

**Investigating the potential of
an ascovirus for biological
control of Diamondback Moth
(DBM), *Plutella xylostella***

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Final Report

Investigating the potential of an ascovirus for biological control of Diamondback Moth (DBM), *Plutella xylostella*

VG06044 (31 October 2009)

Sassan Asgari and Mike Furlong
The University of Queensland

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Media Summary

Diamondback moth (DBM), *Plutella xylostella*, the most destructive pest of Brassica crops, has an unrivalled ability to develop resistance to insecticides and its sustainable management relies on the development of flexible IPM strategies centred on key natural enemies. In this study, the interactions between DBM and an insect pathogenic virus that belongs to the family *Ascoviridae* were investigated. Ascoviruses have predominantly been isolated from larvae of lepidopteran species that belong to the family Noctuidae and are mainly transmitted by parasitoid wasps in the field. The virus, which was originally isolated from *Helicoverpa armigera* larvae, infected DBM and cabbage cluster caterpillar (CCC; *Crociodolomia pavonana*) larvae in the laboratory. It caused mortality in larvae of both species, reduced larval feeding and inhibited the development of pupae. When late instars were infected pupae formed but adult eclosion rates were significantly reduced. The ascovirus was successfully transmitted between susceptible DBM larvae by the common parasitoid *Diadegma semiclausum*, with no detrimental effects on the development or fecundity of the wasp. However, studies indicated that the virus does not replicate efficiently in DBM; this severely impacts on the transmission efficiency of the parasitoid and successful transmission was only recorded when *D. semiclausum* attacked susceptible hosts immediately after oviposition in late instar larvae inoculated with the virus ≥ 6 days previously. In order to fully assess the potential of the virus to contribute to the biological control of DBM further studies under field condition are required to investigate the ecological constraints on both the virus and the parasitoid.

Technical summary

An ascovirus, which was originally isolated from *Helicoverpa armigera* larvae, was characterized at the molecular level and identified as a strain of *Heliothis virescens* ascovirus (HvAV-3e). The main route of ascovirus transmission to susceptible hosts is by parasitoid wasps which mechanically transfer virus particles from infected to healthy larvae. In the laboratory, HvAV-3e was manually transferred to diamondback moth (DBM; *Plutella xylostella*) and cabbage cluster caterpillar (CCC; *Crociodolomia pavonana*) larvae. Pathology studies showed that the virus is able to cause ascovirus-associated pathology in the larvae of both species. Subsequently, several isolates of the virus, collected from various locations in southeast Queensland, were subjected to molecular analysis and pathogenicity tests. Three main isolates (1-3) were identified and these were tested in dose-response bioassays under laboratory conditions. Isolate 1 was found to cause the highest level of mortality in DBM and CCC when larvae were manually inoculated. In addition, feeding damage caused by the larvae inoculated with HvAV-3e was significantly reduced compared to those inoculated with water as control. Ascovirus infection also affected larval weight gain with virus-infected larvae gaining significantly less weight than control larvae.

Since ascoviruses are transmitted by parasitoid wasps in the field, we tested the feasibility of transmission of HvAV-3e from larvae manually inoculated with the virus to healthy larvae by parasitoid wasps. Two endoparasitoid wasps were tested, *Diadegma semiclausum* (Hym: Ichneumonidae) and *Apanteles ippeus* (Hym: Braconidae). Of these, *D. semiclausum* was found to be the more efficient vector of the virus. Examination at the pathological and molecular levels confirmed that the virus can be transmitted from infected larvae to healthy larvae. However, following transmission to healthy larvae virus replication was found to be limited when compared

to its replication in conventional hosts from the family Noctuidae. As a result, the alternative hosts did not produce enough virus particles to initiate a full blown infection. Despite the fact that ascoviruses have been shown to have detrimental effects on parasitoid progeny in other systems, our investigations demonstrated that HvAV-3e does not significantly affect either the development or the fecundity of *D. semiclausum*. In choice experiments, female *D. semiclausum* wasps showed no preference for either ascovirus infected or uninfected host larvae as oviposition sites.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella*, is a notorious pest of crucifer vegetables which causes huge economic crop losses in Australia and worldwide. During the last decade, several new insecticides (eg indoxacarb, chlorfenapyr, spinosad) have been registered for the control of DBM in Australia, providing valuable substitutes for older, outdated chemicals and practical alternatives for rotation with formulations of *Bacillus thuringiensis* (Bt). These compounds, including Bt, are most effectively employed against DBM when incorporated into integrated pest management (IPM) strategies which utilize the capacity of endemic natural enemies to suppress pest populations. DBM has developed field resistance to all groups of chemical insecticides currently used for its management, including δ -endotoxins of Bt and, more recently, to spinosad and indoxacarb in Hawaii and Malaysia, respectively. Such developments seriously threaten the continued field efficacy of these compounds in these regions and serve to emphasize the necessity for the continued development of alternative control measures, resistance management strategies and innovative, flexible IPM programs in Australia.

