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Persistence of invertebrate iridescent virus 6 in tropical artificial aquatic environments

Brief Report

A. Hernández[1], **C. F. Marina**[1,2], **J. Valle**[1], and **T. Williams**[1,3]

[1]ECOSUR, Tapachula, Chiapas, Mexico
[2]Centro de Investigación de Paludismo-INSP, Tapachula, Chiapas, Mexico
[3]Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona, Spain

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Summary. The rate of loss of activity of invertebrate iridescent virus 6 (IIV-6, family *Iridoviridae*) was determined in two artificial aquatic habitats in southern Mexico, using a sensitive insect bioassay technique. IIV-6 placed in trays of water in direct sunlight suffered rapid loss of activity (99.99% reduction) over a period of 36 h, during which water temperatures fluctuated between 24 and 41 °C. No significant deactivation occurred during the hours of darkness. In contrast, IIV-6 placed in trays of water in the shade lost 97% of original activity over a 60 h period, during which water temperatures fluctuated from 24 to 31 °C. Longitudinal analysis involving mixed effects models of time (shade) and cumulative exposure to ultraviolet radiation (sunlight) indicated that the rate of deactivation was best described by third order polynomial equations in both cases. We conclude that the likelihood of transmission of IIVs in aquatic habitats will be mediated by the intensity of UV radiation and water temperature.

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Invertebrate iridescent viruses (IIVs) (family *Iridoviridae*) are icosahedral particles containing a large (~200 kb) dsDNA genome [2]. These viruses infect many invertebrate species worldwide, especially the aquatic larval stages of medically important vectors, such as mosquitoes and blackflies [22, 23]. The ability of IIVs to persist in the environment is poorly understood. Ultraviolet radiation has been used frequently to deactivate iridoviruses for laboratory studies [11, 15], and in aquaculture to sterilize the water of fish farm nurseries [12]. Laboratory studies indicate that moisture appears to be crucial to IIV survival [13, 20] and

microbial activity may also be influential over extended periods in damp soils [19]. Additionally, elevated temperatures can reduce virus persistence [4]; IIV-6 suspension placed in plastic tubes beneath the water surface of a small pond (mean temperature 27 °C) lost activity more rapidly than control samples incubated at 4 °C or 25 °C in the laboratory [8]. Therefore, we determined the rate of inactivation of invertebrate iridescent virus 6 (IIV-6) in full sunlight and shaded conditions, in an aquatic environment of a tropical region, where mosquito-borne diseases are a major human health issue. IIV-6 is the type species of the genus *Iridovirus*, that represents the standard model for the study of this group of viruses [5], and which can cause lethal and sublethal disease in *Aedes aegypti*, the principal vector of dengue and yellow fever [10].

A sample of IIV-6 of uncertain passage history, originally isolated from *Chilo suppressalis* (Lepidoptera: Pyralidae) in Japan, was obtained from Adrian Gibbs (Australian National University, Canberra, Australia) by way of Peter Christian (CSIRO, Canberra) in 1994. IIV-6 was produced by injection in third instar *Galleria mellonella* (Lepidoptera: Pyralidae) obtained from a laboratory colony maintained on a semi-synthetic diet in El Colegio de la Frontera Sur (ECOSUR), Tapachula, Chiapas, Mexico. All insects were maintained in a controlled temperature room at 25 ± 1 °C, 75–85% R.H. and 12 h:12 h L:D. At 7–10 days post-infection, patently infected larvae were sacrificed and triturated in 1 ml sterile distilled water. The homogenate was subjected to three steps of centrifugation at 490 g for 10 min, followed by 15,300 g for 10 min, and then centrifuged through 30% (wt/vol) sucrose at 15,300 g for 30 min and two washes in sterile water. The final suspension was diluted in 68% (vol/vol) isopropanol mixed with polystyrene beads of 460 nm diameter (Sigma-Aldrich Corp. St. Louis, MO). The concentration of each virus stock suspension was then determined by scanning electron microscope (SEM) observation of virus preparations dried onto polylysine coated glass coverslips. A minimum of eight fields of vision from each of five replicate virus-polystyrene mixtures were counted for each virus preparation, representing >1000 virus particles in all cases [3]. Virus suspensions were stored at 4 °C and used within five days of purification.

