El Colegio de la Frontera Sur

Symptoms of Iridovirus Disease in *Aedes aegypti*

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Symptoms of Iridovirus Disease in a Mosquito

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SUMMARY

Recognition is currently growing as to the importance of debilitating effects of insect virus diseases. At the individual level, commonly observed effects of sublethal infection include extended development times, reduced pupal and adult weight and lowered fecundity. At the population level, such effects may translate into changes in the periodicity and amplitude of population cycles and a lowering of the mean population density. 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 patent, lethal disease and covert, non-lethal infection. In the present 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, whereas the frequency of covert infections was not dependent on the inoculum concentration. Exposure 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 R_0 of such insects was calculated at slightly more than 20% relative to controls.

1. 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). 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 & May 1981; Myers 1988) and in the search for effective agents for the biological control of insect pests, attention is invariably focused on virulent diseases that provoke high levels of host mortality. However, the debilitating effects of sublethal virus diseases are newly recognized as being influential in the reproductive rate and population dynamics of insects (Myers & 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 & 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 dsDNA viruses that primarily infect insects in damp or aquatic habitats (Ward & Kalmakoff 1990; Williams 1996). Patent infections are lethal, but using PCR and insect bioassay, 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). It is not known whether these 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 iridescent virus 3 (Linley & Nielsen 1968a,b; Woodard & Chapman 1968).

The present study presents evidence of sublethal infection of a laboratory mosquito by a heterologous iridescent virus and quantifies the impact of disease on the development, reproduction and longevity of the insect host.

2. METHODS

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

The presence of virus in 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, including iridescent virus 6, readily grow in this host and produce a distinctive iridescent lavender blue colour change, symptomatic of patent infection. 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 *Hind* III and *Eco*RI compared against published profiles (Williams & Cory 1994; Williams 1994).

Virus samples were quantified using a direct counting technique with latex beads of 460nm diameter (Day & Mercer 1964; Czuba *et al.* 1994). All experiments were performed at 25±1°C unless otherwise stated. Exposure to virus inoculum occurred in a volume of 200ml of filtered water in all trials.

i) Replication of iridescent virus 6 in *Ae. aegypti*

A proportion of *Ae. aegypti* larvae exposed to high concentrations of iridescent virus 6 developed lethal infections, but to show the presence of covert infection it was also necessary to demonstrate replication of the virus in the absence of patent disease. To do this, groups of 300 first instar larvae were exposed to 2mg/ml of semi-purified virus for 5 hours, followed by 5 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, 10 larvae were taken at random and individually homogenized in 1ml of 0.8% aureomycin solution. The macerate was centrifuged briefly to eliminate insect debris and transferred to a sterile 1ml syringe. Doses of 10μl of this suspension were then injected into groups of 10 *G. mellonella* larvae which were placed on

semisynthetic diet and subsequently monitored for patent infections. Patently infected individuals were immediately removed from the rearing tray. Control mosquitoes were treated identically but were not exposed to virus. The experiment was repeated once.

ii) Concentration-response relationship for patent and covert infections

Groups of 200 first instar *Ae. aegypti* larvae were exposed to one of five concentrations (0, 0.1, 1, 5 or 10 larval equivalents) of purified virus for five hours. The larvae were then washed 5 times as described above and reared through to adulthood. Daily checks were made for patent infections of these larvae. The duration of the larval and pupal stages were recorded and was adult emergence. Thirty adult mosquitoes of each sex were individually bioassayed in *G. mellonella* larvae as described previously. The experiment was replicated four times.

iii) Effect of inoculum presentation

Previous observations had indicated that the form in which virus inocula were presented to larvae may affect the frequency of patent infection (Undeen & 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) homogenized infected *G. mellonella* larvae, b) semi-purified virus, c) semi-purified virus and 0.1g of finely ground sand, d) semi-purified virus and 0.1g of finely ground sand gently agitated using a magnetic stirrer, e) nothing (control). After 5 hours exposure larvae were washed and reared to pupation. The presence of patent infections was noted daily. The test was performed once.

iv) 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.

