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Sublethal effects of iridovirus disease in a mosquito

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Abstract Recognition of the importance of debilitating effects of insect virus diseases is currently growing. Commonly observed effects of sublethal infection at the individual level include extended development times, reduced pupal and adult weights, and lowered fecundity. However, for the most part, sublethal infections are assumed to be present in survivors of an inoculum challenge, rather than demonstrated to be present by microscopy or molecular techniques. Invertebrate iridescent viruses are dsDNA viruses capable of causing disease with symptoms obvious to the naked eye, a "patent" infection, that is lethal. Furthermore, inapparent "covert" infections may occur that are non-lethal and which can only be detected using bioassay or molecular techniques. In this study, replication of *Invertebrate iridescent virus 6* in *Aedes aegypti* larvae was demonstrated in the absence of patent disease. A sensitive insect bioassay (using *Galleria mellonella*) allowed the detection of covert infections, which were more common than patent infections. A concentration-response relationship was detected for the incidence of patent infections. Covert infections were up to 2 orders of magnitude commoner than patent infections, but the prevalence of covert infections did not appear to be related to virus inoculum concentration. Exposure of larvae to virus inoculum resulted in extended juvenile development times. A reduction in the mean and an increase in the variability of fecundity and adult progeny production was observed in females exposed to an inoculum challenge, although formal analysis was not possible. Males appeared capable of passing virus to uninfected females during the mating process. Covertly infected females were smaller and had shorter lifespans than control or virus-challenged females. A conservative estimate for the reduction in the net reproductive rate (R_0) of such insects was calculated at slightly more than 20% relative to controls.

Key words Inapparent virus infection · Juvenile development · Longevity · Female body size · Net reproductive rate

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Introduction

The persistence of viral infections in insect populations has recently begun to attract attention due to two factors: first, the development of sensitive molecular techniques that permit both the detection and identification of latent or sublethal infections (e.g., Hughes et al. 1993), and second, the adoption of a quantitative empirical approach to the study of entomopathogen ecology (Hochberg 1991; Dwyer 1991, 1992; Sait et al. 1994; Goulson et al. 1995; D'Amico et al. 1996). Pathogens have been recognized as major factors affecting the dynamics of insect populations (Anderson and May 1981; Myers 1988). In the search for effective agents for the biological control of insect pests, attention is almost invariably focused on virulent diseases that provoke high levels of host mortality. The debilitating effects of sublethal virus diseases have been suspected to be of importance in the reproduction and population dynamics of insects but, until recently, have been largely ignored because of the difficulty of such studies (Myers and Rothman 1996).

At the level of the individual, sublethal infection has been suspected to cause a reduction in important fitness correlates including the larval developmental rate, pupal and adult weight, egg production, and adult lifespan. Such effects are best documented for baculoviruses and insect cypoviruses (Rothman and Myers 1996). Confirmation of sublethal infection can only be reliably

achieved by observation of virus particles in host tissues or by detecting virus-specific nucleic acid sequences in infected insects (Hughes et al. 1997).

Invertebrate iridescent viruses (family *Iridoviridae*: genus *Iridovirus*) are non-occluded icosahedral double-stranded DNA (dsDNA) viruses that primarily infect insects in damp or aquatic habitats (Ward and Kalmakoff 1991; Williams 1996). Patent infections produce obvious symptoms of disease in the host that are clear to see: the insect changes color to an iridescent lilac-blue due to the accumulation of massive quantities of virus particles that assemble in crystalline arrangements in the cytoplasm of infected cells. Patent infections are invariably lethal. However, using amplification of viral DNA by the polymerase chain reaction (PCR) and insect bioassay, inapparent sublethal infections have been detected in a species of *Simulium* (Diptera: Simuliidae); over 30% of the population may be covertly infected at certain periods (Williams 1993, 1995). For reasons that are not understood, the massive proliferation of virus particles observed in patent infections does not occur in covertly infected hosts. It is not known whether these covert infections provoke disease and reduced viability of afflicted hosts.

Horizontal transmission of iridescent viruses is often believed to occur by cannibalism or predation of patently infected arthropods, as large doses of inoculum are required to produce patent infections. There is some evidence for vertical transmission in *Aedes taeniorhynchus* (Diptera: Culicidae) infected by *Invertebrate iridescent virus 3* (Linley and Nielsen 1968a, 1968b; Woodard and Chapman 1968).

This study demonstrates sublethal infection of a mosquito by a heterologous iridescent virus, and quantifies the impact of disease on the development, reproduction, and longevity of the insect host.