To determine the rate of deactivation of IIV-6 in aquatic habitats, 2 l of dechlorinated tap water were placed in each of three plastic trays (31 cm long $\times$ 20 cm wide $\times$ 9 cm tall). A 2 ml volume of suspension containing 4.8×10^{11} particles of IIV-6 was added to each tray representing a final concentration of 2.4×10^{8} particles/ml. The resulting suspension was agitated thoroughly for two minutes. One tray was placed in direct sunlight on an open grassed area near the laboratory, the second tray was placed 1 m away in the shade of a nearby wall. The third tray was placed away from direct sunlight in the laboratory which was maintained at 25 ± 1 °C. Control trays were prepared in an identical manner, except that virus suspension was not added at any stage. Water loss due to evaporation was corrected by adding the appropriate quantity of clean dechlorinated water to each tray when necessary (indicated by a mark on the side of each tray). Incident ultraviolet light in the range 290–390 nm (UV-A + UV-B) and total solar radiation (300–1100 nm) were measured continuously using a Health Enviromonitor digital weather logger

(Davis Instruments Corp., Hayward, CA). No precipitation was recorded on the days on which the trays were placed outdoors. The water temperature in each tray was recorded hourly.

Samples of 1 ml of water from each tray were taken using a sterile plastic pipette at intervals over a 2 day (tray in sunlight) or 3 day (tray in shade) period. On each occasion, the sample was taken at 5–10 mm below the water surface. Identical samples were taken from control trays. Sampling commenced at 06.00 hrs (sunrise, start of experiment representing time point zero) and continued at intervals of 3–12 h thereafter. The activity of IIV-6 in each sample was then determined by insect bioassay. The experiment was performed three times.

To determine the activity of virus, each sample was serially diluted up to 10^8 fold in tenfold steps in sterile distilled water. Volumes of 8.3 µl of each dilution were individually injected into groups of 30 third instar *G. mellonella* using a manual microinjector fitted with a 1 ml syringe (Burkard Co., UK) [3]. The injected larvae were placed in plastic cups containing a semi-synthetic diet and incubated at 25 °C. The number of larvae displaying signs of patent IIV-6 disease (bluish white coloration of the epidermis with iridescent patches) was noted at 10–12 days post-injection. Where any doubt existed, larvae were triturated and centrifuged. The presence of a iridescent blue pellet of virus was used to determine the presence of an infection.

The activity of the virus in each sample was calculated using the end-point dilution method [18], with the proportion of patently infected *G. mellonella* larvae as the response variable. This bioassay technique is approximately 10 times more sensitive than $TCID_{50}$ assays *in vitro* [3]. The rate of loss of activity in each treatment was then compared by computing linear mixed effects models fit by maximum likelihood of the logarithm of the proportion of virus activity remaining in each sample against time and cumulative incident UV. Cubic, quadratic and straight line models were subjected to pairwise comparisons by calculating the respective log-link ratios, that follow a χ^2 distribution, in S-Plus [17].

The titre of IIV-6 placed in trays of water in the laboratory (25 °C) did not change significantly during the 3 day experimental period ($\chi^2 = 2.02$, d.f. $= 1$, $P = 0.15$). The titre of virus placed in trays of water in the shade declined by approximately 97% (Ln [proportion activity remaining] ≈ -3.5) over a 60 h period (Fig. 1a). This was best described by a cubic regression that was significantly better fit than a simple linear regression ($\chi^2 = 14.9$, d.f. $= 2$, $P < 0.001$) or a quadratic regression ($\chi^2 = 7.57$, d.f. $= 1$, $P = 0.006$). The water temperature of trays placed in the shade during the experimental period varied from 24 °C at night to a maximum of 31 °C during the day, with a mean of 27.0 °C (Fig. 1b).

Virus in trays placed in sunlight was rapidly deactivated during the day but no loss of activity occurred overnight in samples taken between 18.00 and 06.00 hrs the following morning (data not shown). Following exposure to direct sunlight, IIV-6 in trays lost approximately 99.99% of the original activity over a period of 36 h (Ln [proportion activity remaining] ≈ -9.0) (Fig. 2a). The relationship was best described by a cubic equation which was a significantly better fit than a quadratic ($\chi^2 = 14.5$, d.f. $= 2$, $P < 0.001$) or simple linear regression ($\chi^2 = 4.37$,