Ascoviruses are large, double stranded DNA viruses that infect Lepidoptera (Federici, 1983b). They cause a chronic, fatal infection in the larvae of host species and the typical symptoms of infection are loss of appetite, stunted growth and a milky hemolymph (Federici, 1983b; Hamm *et al.*, 1985b). Upon infection, host cells enlarge and eventually cleave into small vesicles that contain virus particles. The milky appearance of the hemolymph is due to the liberation of these vesicles that are released from infected cells (Federici, 1983b; Federici & Govindarajan, 1990b; Federici *et al.*, 1990). Ascoviruses have a relatively low transmissibility via ingestion (Govindarajan & Federici, 1990) and the primary mode of transmission is by parasitoid wasps during oviposition. To date, there are 5 recognised species of ascovirus: *Spodoptera frugiperda* ascovirus (SfAV-1), *Trichoplusia ni* ascovirus (TnAV-2), *Heliothis virescens* ascovirus (HvAV-3), *Diadromus pulchellus* ascovirus (DpAV-4) and *Spodoptera exigua* ascovirus (SeAV-5) (Federici *et al.*, 2005). Four of these species (SfAV-1, TnAV-2, HvAV-3 and SeAV-5) are phylogenetically closely related and have predominantly been reported from the Noctuidae. The fifth species, DpAV-4, is the only AV named after its parasitoid vector, *D. pulchellus*, and infects pupae of its host *Acrolepiopsis assectella* (Lepidoptera: Plutellidae) (Bigot *et al.*, 1997).

In this project, we sought to determine if DBM and cabbage cluster caterpillar (CCC; *Crociodolomia pavonana*) are susceptible to HvAV-3e. Based on the outcome, we then examined the effects of infection on the pests and the DBM-specific parasitoid, *Diadegma semiclausum* and investigated whether *D. semiclausum* could transmit the virus between infected and healthy individuals.

2. Materials & Methods

A series of experiments was carried out to investigate the effect of *Heliothis virescens* ascovirus (HvAV-3e), originally isolated from *Helicoverpa armigera* larvae in southeast Queensland, on DBM and CCC. Isolates of HvAV-3e were also analysed at the molecular level and their efficacies were investigated in dose-response assays to determine the most virulent strain.

2.1. Insects

A laboratory culture of *P. xylostella* was established from larvae collected in an experimental broccoli crop in the Lockyer Valley, southeastern Queensland. Insects were reared on leaves of glasshouse grown potted cabbage plants (*B. oleracea* var. *capitata* cv Warrior) in ventilated plastic containers (30 by 20 by 15 cm) in a controlled temperature cabinet (23 ± 2 °C, photoperiod of 12:12 [L:D] h, 60% RH). Pupae were collected from containers and stored at 4 °C for a maximum of 14 d before they were transferred to an oviposition cage (60 × 45 × 45 cm) for emergence. Adults were fed on aqueous honey solution [10% (wt: vol)] and supplied with fresh potted cabbage plants for oviposition daily.

A laboratory culture of *C. pavonana* was established from larvae collected in a cabbage crop in the Lockyer Valley in south-east Queensland, Australia and maintained in an incubator under controlled conditions (25 ± 2 °C; L:D 12:12hr). Larvae were reared in ventilated plastic boxes (15 × 20 × 30 cm) lined with tissue paper and supplied with fresh glasshouse grown common cabbage plants (*Brassica oleracea* var Warrior) daily. Waste leaf material was removed daily and tissue paper was replaced as required. Pupae, which formed between layers of tissue paper, were collected within 1-2 days of formation and transferred to Petri dishes (9 cm diameter) to develop. Just before eclosion (5-6 days at 25°C), approximately 50 pupae were placed in the bottom of a Petri dish (9 cm diameter) and transferred to the base of an oviposition cage (50 cm × 50 cm × 50 cm) containing a potted cabbage plant for oviposition and 10% honey solution as a food source. Once oviposition commenced, potted plants on which egg masses were laid were removed from the cage and replaced with fresh plants. Egg masses were allowed to hatch *in situ* and neonate larvae fed on the plant until they reached the 2nd instar (L2), at which point the cabbage was cut off below the first leaf and transferred to a ventilated container (15 × 20 × 30 cm) lined with tissue paper and fed on fresh cabbage leaves as described above.