Methods

Organisms

Insects were obtained from laboratory cultures of *Aedes aegypti* (Diptera: Culicidae) and *Galleria mellonella* (Lepidoptera: Pyralidae) maintained in the Centro de Investigación de Paludismo and ECOSUR, Mexico, respectively. *Invertebrate iridescent virus 6* (IIV-6), originally isolated from *Chilo suppressalis* (Lepidoptera: Pyralidae), was grown by injection into *G. mellonella* larvae and stored as frozen infected insects at −20°C until required.

Patent infections of mosquito larvae are obvious because infected larvae change color to a striking opalescent lavender blue. However, the presence of virus in covertly infected mosquito larvae and adults was detected by bioassay of insect samples in third instar *G. mellonella* larvae as described previously (Williams 1993, 1995). The majority of iridescent viruses grow readily in this host and produce the distinctive color change, symptomatic of patent infection. *G. mellonella* is very sensitive to infection by iridescent viruses; the LD_{50} for IIV-6 injected into *G. mellonella* larvae has been determined as 1.92 particles (C.F. Marina, J.M. Feliciano, J. Valle and T. Williams, unpublished work), similar to previous observations made with an IIV from a coleopteran host (Day and Gilbert 1967). IIV-6 was chosen over the IIV-3 from *A. taeniorhynchus* because IIV-6 has a broad host range and replicates well in *G. mellonella*, whereas IIV-3 has a very restricted host range and will not replicate in *G. mellonella* (Williams 1998). The identity of virus in bioassay samples was confirmed by isolation of virus from infected *G. mellonella* larvae, followed by DNA extraction, phenol-chloroform purification and restriction endonuclease analysis using *Hin*d III and *Eco*RI.

Virus purification was achieved by trituration of patently infected *G. mellonella* larvae in cold sterile distilled water followed by four steps of centrifugation at 490 *g*, 960 *g*, 1250 *g*, each for 10 min, to remove insect debris, and 15,300 *g* to pellet virus. The pellet was resuspended in 300 μl of water, layered onto 30% w/v sucrose and spun at 15,300 g for 30 min. The resulting pellet was washed once and resuspended in a final volume of 1 ml of sterile distilled water.

Virus samples were quantified using a direct counting technique with latex beads of 460 nm diameter (Day and Mercer 1964; Czuba et al. 1994; C.F. Marina, J.M. Feliciano, J. Valle and T. Williams, unpublished work). All experiments were performed at 25 ± 1°C unless otherwise stated. Exposure to virus inoculum occurred in a volume of 200 ml of filtered water in all trials.

Replication of IIV-6 in *A. aegypti*

To show the presence of covert infection it was necessary to demonstrate replication of the virus in the absence of patent disease. To do this, two patently infected *G. mellonella* larvae were triturated in a volume of 2 ml of deionized water. Groups of 300 first instar *A. aegypti* larvae were exposed to the homogenate (a concentration of approximately 2.6×10^9 particles ml^{-1}) for 5 h, followed by five washes in clean chlorinated tap water. Larvae were then transferred to a clean rearing tray and fed with a powdered yeast and soya mixture. On each of the following 7 days, ten larvae were taken at random and individually homogenized in 1 ml of 0.8% aureomycin solution. The macerate was centrifuged briefly to eliminate insect debris and transferred to a sterile 1-ml syringe. Doses of 10 μl of this suspension were then injected into groups of ten *G. mellonella* larvae that were placed on semisynthetic diet and subsequently monitored for patent infections. Patently infected mosquito larvae were not observed in this experiment although all insects were followed through until pupation. Control mosquitoes were treated identically but were not exposed to virus. The experiment was performed twice.

Concentration-response relationship for patent and covert infections

Groups of 200 first instar *A. aegypti* larvae were exposed to one of five concentrations (0, 0.1, 1, 5, or 10 larval equivalents) of purified virus for 5 h. One larval equivalent, the yield of particles from one patently infected *G. mellonella* larva, was estimated to be approximately 2.6×10^{11} particles (see results section) or 1.3×10^9 particles ml^{-1} in 200 ml of water used for inoculation. The larvae were then washed five times as described above and reared through to adulthood. Daily checks were made for patent infections of these larvae. The durations of the larval and pupal stages were recorded, as was adult emergence. A total of 30 adult mosquitoes, 15 of each sex, were individually bioassayed in *G. mellonella* larvae as described previously. The experiment was replicated four times.