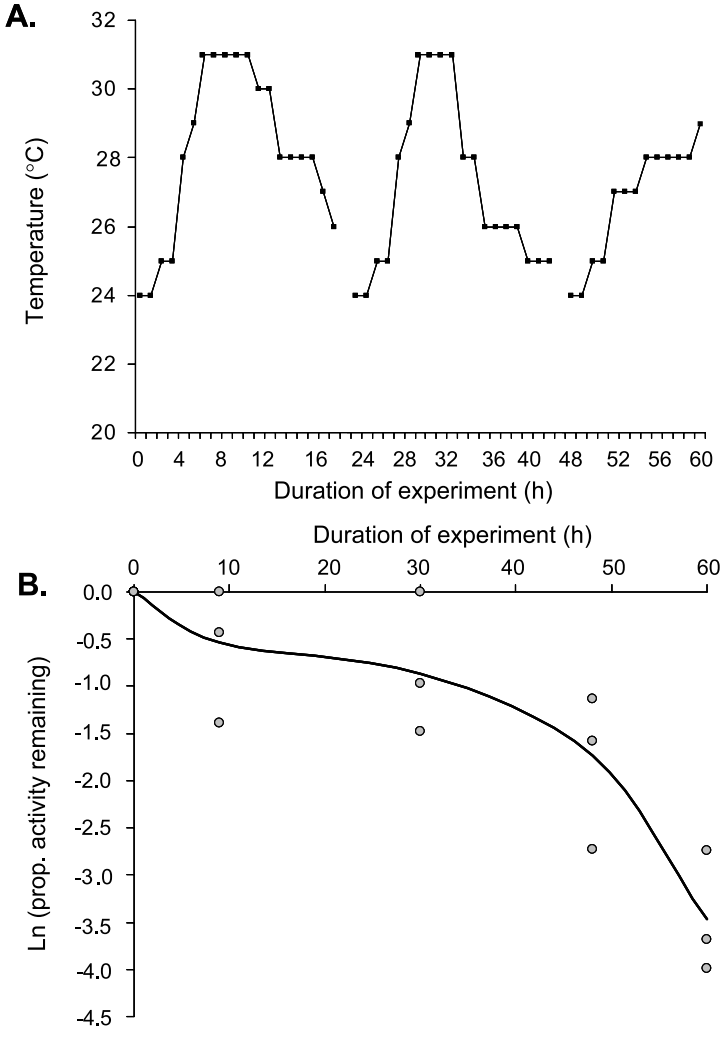

Fig. 1. A Fluctuation in water temperatures in trays placed in shade during experimental period. **B** Mixed effects model of Ln (proportion of virus activity remaining) over time (36 h) for IIV-6 placed in trays in the shade. The minimal significant model fitted by maximum likelihood was $y = -0.000047x^3 + 0.003258x^2 - 0.084398x$. Grey dots indicate data points of individual replicates

d.f. = 1, P = 0.036). During this time the trays received 45 W/m^2 of incident UV radiation and temperatures that ranged from 24 to 41 °C, with a mean of 31.9 °C (Fig. 2b). None of the control larvae developed iridescent virus infections when inoculated with water samples from control trays placed in sunlight or shade.

Exposure to direct sunlight and high temperatures (up to 41 °C) resulted in rapid loss of activity of IIV-6 in water, whereas the virus persisted longer in trays of water placed in the shade that were subjected to no direct UV radiation and warm temperatures (up to 31 °C). It was not possible to separate the effects of temperature and UV radiation because exposure to the sun inevitably resulted