Diadegma semiclausum and *Apanteles ippeus* pupae were also collected from an experimental broccoli crop in the Lockyer Valley. Upon emergence, adults were released into oviposition cages (60 × 45 × 45 cm) and supplied with aqueous honey solution [10% (wt: vol)] as a food source. Cabbage plants infested with approximately 200 2nd (L2) and 3rd (L3) instar larvae were placed in the cages and 6-10 female wasps were left to forage for 24-48 h. The plants were then cut at the base and the parasitised larvae reared in ventilated plastic boxes as described above.

2.2. Virus

Isolates of *Heliothis virescens* ascovirus (HvAV-3e) were isolated from *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) collected from cotton farms in southeast Queensland. Three main isolates were identified among those and are referred to as isolates 1-3. Isolate-1 was the original isolate previously reported (Asgari *et al.*, 2007).

Laboratory stocks of each HvAV-3e isolate were amplified in *Spodoptera litura* F. (Lepidoptera: Noctuidae) larvae. The inoculum used for subsequent infections was produced by collecting the hemolymph from *S. litura* larvae 6-7 days post ascovirus infection. To collect hemolymph, larvae were surface sterilized in 70% ethanol and dried. One of the prolegs was cut using a pair of fine forceps and the hemolymph was collected in an Eppendorf tube (1.5 ml) and then stored at -80°C until used in experiments.

2.3. Ascovirus DNA isolation and RFLP analysis

Thirty *H. armigera* L3 larvae were inoculated with HvAV-3e by dipping a fine needle into the virus inoculum and pricking larvae. Seven days after inoculation hemolymph containing viral vesicles was collected from the larvae previously described. In separate extractions, total genomic DNA was isolated from the collected hemolymph and a sample of the original inoculum using a QIAamp DNA mini kit (Qiagen). Subsequently, a restriction fragment length polymorphism (RFLP) analysis was conducted on each of the HvAV-3e isolates. Four µg of viral genomic DNA was used in single enzyme digests with both *Bam*HI and *Hind*III restriction enzymes. The digests were run overnight on an 8% agarose gel containing ethidium bromide and viewed under UV light.

2.4. Ascovirus dose response assays

The concentration of virus-containing vesicles in stock solutions of each of the three ascovirus isolates was determined by making a 1000-fold dilution of viral stock in sterile water and then counting the virus containing vesicles (vcv) in the solution in a hemocytometer (strain-1= 3.3×10^8 vcv ml⁻¹; strain-2= 2.7×10^8 vcv ml⁻¹; strain-3= 1.0×10^9 vcv ml⁻¹). Five 10-fold dilutions of the viral stock of each isolate were then made in sterile water and used in the assays. Fourth instar (L4) larvae were anaesthetised on a cooling table (surface temperature -2 °C) and then individuals were inoculated with one test concentration by pricking the larva along its dorsal surface with a UV-sterilised minuten pin that had been dipped into the inoculum. For isolates 1 and 2, 60 insects were treated with each test concentration and a further 60 were treated with sterile water in the same manner to serve as a control. For isolate 3, 30 insects were treated with each test concentration and sterile water as the control. Treated and control larvae were reared in groups of 10 in Petri dishes (9 cm diameter) containing a moistened filter paper and incubated (25 ± 2 °C; L:D 12:12 h). Larvae were supplied with fresh cabbage leaf as required and monitored daily until death or the eclosion of adult moths.

