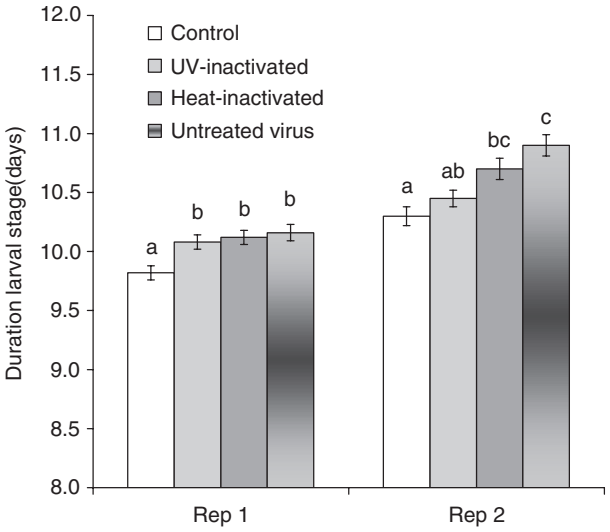

Fig. 1. Comparison of mean development times, from inoculation to pupation, for mosquito larvae exposed to untreated virus inoculum, virus deactivated by UV or heat, and non-inoculated controls. Columns labelled with identical letters do not differ significantly for comparisons within each replicate (correspondence analysis, $P > 0.05$). Bars indicate SEM.

of larval development in the first replicate was shortest in the control larvae (9.8 ± 0.06 days) and significantly longer in larvae exposed to untreated and UV or heat-inactivated virus, with the longest development time (10.2 ± 0.07 days) observed in larvae exposed to untreated virus ($\chi^2 = 31.9$; d.f. = 15; $P = 0.006$). Similarly, in the second replicate, larval development was shortest in control larvae (10.3 ± 0.08 days), longest in larvae inoculated with untreated virus (10.9 ± 0.09) and intermediate in larvae exposed to UV or heat-inactivated virus ($\chi^2 = 43.2$, d.f. = 15, $P < 0.001$) (Fig. 1).

Emergence of adult mosquitoes followed a similar pattern to that seen in larval development times. The interval between inoculation and adult female emergence was shortest in control females (13.0 ± 0.07 days) and longest in females inoculated with untreated virus (13.7 ± 0.09) (data from both replicates pooled), mean female emergence times from UV (13.3 ± 0.08) and heat-inactivated virus (13.6 ± 0.08) were intermediate ($F = 12.5$, d.f. = 3, 651, $P < 0.001$). Emergence of males occurred approximately 1 day prior to that of females but followed the same pattern (data not shown).

Of the 386 females tested, a total of 34 female mosquitoes were identified as being covertly infected by insect bioassay. Infected females were considered as a separate group in the following analyses to distinguish them from females that had been inoculated with virus but had not become infected (exposed females).

Univariate ANOVA indicated that mean fecundity ($\pm$ SE) among females inoculated with untreated or UV/heat-deactivated virus did not differ significantly from that of control females (226 ± 14.6 eggs per female), but was significantly reduced in covertly infected mosquitoes (144 ± 13.9) ($F = 3.14$, d.f. = 4, 357, $P = 0.015$) (Table 1). Consequently, covertly infected females produced significantly fewer progeny (73 ± 10.3 offspring per female) than control females (111 ± 7.2), whereas progeny production in females from untreated virus or UV/heat-deactivated virus treatments was intermediate ($F = 3.01$, d.f. = 4, 357, $P = 0.018$).

The longevity of covertly infected females (41.5 ± 1.6 days) was also significantly reduced compared to control mosquitoes (49.2 ± 1.6 days) or females ino2culated with untreated virus or UV/heat-deactivated virus ($F = 9.69$, d.f. = 4, 355, $P = 0.001$) (Table 1). Female wing length, used as an indicator of body size, was significantly smaller in mosquitoes exposed to heat-deactivated virus (2.84 ± 0.001 mm) compared to control females (2.92 ± 0.001 mm), wing length of mosquitoes from all other treatments were intermediate for this variable ($F = 4.16$, d.f. = 4, 306, $P = 0.003$).

Multivariate ANOVA of the above parameters analysed as a group of dependent variables confirmed the univariate analyses in detecting significant treatment effects ($F = 2.78$, d.f. = 16, 1220, $P < 0.001$). Examination of the standardized canonical coefficients revealed that fecundity and progeny production were more influential than longevity or wing length in their contribution to the observed differences among treatments (Table 2).