Effect of inoculum presentation

Previous observations had indicated that the form in which virus inocula were presented to mosquito larvae may affect the frequency of patent infection (Undeen and Fukuda 1994). In an effort to achieve elevated levels of patent and covert infection, batches of 200 first instar larvae were exposed to two larval equivalents in one of the following treatments: (a) triturated infected *G. mellonella* larvae, (b) purified virus, (c) purified virus and 0.1 g of finely ground sand, (d) purified virus and 0.1 g of finely ground sand

gently agitated using a magnetic stirrer, (e) no exposure (control). After 5 h exposure larvae were washed and reared to pupation. The presence of patent infections was noted daily. The test was performed once.

Effects of covert infection

To determine whether covert infection affected the potential reproductive success of infected individuals a number of fitness components were compared for mosquitoes exposed to virus and unexposed individuals.

Groups of 200 mosquito larvae were exposed as third instars to 2 larval equivalents of purified virus in the presence of finely ground sand and gently agitated for 5 h. Control insects were treated identically, i.e., were agitated with fine sand, but were not exposed to virus. Larvae were washed five times and reared through to adulthood as normal. Development time from first instar larva to pupa was noted, as were the duration of the pupal stage, larval and pupal mortality, the occurrence of patent virus infections, and the percentage of adult emergence. From each treatment 30 adult females were randomly selected, numerically coded, and assigned to one of four mating categories: virus-exposed females × virus-exposed males, virus-exposed females × control males, control females × virus-exposed males, or control females × control males.

After mating, each female was allowed one human blood-meal and then placed individually in a plastic cup with water and an oviposition substrate (a wooden spatula) for 48–72 h. After laying one egg batch, female mosquitoes were transferred to individual holding cups held at ambient temperatures (26–32°C) and offered sugar solution (10%) until death. Upon death, the female was individually placed in a 1.5-ml Eppendorf tube and stored at −20°C. The egg batch produced was counted and larval emergence was observed. The development time, mortality, and incidence of patent iridescent virus infections for each stage of these progeny insects were noted daily.

Dead frozen female mosquitoes were thawed, measured for wing length to an accuracy of ±0.02 mm using a standard protocol (Xue and Ali 1994) and subsequently bioassayed in *G. mellonella* as described above. In this way it was possible to confirm the presence of a covert iridovirus infection in experimental insects and thereby classify insects that had been exposed to virus as "challenged" (i.e., the uninfected survivors of an inoculum challenge) or "truly infected" (i.e., mosquitoes that had become covertly infected as a result of the inoculum challenge). Each treatment was replicated three times.

Results

Restriction endonuclease analysis confirmed that covert infections detected by insect bioassay were identical to the inoculum virus and were not due to the activation of latent infections in mosquitoes. One patently infected larva of *G. mellonella* [222.1 ± 7.7 mg wet weight, 79.5 ± 3.2 mg dry weight (mean ± SE), n = 20] was estimated to contain 2.597×10^{11} virus particles ($\pm 7.263 \times 10^{10}$ SE, n = 10), or 3.267×10^{9} particles mg^{-1} of dry insect tissue. These values are lower than previous observations (Day and Mercer 1964) because patent infections of *G. mellonella* in the present study were not allowed to progress to death.

Replication of IIV-6 in *A. aegypti*

Replication of IIV-6 in *A. aegypti* larvae in the absence of patent infection was confirmed by insect bioassay (Fig. 1). The observed infection level of 27% at 24 h post-infection (p.i.) was undoubtedly an artifact due to "carry-over" of inoculum (e.g., virus present in the insect gut) that could not be diluted away by the serial washing process. The subsequent increase in patently infected *G. mellonella* larvae from 12% infection at 2 days p.i. to 50% infection at 6 days p.i. was highly significant (χ^2 = 68.56, df = 6, P = 0.008) and can only have been due to an increase in the concentration of virus particles in mosquito tissues. All control larvae proved negative for infection in the bioassay. In this test, none of the mosquito larvae exposed to virus developed patent infections, indicating that the increase in *G. mellonella* infection shown in Fig. 1 was a result of inapparent viral replication in the mosquito rather than an early stage of what would later become a patent infection.