2.5. Effect of ascovirus infection on DBM and CCC larval feeding and growth

One hundred and fifty L2 DBM larvae were selected without bias from a rearing box. They were randomly split into groups of 5 larvae and each group was then weighed. Fifty L2 were inoculated with HvAV-3e isolate-1 as described above, 50 were treated with sterile water and the remaining 50 were left untreated. Each group of 5 larvae was then placed in a Petri dish (3.5 cm diameter) containing 5 ml of 2% (w/v) tap-water agar and a weighed freshly cut common cabbage leaf disc (2 cm diameter). Each day each group of 5 larvae and the leaf disc upon which they had been feeding was weighed. Larvae were then returned to the Petri dish and supplied with a fresh, weighed leaf disc. The experiment was terminated when larvae ceased feeding and entered the pre-pupal stage.

The effect of ascovirus infection on the fate of L2 CCC larvae was also investigated. Second instar *C. pavonana* larvae were selected from infested cabbage plants. Batches of larvae were anaesthetised on ice and then 25-50 insects were inoculated with ascovirus or pricked with a sterile minuten pin as described above. An additional group of 15 L2 larvae, which were anaesthetised but not exposed to ascovirus or wounding, were set up as an untreated control. Batches of 5 larvae from each of the controls and the ascovirus treatment were reared in a Petri dish (9 cm diameter) containing a moistened filter paper and fresh cabbage leaves. Larvae were incubated (25±2°C; L:D 12:12hr) and monitored daily until death or the emergence of adult moths.

2.6. Effect of larval infection on adult DBM fecundity

One hundred female and 100 male DBM L4s (sex determined by the presence or absence of visible male gonads) were selected from a rearing box. Sexed larvae were then separated into single sex cohorts of 10 insects and held in Petri dishes (9 cm diameter) lined with a moistened filter paper. From these cohorts 20 female L4s and 50 male L4s were left untreated, 20 of each sex were treated with sterile water and 60 female L4s and 30 male L4s were inoculated with isolate-1 as described above. Larvae were supplied with a portion of fresh common cabbage leaf and incubated at (25 ± 2 °C; L:D 12:12 h). Insects were monitored daily, mortality was recorded and larvae were supplied with fresh leaves as required. As soon as larvae developed to the pupal stage they were removed from the Petri dish, weighed and transferred singly to Petri dishes (5 cm diameter). Upon emergence female insects from each treatment were paired with a male insect from the control treatment in a clean Petri dish (5 cm diameter) and supplied with a small ball of cotton wool saturated with aqueous honey solution [10% (wt:vol)] as a food source and a common cabbage leaf disc (4.5 cm diameter) as an oviposition substrate. After 24 h the leaf disc was removed and replaced with a fresh leaf disc. The number of eggs laid upon the first leaf disc was then counted under a binocular microscope. This process was repeated daily until the death of each female moth when the total number of eggs laid by each during its lifetime was calculated.

2.7. Effect of larval infection on host selection by *D. semiclausum*

The preference of *D. semiclausum* female wasps for AV-inoculated or non-infected healthy larvae was investigated in choice tests. One hundred and fifty L2 DBM larvae were inoculated with HvAV-3e isolate-1 by pricking with an inoculated minuten pin as described above. Single larvae were then transferred to single Petri dishes (3.5 cm diameter) containing a small (2.0 cm diameter) cabbage leaf disc and incubated (20 °C; L:D 12:12 h). One hundred and fifty untreated L2 larvae were also transferred singly to Petri dishes (3.5 cm diameter) containing a small (2.0 cm) cabbage leaf disc and incubated under the same conditions. After 3 days, 50 inoculated larvae and 50 untreated larvae were randomly selected from each group. The abdomen of each untreated larva was then delicately marked with a fine-pointed red coloured permanent marker pen so that they could be readily identified. Fifty Petri dishes (5.0 cm diameter) were lined with a filter paper and furnished with a cabbage leaf disc (3.0 cm diameter). A single AV-treated larva and a marked untreated larva were then introduced into five of the dishes and they were arranged on a white plastic tray on the laboratory bench and larvae were allowed to establish feeding sites (approximately 10 min after introduction). A single mated *D. semiclausum* female (1-3 days post eclosion) was then introduced into each dish and observed by two experimenters for a maximum of 10 min. The first larva to be attacked by each *D. semiclausum* in each dish was recorded. Dishes in which the parasitoid did not attack a larva within 10 min were discarded. Each parasitoid was used only once and all were discarded at the end of the experiment. The following day, four days after inoculation of the larvae, the experiment was repeated with another 50 of the inoculated and 50 of control larvae and a fresh batch of parasitoids. Six days after the inoculation of larvae the experiment was conducted for a third time using the remaining larvae and a fresh batch of parasitoids.