Table 1. Mean ($\pm$ SE) fecundity, progeny production, longevity and wing length of *Aedes aegypti* females that had been exposed to untreated virus or to deactivated virus. Control mosquitoes had no prior exposure to virus. Data were subjected to univariate ANOVA

	Treatment*				
Parameter	Control	UV-inactivated virus	Heat-inactivated virus	Untreated virus (non-infected mosquitoes)	Covertly infected mosquitoes
Fecundity (eggs per female)	226.7 ± 14.6^{a}	211.1 ± 12.2^{a}	208.8 ± 12.1^{a}	223.1 ± 13.5^{a}	144.2 ± 13.9^{b}
Progeny production (offspring per female)	111.1 ± 7.2^{a}	91.7 ± 7.1^{ab}	98.4 ± 6.9^{ab}	105.4 ± 7.4^{ab}	73.6 ± 10.3^{b}
Longevity (d)	49.2 ± 1.6^{a}	45.1 ± 1.5^{a}	44.8 ± 1.5^{a}	44.9 ± 1.4^{a}	41.5 ± 1.6^{b}
Wing length (mm)	2.92 ± 0.001^{a}	2.89 ± 0.001^{a}	2.84 ± 0.001^{b}	2.88 ± 0.001^{ab}	2.89 ± 0.002^{ab}

*Figures followed by the same letter are not significantly different for comparisons between treatments within a single row (ANOVA, Tukey test $P > 0.05$).

Table 2. Multivariate analysis of fitness correlates of mosquitoes, *Aedes aegypti*, that had been exposed to untreated virus, or to virus deactivated by UV or heat during the larval stage. Control mosquitoes were not exposed to virus. Examination of standardized canonical coefficients indicates that fecundity and progeny production were the most influential variables in generating the observed treatment differences

Source	F	d.f.	*P*
Experiment (Pillai's Trace)	2.78	16, 1220	0.0002
Standardized Canonical Coefficients			
Fecundity	Progeny production	Longevity	Wing length
−1.3096	1.0997	−0.7379	0.7324

Discussion

In this study our hypothesis was that sublethal effects observed in mosquitoes exposed to UV-inactivated virus should be due solely to the toxic viral protein, as the genome was rendered non-functional in UV treated virus. Heat-inactivated virus was expected to produce no sublethal effects whatsoever, because the toxic protein was denatured by heating and this protein is essential for replication and expression of the viral genome. Differences in the magnitude of sublethal effects observed between insects exposed to UV-inactivated virus and insects that became covertly infected following exposure to untreated virus should therefore be due to virus infection and replication in host cells.

Our results indicate that covert IIV infection is principally responsible for the sublethal effects on fitness correlates observed in mosquitoes. The sublethal effects were more consistent and of greater magnitude in insects that were covertly infected than in insects exposed to denatured inocula or virus inoculated insects that did not become infected. By contrast, the low prevalence of patent infection during the larval stage, although fatal for the infected individual, had little impact on mosquito demography at the population level.

It was evident, however, that exposure to virus, whether UV/heat-deactivated or not, was associated with a number of effects on the fitness correlates of the insect, although in the great majority of cases, these were less extreme than those observed in covertly infected individuals.

Covert infection caused a reduction in the fecundity, progeny production, longevity and body size (wing length) of infected mosquitoes compared to controls. Insects that were inoculated with untreated virus but did not become infected suffered a small decrease in the same parameters, which may have been caused by the toxic virus protein or may have resulted from some metabolic or physiological cost associated with avoiding infection or mounting an immune response against a viral challenge (Lowenberger, 2001).

Heat-deactivated virus was predicted to have no effect on mosquitoes because the heating process denatures proteins. Indeed, the insect bioassay demonstrated that the heat-treated virus had lost 8 logarithms of activity. This was a result of the inactivation of at least two essential trigger proteins: the first being the cytotoxic protein that shuts down host macromolecular synthesis and the second, a polypeptide that is believed to interact with host RNA polymerase II or bind to viral template DNA in order to initiate viral transcription in the host nucleus (Goorha, 1981; Willis & Granoff, 1985). The fecundity, progeny production and longevity of mosquitoes exposed to heat-treated virus were all statistically similar to control insects, whereas body size (wing length) was significantly smaller in insects from this treatment. Wing length is generally considered to be a conservative indicator of body size in mosquitoes (Briegel, 1990; Siegel *et al*., 1992).

Similarly, compared to control mosquitoes, insects exposed to UV-inactivated virus did not show significant differences in any of the variables that we measured in the adult mosquitoes. Moreover, direct toxin-induced mortality in mosquito larvae treated with UV-inactivated virus was not observed.

We suggest that the effects observed in heat and UV-deactivated virus are related to the cost of dealing with cells that have become infected by virus particles. Heat and UV-deactivated virus particles will still penetrate host cells and the presence of viral components in the host may have induced apoptosis leading to shedding of gut epithelial cells and a possible reduction in feeding or a reallocation of host resources to repair damaged gut cells at the cost of larval growth. Apoptosis, genetically regulated cell suicide, serves as an important host defence strategy in response to viral invasion (O'Brien, 1998). Consequently, a number of viruses, including insect baculoviruses and iridescent viruses, express genes that block the cell death program (Birnbaum *et al*., 1994; Miller, 1997). The occurrence of viral-induced apoptosis in mosquito gut cells would have occurred to the same degree in mosquitoes exposed to untreated virus, the difference being that untreated virus may have been able to transcribe antiapoptosis genes and inhibit cell death, even though only a portion of these insects went on to develop an inapparent infection. By contrast, transcription of heat-deactivated virus genes is likely to have been minimal or absent, because of the lack of functional trigger polypeptides, and transcription of UV-inactivated viral DNA would be inhibited because UV treatment causes severe nucleic acid damage and renders the genome non-functional (Pearlman *et al*., 1984). The inability to block apoptosis may have led to cell sloughing, reduced feeding, extended larval development and reduced adult body size. Further studies on gut cell turnover would be required to support this hypothesis.