Mosquito larvae were exposed as third instars to 2 larval equivalents of semi-pure virus in the presence of finely ground sand and gently agitated for 5 hours. Control insects were treated identically, i.e. were agitated with fine sand, but were not exposed to virus. Larvae were washed 5 times and reared through to adulthood as normal. Development time 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 x virus-exposed males, virus-exposed females x control males, control females x virus-exposed males, or control females x 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 48-72h. Female mosquitoes were subsequently 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 1.5ml Eppendorf tube and stored at -20°C. The first egg batch produced was counted and larval emergence was noted. 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 ±0.02mm using a standard protocol (Xue & 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" or "truly infected". Each treatment was replicated three times.

3. RESULTS

Restriction endonuclease anaylsis 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 larva of *G. mellonella* (218 ± 5.0mg mean weight ± SE, n=35) was estimated to contain 5.11×10^{12} virus particles ($\pm 1.44 \times 10^{11}$ particles standard error), or 2.34×10^{10} particles per mg of insect tissue, values similar to previous observations (Day & Mercer 1964).

i) Replication of iridescent virus 6 in *Ae. aegypti*

Replication of IV6 in *Ae. aegypti* larvae in the absence of patent infection was confirmed by insect bioassay (Fig. 1). The 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 ($\chi^2 = 68.56$, d.f. = 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.

ii) Concentration-response relationship for patent and covert infections

A consistent relationship was observed between inoculum concentration and patent virus infection ($\chi^2 = 12.39$, d.f.$=4$, $P=0.015$), 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 2.43% 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 (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 interval larva-adult 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.

iii) 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 ($\chi^2 = 15.59$, d.f$=4$, $P<0.004$), compared to 1.4%

for virus with unstirred sand, 1.0% for macerated infected *G. mellonella* cadavers. No patent infection occurred when semi-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.

iv) Effects of covert infection

Mosquitoes exposed to virus inoculum showed a number of differences in selected fitness components compared to controls. Numerically, the mean fecundity of IV 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 due to a consistent increase in the variability and a non-normal distribution of these variables in virus treatments (Fig. 3). Overall, 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 ($\chi^2 = 2.668$, d.f.=3, P=0.446). There was also 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 x virus-exposed males which had a significantly male biased sex ratio ($\chi^2 = 5.27$, d.f. = 1, $P < 0.025$) (Table 2).

By way of 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 females x virus-exposed males there were 10 virus-positive females (12.7%). Covertly infected females lived 37.8 ± 2.79 days (mean $\pm$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. 4). Covertly infected females were smaller (2.73 $\pm$ 0.04 mm) than control (2.85 ± 0.017 mm) or virus-exposed conspecifics (2.84 ± 0.016 mm) in terms of wing length ($F_{2,159} = 3.89$, d.f. = 2, $P = 0.02$).

4. DISCUSSION

Invertebrate iridescent virus 6 replicated in larvae of *Ae. agypti* and caused low levels of infection in the absence of patent disease. There was a positive

correlation between inoculum concentration and the frequency of patent infection of *Ae. aegypti*. Covert infections were generally more abundant than patent infections but were not sensitive to inoculum concentration. 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 *Ae. taeniorhynchus* did not participate in the vertical transmission of IV3 although diagnosis of infection in progeny larvae was restricted to patent infection and no sublethal effects were considered.

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 if not more probable. First, that such effects result from costs incurred fighting viral infection or second, 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 & Richer 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 hour of exposure of mosquito cells to IV6 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 (Cerrutti & 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 R_0 values 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 & 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 was not so for cypoviruses. Only one third of the studies analyzed by Rothman & Myers 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 the present study it was possible to distinguish between the effects observed in survivors of an inoculum challenge and those of truly infected insects. Covert infection resulted in a reduction in mean progeny production of more than 20%, a value similar to those observed in Lepidoptera sublethally infected with other insect DNA and RNA viruses (Rothman & 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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Table 1. Incidence of patent infection of mosquito larvae and covert infection of adult mosquitoes following exposure to different concentrations of IV6 (all figures are mean percentage).