2.8. Transmission assay

Approximately 30 L2 DBM larvae were inoculated with HvAV-3e isolate-1 and 30 control larvae

were treated with sterile water as previously described. Larvae were transferred in groups of 5 to Petri dishes (9 cm diameter) containing a portion of cabbage leaf and a moistened filter paper and incubated (20 °C; L:D 12:12 h) for 6 days. Ten surviving inoculated larvae (infection confirmed by examination under a light microscope on completion of the experiment) and 10 surviving control larvae were selected and then transferred singly to Petri dishes (5 cm diameter) which contained a portion of cabbage leaf. A single newly emerged mated *D. semiclausum* female was then introduced to each dish and observed until the provided host was attacked. The parasitoid was then immediately removed and placed in a second oviposition arena (5 cm diameter) containing three healthy L2 DBM larvae. The parasitoid was again observed until one of the healthy hosts was attacked and then both the parasitoid and the freshly attacked host were isolated. The attacked host larva was reared for 6 days (20 °C; L:D 12:12 h) and then stored at -80 °C prior to DNA analysis to confirm the presence or absence of AV infection. Total DNA extraction was performed for each insect as described previously (Glatz *et al.*, 2003) and then PCR was performed using primers specific to *orf28* of HvAV-3e. PCR conditions were 95 °C for 5 min initial denaturation followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min followed by the final extension at 72 °C for 5 min.

2.9. Effect of ascovirus on *D. semiclausum* development

To provide AV infected hosts, 20 L2 DBM were inoculated with isolate-1 as previously described and then incubated at 20 °C (L:D 12:12 h) for 6 days. Five days after these insects were inoculated a further 40 L2 DBM larvae were inoculated with isolate-1 in the same manner. These larvae, together with a further 45 untreated L2s, were then transferred singly to Petri dishes (3.5 cm diameter) containing 5 ml of 2% (w/v) tap-water agar and a freshly cut common cabbage leaf disc (2 cm diameter) and incubated at 20 °C. After 24 h larvae were removed from the incubator and examined, any larvae which had died as a result of inoculation were discarded. Fifteen of the larvae inoculated 6 days previously were selected and transferred singly to Petri dishes (3.5 cm diameter) containing a moistened filter paper and a common cabbage leaf disc (2 cm diameter). Larvae were allowed 5 min to settle and then a single female *D. semiclausum* (1-3 days post eclosion) was introduced into each dish. Each parasitoid was observed carefully and as soon as it attacked the infected host larva it was removed and transferred to one of the Petri dishes containing an untreated L2. Each parasitoid was again watched carefully until it attacked the L2 and then it was removed and discarded. Next, a single fresh female *D. semiclausum* (1-3 days post eclosion) was introduced into each of the Petri dishes containing an L2 that was inoculated with isolate-1 24 h previously. Each parasitoid was observed carefully until it attacked the host and then it was removed from the dish and discarded. Finally, a single fresh female *D. semiclausum* (1-3 days post eclosion) was introduced into each of the Petri dishes containing a healthy untreated L2 and observed carefully until it attacked the host whereupon it was removed from the dish and discarded. All DBM L2s attacked (15 healthy larvae attacked by a parasitoid immediately after oviposition into an infected host, 15 healthy larvae attacked by a fresh uncontaminated parasitoid and 15 larvae manually inoculated with isolate-1 24 h previously and then attacked by a fresh uncontaminated parasitoid), 15 manually inoculated larvae not exposed to *D. semiclausum* and 15 larvae which were neither inoculated nor exposed to *D. semiclausum* were incubated at 20 °C. Larvae were examined twice daily and supplied with fresh cabbage leaf discs if required. For each individual, mortality, the time to parasitoid cocoon development, the duration of the parasitoid pupal period and the longevity and dry weight of adults was recorded.