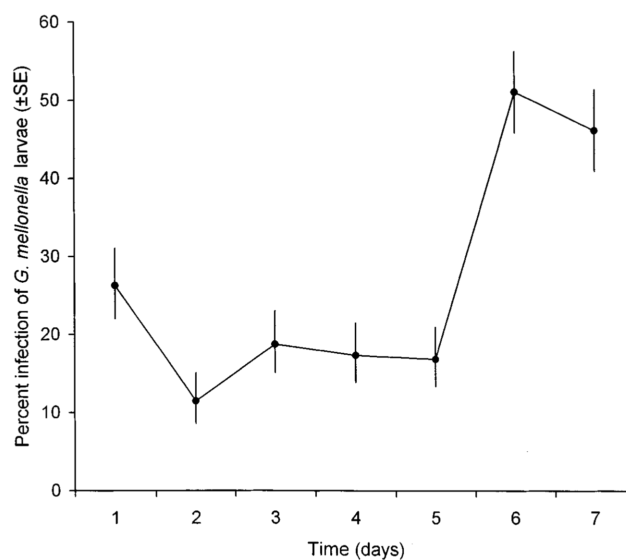

Fig. 1 Replication of *Invertebrate iridescent virus 6* (IIV-6) in *Aedes aegypti* detected by bioassay in *Galleria mellonella* larvae at 1–7 days post inoculation. *Data points* are means with SEs calculated assuming a binomial error structure

Concentration-response relationship for patent and covert infections

A consistent relationship was observed between inoculum concentration and patent virus infection (χ^2 = 12.39, df = 4, P = 0.015), although this was very low (maximum 0.65%) even at the highest concentration (Table 1). The incidence of covert infection detected in bioassays varied between 0 and 10.0% but did not appear to be related to virus concentration or the frequency of patent infections. Overall, covert infections were more common than patent infections by almost 2 orders of magnitude (Table 1).

Exposure to virus increased the duration of larval development by approximately 10% from 8.29 ± 0.05 days in control insects compared to 9.20 ± 0.04 days in larvae exposed to inoculum ($F_{4,2902}$ = 299.3, P < 0.001). There was also a significant increase in the

Table 1 Incidence of patent infection of mosquito larvae and covert infection of adult mosquitoes following exposure to different concentrations of *Invertebrate iridescent virus 6* (IIV-6) (all figures are mean percentages with range of percent infection observed in each replicate given in parentheses; *L.E.* larval equivalent)

	Control	Inoculum concentration (L.E.)			
		0.1	1	5	10
Patent infection of larvae	0	0 (0.0–0.0)	0.13 (0.0–0.5)	0.14 (0.0–0.5)	0.65 (0.0–1.5)
Covert infection of adult females	0	5.0 (0.0–13.3)	8.3 (0.0–20.0)	3.3 (0.0–6.7)	1.7 (0.0–6.7)
Covert infection of adult males	0	8.3 (0.0–20.0)	10.0 (6.7–20.0)	0 (0.0–0.0)	5.0 (0.0–13.3)

interval first instar larva to adult emergence: 10.45 ± 0.05 days for control insects and 11.35 ± 0.04 days for insects exposed to virus ($F_{4,2993} = 216.4$, $P < 0.001$) (all figures are means ± SE) (Fig. 2). There was no consistent concentration-response effect for either of these variables.

Effect of inoculum presentation

Virus formulation affected the incidence of patent disease. The highest incidence of patent infection, 4.4% was observed when virus was gently agitated in the presence of finely ground sand ($\chi^2 = 15.59$, $df = 4$, $P < 0.004$), compared to 1.4% for virus with unstirred sand, and 1.0% for macerated infected *G. mellonella* cadavers. No patent infection occurred when purified virus was offered as inoculum. This simple observation on virus formulation and patent infection lead to the selection of virus and stirred sand for the experiment described in the following section.

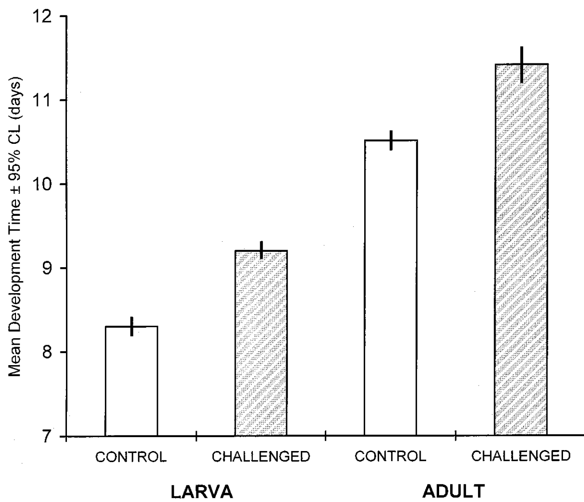

Fig. 2 Effect of exposure to virus inoculum on the development of *A. aegypti* given as duration of larval stage and duration of larval + pupal stages (*adult emergence*) (mean ± 95% C.L.)