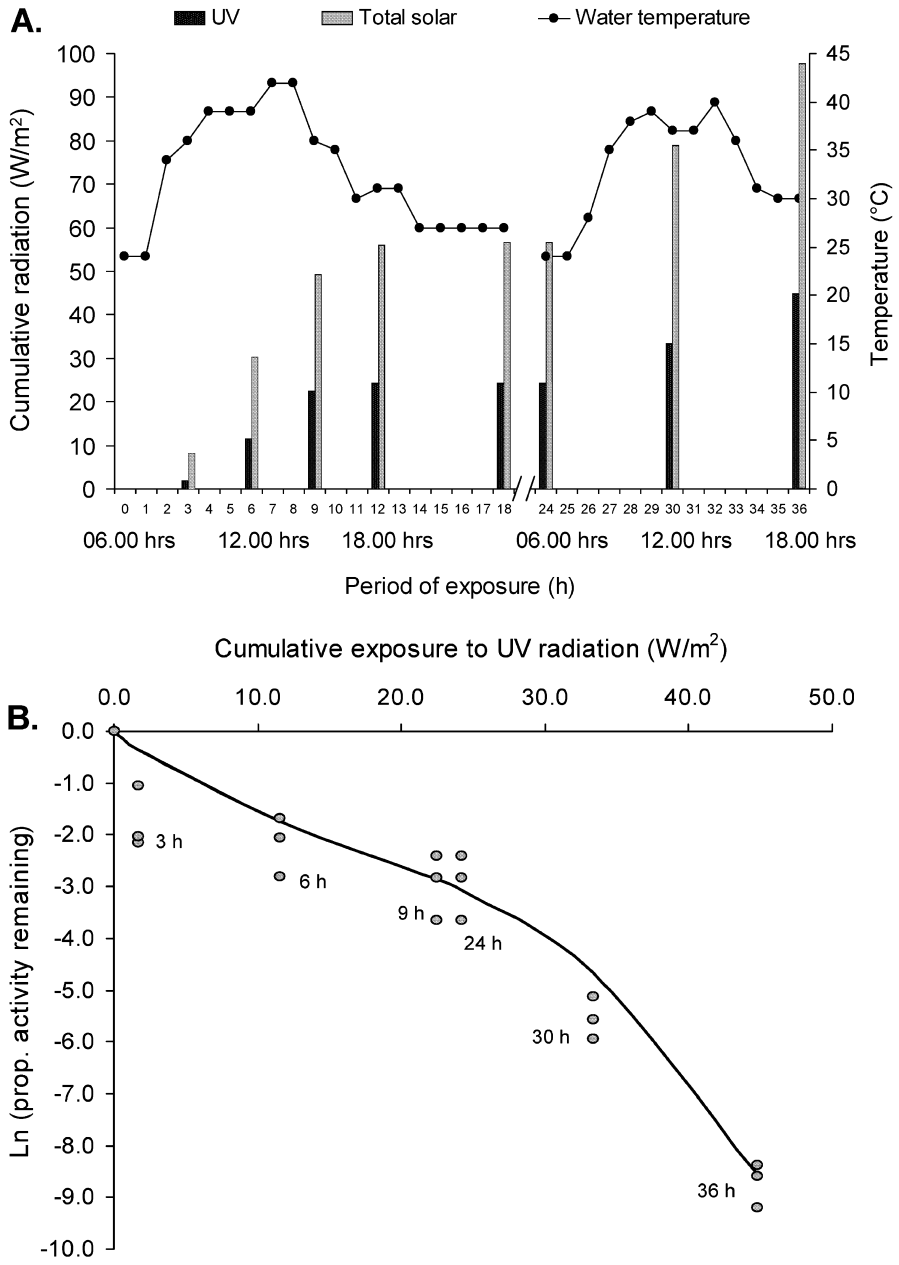

Fig. 2. **A** Cumulative exposure to total solar ($\times 10^{-2}$) and ultraviolet radiation (W/m^2) and fluctuation in water temperatures in trays exposed to sunlight during experimental period. **B** Mixed effects model of Ln (proportion of virus activity remaining) over time (18 h) for IIV-6 placed in trays in direct sunlight. The minimal significant model fitted by maximum likelihood was $y = -0.000149x^3 + 0.007168x^2 - 0.213269x$. Grey dots indicate data points of individual replicates

in an increase in water temperature. Moreover, the objective of the study was to determine IIV persistence in water under natural conditions in which solar UV radiation is invariably concurrent with an infra-red heating effect. Preliminary

experiments in trays containing clay particles indicated that daytime warming resulted in convection currents in the trays that would ensure thorough mixing and avoid sedimentation of virus particles during the day.

Solar UV has been clearly identified as a key factor in the decay of viruses in marine [14, 24] and terrestrial ecosystems [1]. The results of this study provide the first quantitative evaluation of the ability of IIVs to persist outside the host in an aquatic habitat. In contrast, studies of other families of virus pathogens of insects have almost exclusively focused on ways in which virus particles sprayed onto plants as biological pesticides can be protected from the degrading effects of solar radiation [16, 21].

The routes by which IIVs are transmitted are uncertain. Cannibalism and aggressive interactions can result in transmission in mosquito larvae [6, 9]. However, IIVs are extremely infectious by injection and parasite mediated transmission has been demonstrated in Lepidoptera [7] and in aquatic chironomid larvae exposed to virus suspensions in the presence of mermithid nematode parasites [13]. The parasites appear to introduce IIV particles during the act of host penetration, resulting in a dramatic increase in the prevalence of infection. The results of this study suggest that the probabilities of such transmission are mediated by the dose of UV radiation received and prevailing water temperatures [8] in the interval between release of virus particles from an infected insect corpse and acquisition of the infection by a susceptible host.