2.10. Effect of ascovirus on DBM and immature *D. semiclausum* under semi-field conditions

Forty five potted Chinese cabbage plants (5 leaf stage) were each infested with 10 DBM L2s. Five plants were then placed inside a large field cage (1.5 m × 1.5 m × 1m); a single plant was placed in each corner of the cage and the fifth plant was placed in the centre of the cage. Nine cages were prepared and then arranged in a 3 × 3 grid, with a 2 m spacing between cages on a flat concreted area in the open air. A single DBM larva which had been inoculated with ascovirus 6 days previously was placed on the central plant in three of the cages. A single mated *D. semiclausum* female was then introduced into each of the three cages. Similarly, a single mated *D. semiclausum* female was introduced into three of the remaining cages. Finally single mated *D. semiclausum* females which had previously oviposited into an infected DBM larva (6 days post inoculation) were introduced into each of the three remaining cages. The parasitoids were allowed to forage for larvae within the cages for 24 h when they were removed. Larvae were carefully removed from each plant in each cage and introduced singly to individual labelled Petri dishes (3.5 cm diameter). Larvae were supplied with fresh Chinese cabbage foliage as required and the insects monitored daily. The rate of *D. semiclausum* parasitism, the development time of *D. semiclausum* from egg- pupa and the dry weight of adult wasps was recorded.

2.11. Statistical analysis

Dose response data were subject of logit analysis in Poloplus (LeOra software, 2002). All other statistical tests were conducted in Statview (SAS Insitute Inc. 1998). Where appropriate, treatments were compared by ANOVA and means separated by LSD. When data were not normally distributed they were compared by the Mann-Whitney U-test when there were two treatment groups and by the Kruskal-Wallis test when there were three treatment groups. Choice data were compared by the χ^2 test.

3. Results

3.1. Restriction fragment length polymorphism (RFLP) analysis

We carried out restriction fragment length polymorphism (RFLP) studies screening a pool of ascovirus isolates from the field. We identified three strains (1-3) that showed clear and different RFLP patterns when tested with two restriction enzymes (*Bam*HI and *Hind*III) (Fig. 1).

3.2. Ascovirus dose response assays

DBM larvae were readily infected by HvAV-3e and enlarged vesiculated cells were detected in hemolymph smears (Fig. 2A). The pathology was not observed in any smears prepared from control larvae pricked with sterile water (Fig. 2B). Similar observations were made in CCC. The dose-response assays revealed that DBM is susceptible to HvAV-3e, but that the pathogenicity of each isolate to DBM varied significantly (Table 1). Isolate-1 was the most pathogenic of the three isolates and comparison of LC_{50} values indicated that it was approximately 261-times as pathogenic as isolate-2 and 31-times as pathogenic as isolate-3.

In CCC, when L2 larvae were inoculated with isolate 1 (n=50) all were killed; 6% as larvae and the remaining 94% as pupae (Fig. 3). For untreated control larvae (n=15) and larvae pricked with sterile water (n=25) mortality rates were only 20% and 28% respectively, although 7% and 12% of adults emerging from the control and water-pricked treatments were deformed.

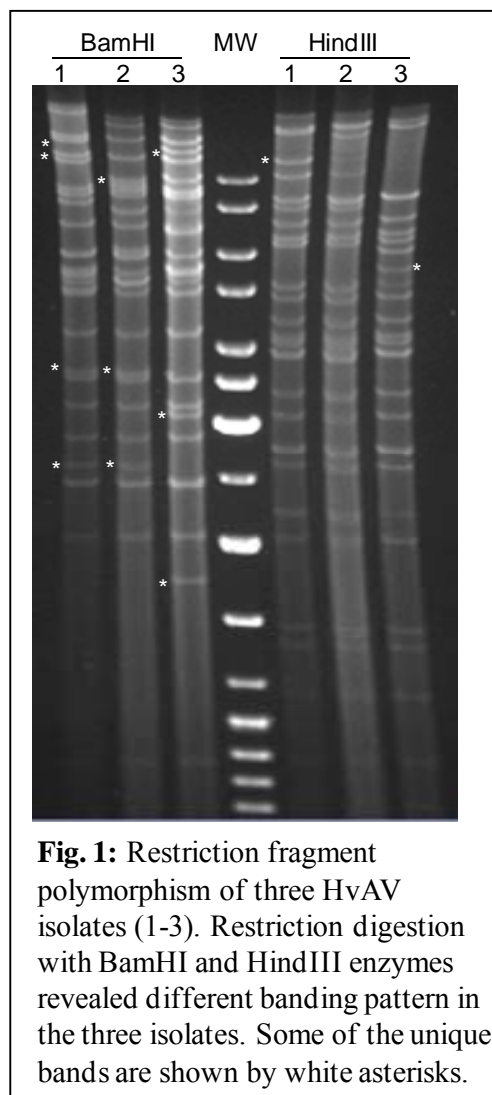


Fig. 1: Restriction fragment polymorphism of three HvAV isolates (1-3). Restriction digestion with BamHI and HindIII enzymes revealed different banding pattern in the three isolates. Some of the unique bands are shown by white asterisks.