Effects of covert infection

Mosquitoes exposed to virus inoculum showed a number of differences in selected fitness components compared to controls. Numerically, the mean egg production of IIV-exposed mosquitoes was lower than controls. This resulted in fewer larvae, pupae, and adults in insects exposed to virus and the crosses of control males or females with virus-exposed mosquitoes. However, no formal analysis was possible, because of a consistent increase in the variability and a non-normal distribution of these variables in virus treatments. Females exposed to inoculum produced 14.8–22.3% less progeny than control females (Table 2).

There was no significant difference in the frequency of females from different treatments that failed to oviposit: with between 14.9–19.9% of virus exposed females and 12.4% of control females failing to lay eggs ($\chi^2 = 2.668$, $df = 3$, $P = 0.446$). Non-ovipositing females were not dissected and the status of egg development in these females is unknown.

There was no significant difference in the tertiary sex ratio when comparisons were made among treatments or compared to a 1:1 sex ratio, with the exception of the treatment of virus-exposed females × virus-exposed males which had a significantly male biased sex ratio ($\chi^2 = 5.27$, $df = 1$, $P < 0.025$) (Table 2).

By using bioassay, it was possible to classify female mosquitoes as covertly infected or simply virus-exposed. Bioassays were performed on a total of 271 females from all treatments. In virus-exposed females, 11 cases (8.6%) of covert infection were detected and in control

Table 2 Production of eggs, adult progeny and sex ratio (proportion male) in mosquitoes exposed to IIV-6, classified according to mating treatment, or as covertly infected or exposed to inoculum but not infected (means ± SE)

Treatment	Egg production	Adult progeny	Sex ratio
Control	54.78 ± 3.15	47.22 ± 2.91	0.512
Virus-exposed female × virus-exposed male	48.50 ± 3.61	40.25 ± 3.30	0.532
Virus-exposed female × control male	46.65 ± 3.34	37.05 ± 3.14	0.520
Control female × virus-exposed male	54.15 ± 3.41	43.79 ± 3.07	0.518
Virus-exposed females	48.55 ± 2.56	39.38 ± 2.40	0.524
Covertly infected females	42.57 ± 5.96	36.67 ± 24.83	0.507

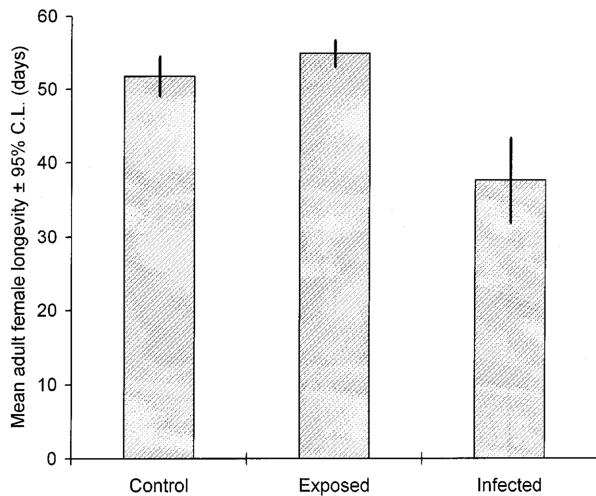

Fig. 3 Reduced longevity in covertly infected female mosquitoes compared to the uninfected survivors of a virus challenge (*exposed*) or control females (mean ± 95% C.L.)

females × virus-exposed males there were 10 virus-positive females (12.7%). Covertly infected females lived 37.8 ± 2.79 days (mean ± SE) while control females and virus-exposed but uninfected females lived 51.7 ± 1.38 and 54.7 ± 0.94 days respectively ($F_{2,197} = 22.3$, $P < 0.001$; Fig. 3). Covertly infected females were smaller (2.73 ± 0.04 mm, $n = 20$) than control (2.85 ± 0.017 mm, $n = 46$) or virus-exposed conspecifics (2.84 ± 0.016 mm, $n = 96$) in terms of wing length ($F_{2,159} = 3.89$, $P = 0.02$).