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References

1. Caldwell MM, Ballaré CL, Bornman JF, Flint SD, Björn LO, Teramura AH, Kulandaivelu G, Tevini M (2003) Terrestrial ecosystems, increased solar ultraviolet radiation and interactions with other climatic change factors. Photochem Photobiol Sci 2: 29–38
2. Chinchar VG, Essbauer S, He JG, Hyatt A, Miyazaki T, Seligy V, Williams T (2005) Iridoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds), Virus taxonomy: Eighth Report of the International Committee on the Taxonomy of Viruses. Elsevier, London, pp. 163–175
3. Constantino M, Christian P, Marina CF, Williams T (2001) A comparison of techniques for detecting invertebrate iridescent virus 6. J Virol Methods 98: 109–118
4. Day MF, Mercer EH (1964) Properties of an iridescent virus from the beetle *Sericesthis pruinosa*. Aust J Biol Sci 17: 892–902
5. Jakob NJ, Muller K, Bahr U, Darai G (2001) Analysis of the first complete DNA sequence of an invertebrate iridovirus: coding strategy of the genome of Chilo iridescent virus. Virology 286: 182–196
6. Linley JR, Nielsen HT (1968) Transmission of a mosquito iridescent virus in *Aedes taeniorhynchus*. I. Laboratory experiments. J Invertebr Pathol 12: 7–16
7. López M, Rojas JC, Vandame R, Williams T (2002) Parasitoid-mediated transmission of an iridescent virus. J Invertebr Pathol 80: 160–170

8. Marina CF, Feliciano JM, Valle J, Williams T (2000) Effect of temperature, pH, ion concentration and chloroform treatment on the stability of invertebrate iridescent virus 6. J Invertebr Pathol 75: 91–94
9. Marina CF, Fernández-Salas I, Ibarra JE, Arredondo-Jiménez JI, Valle J, Williams T (2005) Transmission dynamics of an iridescent virus in an experimental mosquito population: the role of host density. Ecol Entomol (in press)
10. Marina CF, Ibarra JE, Arredondo-Jiménez JI, Fernández-Salas I, Liedo P, Williams T (2003) Adverse effects of covert iridovirus infection on life history and demographic parameters of *Aedes aegypti*. Entomol Exp Appl 106: 53–61
11. Marina CF, Ibarra JE, Arredondo-Jiménez JI, Fernández-Salas I, Valle J, Williams T (2003) Sublethal iridovirus disease of the mosquito *Aedes aegypti* is due to viral replication not cytotoxicity. Med Vet Entomol 17: 187–194
12. Miocevic I, Smith J, Owens L, Speare R (1993) Ultraviolet sterilisation of model viruses important to finfish aquaculture in Australia. Aust Vet J 70: 25–27
13. Mullens BA, Velten RK, Federici BA (1999) Iridescent virus infection in *Culicoides variipennis sonorensis* and interactions with the mermithid parasite *Heleidomermis magnapapula*. J Invertebr Pathol 73: 231–233
14. Noble RT, Fuhrman JA (1997) Virus decay and its causes in coastal waters. Appl Environ Microbiol 63: 77–83
15. Ohba M, Aizawa K (1982) Mammalian toxicity of an insect iridovirus. Acta Virol 26: 165–168
16. Petrik DT, Iseli I, Montelone BA, Van Etten JL Clem RJ (2003) Improving baculovirus resistance to UV inactivation: increased virulence resulting from expression of a DNA repair enzyme. J Invertebr Pathol 82: 50–56
17. Pinheiro JC, Bates DM (2000) Mixed-Effects Models in S and S-PLUS. Springer, Heidelberg, Germany
18. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27: 493–497
19. Reyes A, Christian P, Valle J, Williams T (2004) Persistence of invertebrate iridescent virus 6 in soil. BioContr 49: 433–440
20. Ricou G (1975) Production de *Tipula paludosa* Meig. en prarie en function de l'humiditié du sol. Rev Ecol Biol Sol 12: 69–89
21. Shapiro M, Domek J (2002) Relative effects of ultraviolet and visible light on the activities of corn earworm and beet armyworm (Lepidoptera: Noctuidae) nucleopolyhedroviruses. J Econ Entomol 95: 261–268
22. Williams T (1998) Invertebrate iridescent viruses. In: Miller LK, Ball LA (eds), The Insect Viruses. Plenum Press, New York, pp. 31–68
23. Williams T, Barbosa-Solomieu V, Chinchar VG (2005) A decade of advances in iridovirus research. Adv Virus Res (in press)
24. Wilhelm SW, Jeffrey WH, Dean AL, Meador J, Pakulski JD, Mitchell DL (2003) UV radiation induced DNA damage in marine viruses along a latitudinal gradient in the southeastern Pacific Ocean. Aquat Microb Ecol 31: 1–8

Author's address: Trevor Williams, Depto. Producción Agraria, Universidad Pública de Navarra, Pamplona 31006, Spain; e-mail: trevor.williams@unavarra.es