Table 1: Dose response of L4 DBM larvae to three isolates of HvAV-3e following manual inoculation with a minuten pin

Isolate ^a	n	LC_{50} (95% confidence interval)	Slope (\pm SE) ^b	Heterogeneity (df)
1	360	8.38×10^5 (1.71×10^5 - 2.66×10^6)	0.60 (\pm 0.12)	0.151(28)
2	360	2.19×10^8 (1.05×10^8 - 6.86×10^8)	1.64 (\pm 0.42)	0.195 (28)
3	180	2.62×10^7 (4.75×10^6 - 1.48×10^9)	0.93 (\pm 0.20)	1.825 (13)

^a Regression lines for the dose response of *P. xylosotella* larvae to each AV isolate were significantly different from each other (χ^2 (df=4)= 85.84; P <0.001).

^b Slopes significantly different (χ^2 (df=2)= 10.03; P =0.006)

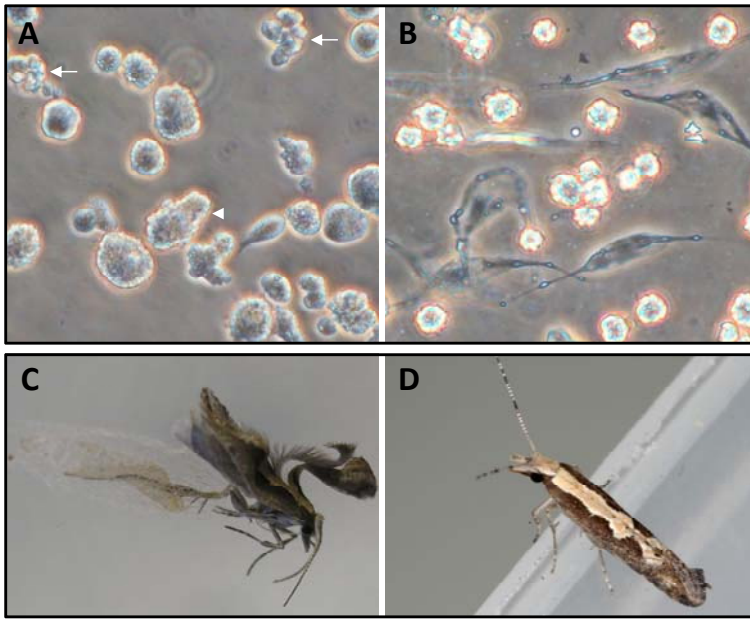


Fig. 2: Hemolymph smears from DBM larvae: (A) inoculated with HvAV-3e isolate-1 and incubated for 6 days; and (B) non-infected (pricked with sterile water). 40× magnification. DBM adults developing from (C) L4 larvae infected with HvAV-3e isolate-1 and (B) control (water).

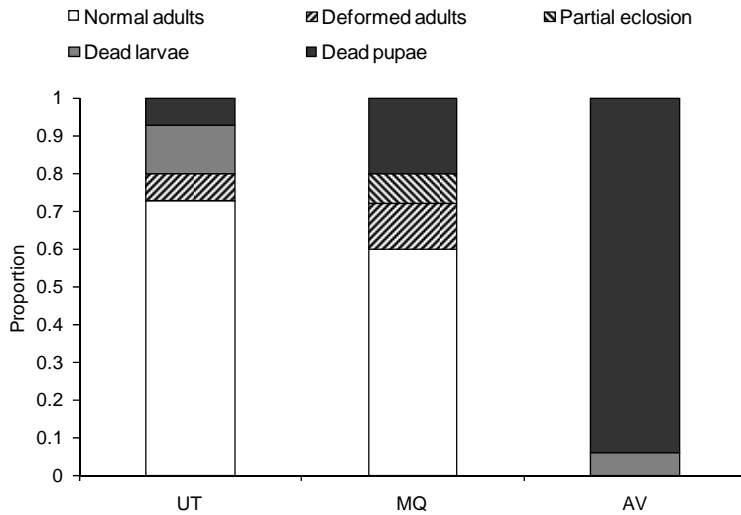


Fig. 3: Fate of untreated *C. pavonana* larvae and *C. pavonana* larvae pricked with water or inoculated with HvAV-3e as L2 larvae.