Discussion

IIV-6 replicated in larvae of *A. aegypti* and caused low levels of infection in the absence of patent disease. The shape of the replication curve (Fig. 1) is not typical of those normally observed for viruses replicating in cell culture, as there appears to be a lag in the time between inoculation and replication. However, as far as we are aware, this represents the only evidence for an insect DNA virus replicating *in vivo* leading to an inapparent infection, so a "typical" replication curve may not necessarily be expected.

There was a positive correlation between inoculum concentration and the frequency of patent infection of *A. aegypti*. Patent infections of mosquito pupae did occur, but were extremely rare; only 2 such pupae were observed out of a total of 4,400 virus-challenged insects used in the experiments described here. Covert infections were generally far more abundant than patent infections, but there was no obvious dose-response relationship between inoculum concentration and the prevalence of covert infection. Exposure to virus significantly prolonged larval development time and adult eclosion. It also caused a consistent decrease in female fecundity and progeny production although in all cases an increase occurred in the variability of these parameters relative to controls. Sublethal infection reduced the size and longevity of infected females.

The observation of covert infections in control females crossed with virus-exposed males is intriguing and suggests that males may be able to participate in virus transmission via infected sperm or seminal fluids. This observation contrasts with that of Hembree (1979), who asserted that males of *A. taeniorhynchus* did not participate in the vertical transmission of IIV-3, although diagnosis of infection in progeny larvae was restricted to patent infection and no sublethal effects were considered.

The bioassay technique used here is subject to the criticism that the possibility of covert infection of *G. mellonella* was not taken into account. However, with an LD_{50} of just 2 virus particles (C.F. Marina, J.M. Feliciano, J. Valle and T. Williams, unpublished work), the technique is so sensitive that the frequency of inapparent infections in *G. mellonella* seems likely to be small. An alternative technique, the use of cell culture plaque assay, has been shown to be approximately 10^5 times less sensitive than the insect bioassay (Czuba et al. 1994).

Previous reports had noted that covert iridescent virus infections of *Simulium variegatum* were not lethal and a number of genetically distinct strains, possibly distinct virus species, were isolated from natural covert and patent infections (Williams 1993, 1995). However, laboratory rearing of simuliids is extremely difficult, so that studies of the symptoms of natural sublethal disease were not possible.

Whereas many similar studies declare that changes in fitness correlates observed in survivors of an inoculum challenge are due to sublethal infection, very few demonstrate infection per se. A number of alternative explanations are just as, if not more, probable: for example, that such effects result from costs incurred fighting viral infection or that selection of resistant individuals from the experimental population has occurred, and that resistance is correlated with changes in a number of fitness correlates (Fuxa and Richter 1989; Milks 1997). In the case of iridescent viruses, an additional explanation may be that viral proteins exhibit direct cytotoxic effects which are not dependent on viral replication or gene expression (Lorbacher de Ruiz 1990). Exposure to virions elicits a rapid shutdown of host cell macromolecular synthesis. This occurred within 1 h of exposure of mosquito cells to IIV-6 and the rate of shutdown was dependent on the multiplicity of infection. A soluble, heat-stable viral protein is believed to be responsible for this effect (Cerutti and Devauchelle 1990). Production of viral macromolecules is not affected by the shutdown process.

Results from studies with cypoviruses or nucleopolyhedroviruses showed a reduction of 32–34% in the net reproductive rate (R_0) of Lepidoptera due to debilitating effects of sublethal infection, whereas sublethal effects caused a reduction of 5–25% in R_0 for insects inoculated by granuloviruses, entomopoxviruses,

and a number of insect small RNA viruses (Rothman and Myers 1996). Generally, there was little evidence for debilitating effects as being dose-dependent for any virus, whereas insect instar was important in the probability of debilitating effects for nucleopolyhedroviruses, but not for cypoviruses. Only one-third of the studies analyzed by Rothman and Myers (1996) confirmed the presence of virus infection in test insects, which calls for caution when speculating upon possible causes of changes in R_0 values.

In this study it was possible to distinguish between the effects observed in survivors of an inoculum challenge and those of truly infected insects. Covert infection was associated with a reduction in net progeny production (R_0) of approximately 22% (mean offspring per female was 36.67 in covertly infected mosquitoes and 47.22 in controls). This value is similar to those observed in Lepidoptera sublethally infected with other insect DNA and RNA viruses (Rothman and Myers 1996). We would argue that this is a conservative estimate as females in this study were only given one oviposition opportunity. It seems probable that lifetime fecundity would be further reduced due to a combination of lower egg production and shortened lifespan, leading to a marked reduction in R_0 in covertly infected female mosquitoes.

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