	Inoculum concentration (L.E.)				
	Control	0.1	1	5	10
Patent infection of larvae	0	0	0.13	0.14	0.65
Covert infection (adult females)	0	0.54	0.89	0.39	0.18
Covert infection (adult males)	0	0.92	2.43	0	0.52

Table 2. Production of eggs, adult progeny and sex ratio (proportion male) in mosquitoes exposed to IV6, 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 x virus-exposed male	48.50 ± 3.61	40.25 ± 3.30	0.532
Virus-exposed female x control male	46.65 ± 3.34	37.05 ± 3.14	0.520
Control female x virus-exposed male	54.15 ± 3.41	43.79 ± 3.07	0.518
Virus-exposed (all females)	48.55 ± 2.56	39.38 ± 2.40	0.524
Covertly infected	42.57 ± 5.96	36.67 ± 24.83	0.507

Figure Legends

Fig. 1 Replication of IV6 in *Ae. aegypti* detected by bioassay in *G. mellonella* larvae at 1 to 7 days post inoculation. Data points are means with standard errors caculated assuming a binomial error structure.

Fig. 2 Effect of exposure to virus inoculum on the development of *Ae. aegypti* given as duration of larval stage and duration of larval+pupal stages (adult emergence).

Fig. 3 Egg production by females exposed to virus inoculum or crossed with virus-exposed males showing increase of variation in fecundity in virus-exposed insects. Points indicate mean, boxes indicate standard error and lines indicate interquartile range.

Fig. 4 Reduced longevity in covertly infected female mosquitoes compared to virus-exposed and controls. Points, boxes and lines as in figure 3.