Assuming that the mean number of progeny produced per female represents a reasonable estimate of the net

reproductive rate (R_0), i.e. viral infection does not differentially affect survival of the sexes or secondary sex ratio (Marina *et al*., 1999), it is apparent that viral infection was associated with a 34% decrease in the R_0 of covertly infected mosquitoes compared to control insects. This is greater than the 22% decrease reported previously (Marina *et al*., 1999). Between 5 and 17% decrease was observed in the R_0 of insects from other treatments compared to controls, presumably related to the effects described above.

Effects similar to those observed in the present study have also been reported in insects, principally Lepidoptera, treated with baculoviruses and cypoviruses (Goulson & Cory, 1995; Rothman & Myers, 1996). Other effects include a reduction in the weight of pupae and adults (Myers *et al*., 2000) and a reduced reproductive capacity (Sait *et al*., 1994b, 1998). However, many studies have failed to demonstrate the presence of virus particles in the survivors of sublethal doses of virus, placing in doubt the causal nature of the relationship between the pathogen and the observed effects (Cory *et al*., 1997).

A number of other viruses also cause non-lethal infections in mosquitoes, although their effects on the reproductive capacity of the insect remain uncertain (Bird *et al*., 1972; Chapman, 1974; Kawase & Kurstak, 1991). By contrast, microsporidian pathogens of mosquitoes have consistently been reported to cause marked sublethal effects including reduced fecundity, fertility, longevity and body size (Nasci *et al*., 1992; Seif, 2000) or a decrease of up to 98% in the R_0 of infected *Ae. aegypti* females (Becnel *et al*., 1995). The presence of pathogens may also be repellent to ovipositing mosquitoes, resulting in a decrease in the prevalence of oviposition in pathogen-contaminated water sources (Zahiri & Rau, 1998).

Sublethal effects of viruses are notoriously difficult to observe because of heterogeneity in the degree to which individuals respond to infection (Sait *et al*., 1998; Vargas-Osuna, 2001). This limits the power of statistical tests applied to such data because a reduction in mean parameter values may be associated with an increase in variance. This phenomenon was particularly evident in a previous study of sublethal effects of IIV-6 on *Ae. aegypti* wherein reduced fecundity of covertly infected females was accompanied by a marked increase in variance and non-normal distribution of data that prevented formal statistical analysis (Marina *et al*., 1999). In the present study, the univariate ANOVA gave clearer results and was easier to interpret in terms of the biology of the experimental system. The multivariate ANOVA was useful in identifying which of the variables made the greatest contribution to the F statistic (i.e. the importance of the treatment effect) and has the advantage of a reduced risk of type I and II errors arising from multiple independent analyses (Scheiner, 1993).

The reason to use a lepidopteran virus (IIV-6) for this study may not be obvious: IIV-6 has advantages over the only recognized mosquito isolate (IIV-3) because sensitive biosassay and specific PCR detection systems have been developed for IIV-6. Moreover, IIV-3 is apparently specific to the mosquito *Ochlerotatus taeniorhynchus* (Wiedemann), whereas IIV-6 has a much broader host range and a great deal more information is available concerning its genome, structure and biology than is available for IIV-3 (Williams *et al*., 2000).

The mode of action of the iridovirus toxin is believed to involve inhibition of DNA, RNA and protein synthesis (Cerutti & Devauchelle, 1980; Lee & Brownrigg, 1982). Ribosomes purified from livers of mice that had been injected with a lethal dose of virus were unable to translate mRNA *in vitro* (Elharrar & Kirn, 1977). Cells treated with UV-inactivated, antiserum-inactivated virus or a solubilized virus extract die within 24 h unless superinfected by viable virus, whereupon macromolecular synthesis is restored and a normal viral replication cycle is observed (Drillien *et al*., 1977; Cerutti & Devauchelle, 1990).

If IIV-6 were to be applied for mosquito control purposes, despite its very limited impact on mosquito bionomics, such effects would be largely due to active infection. Mosquito exposure to deactivated virus induced various biological effects which cannot be due to viral replication, of lesser magnitude than observed in covertly infected insects. Moreover, viable IIV-6 may replicate without causing visible signs of disease in covertly infected mosquitoes (Marina *et al*., 1999). A reverse transcriptase PCR assay (to demonstrate the presence of viral RNA transcripts) is needed to detect IIV activity in covertly infected insects, and this is currently being developed.

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