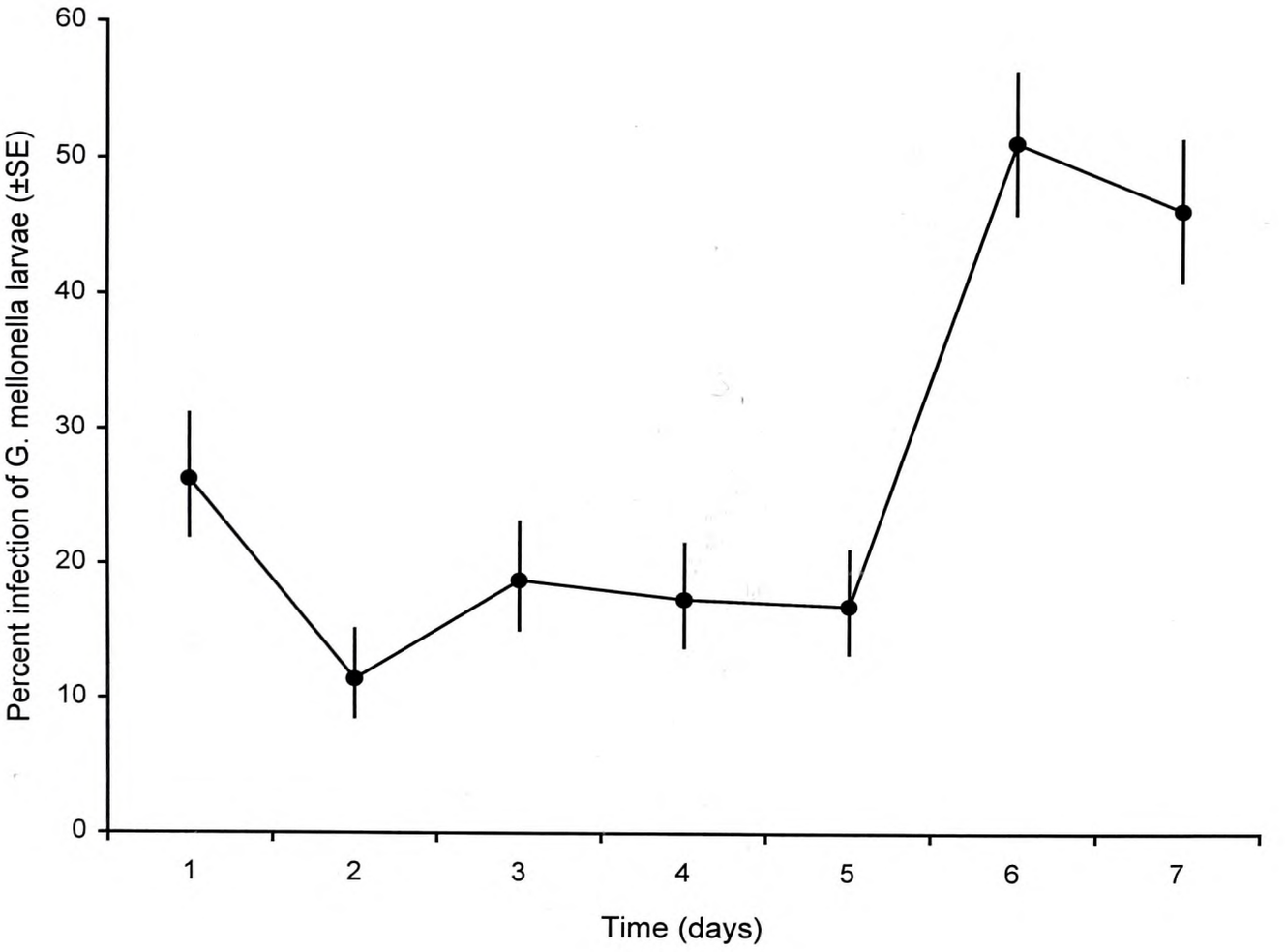

Percent infection of G. mellonella larvae (±SE)
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Time (days)

Fig 1.

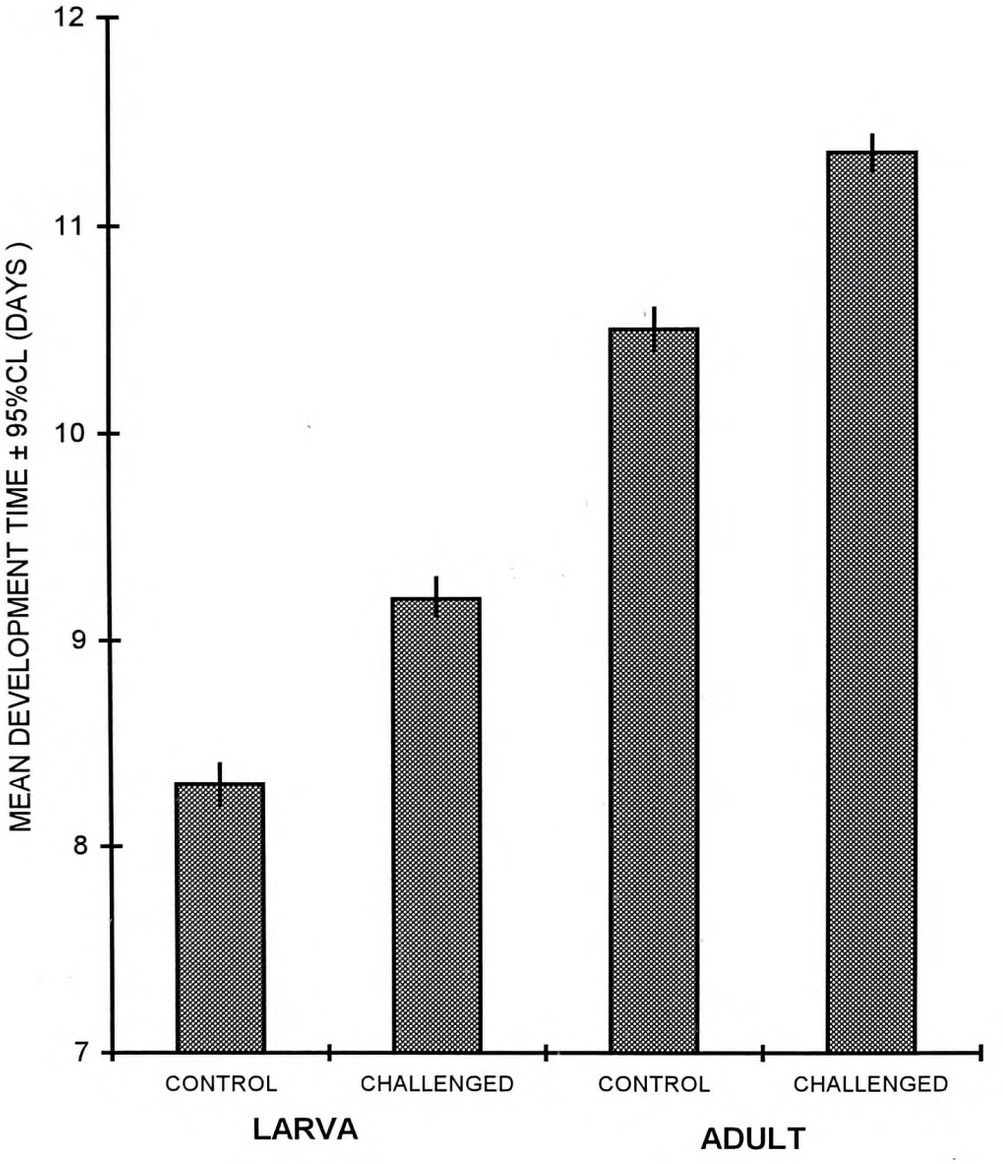
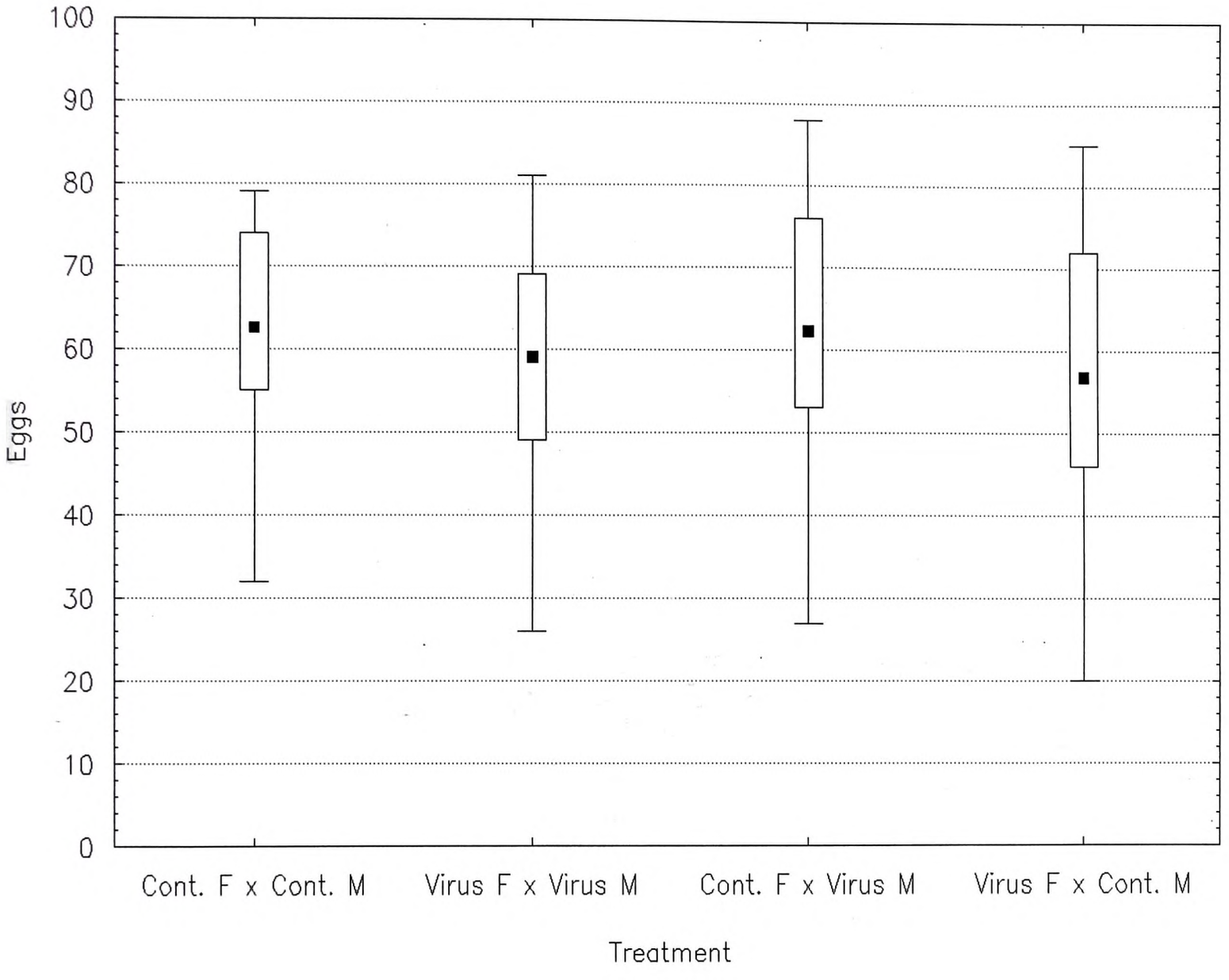

12
11
10
9
8
7
MEAN DEVELOPMENT TIME ± 95%CL (DAYS)
CONTROL
CHALLENGED
CONTROL
CHALLENGED
LARVA
ADULT

Fig. 2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90
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70
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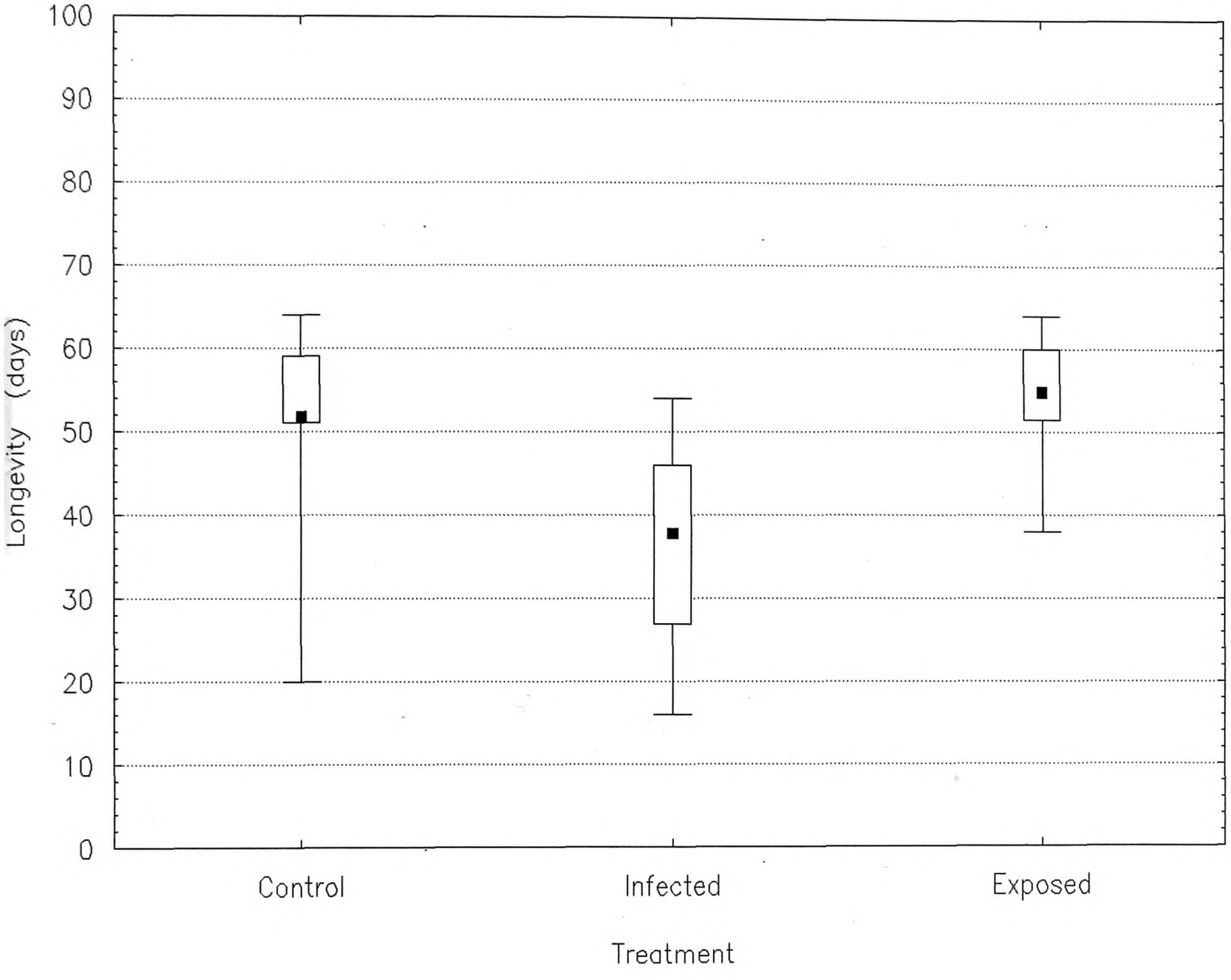
Eggs
Cont. F x Cont. M
Virus F x Virus M
Cont. F x Virus M
Virus F x Cont. M
Treatment

Fig 3


100
90
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40
30
20
10
0
Longevity (days)
Control
Infected
Exposed
Treatment

Fig 4