3.3. Effect of ascovirus infection on DBM larval feeding and growth

Control larvae and larvae treated with sterile water completed development and ceased feeding by day 6 whereas larvae inoculated with isolate-1 did not cease feeding and pupate until day 8 after inoculation at the earliest. Despite this longer development period the total amount of leaf material eaten by larvae inoculated at L2 was significantly lower than that consumed by control larvae and larvae treated with sterile water ($F_{2,17} = 4.76$; $P < 0.023$) (Fig. 4A). There was no significant difference between the amount of leaf material consumed by control and sterile water treated larvae (LSD, $P > 0.05$; Fig. 4A). Similarly, by day 6 after treatment larvae inoculated with isolate 1 weighed significantly less than larvae treated with sterile water or larvae which were left untreated ($F_{2,17} = 9.70$; $P = 0.0025$; Fig. 4B). There was no significant difference between the weights of control and sterile water treated larvae (LSD, $P > 0.05$; Fig. 4B).

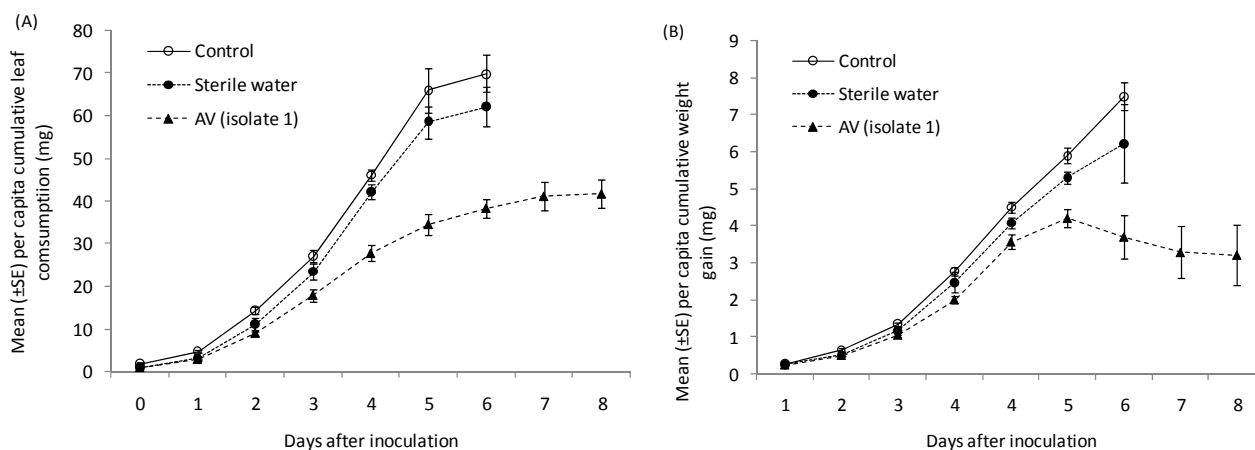


Fig. 4: Effect of HvAV-3e isolate-1 on DBM larvae. (A) cumulative *per capita* food consumption prior to pupation. (B) cumulative *per capita* weight gain of DBM larvae following inoculation.

3.4. Effects of ascovirus infection on *C. pavonana*

In the 24 h period immediately after pricking with sterile water or inoculating with HvAV-3e, the treatments had no significant impact on the weight gain of *C. pavonana* larvae when compared with untreated controls ($F_{2,18}=2.55$; $P=0.106$; Fig. 5A). On the second, third and fourth days after inoculation with HvAV-3e daily weight gains of larvae were significantly reduced when compared with untreated and water pricked controls (24-48h, $F_{2,18}=10.94$, $P=0.0008$; 48-72h, $F_{2,18}=61.40$, $P<0.0001$; 72-96h, $F_{2,18}=80.04$, $P<0.0001$; Fig. 5A). On the fifth day after inoculation with HvAV-3e, inoculated larvae gained significantly less weight than water pricked controls (96-120h, $F_{2,18}=4.10$, $P=0.0349$; Fig. 5A) but there was no significant difference between the weight gain of HvAV-3e inoculated larvae and untreated controls (LSD $P=0.095$; Fig. 5A). Similarly, inoculation with HvAV-3e reduced total food consumption by larvae by 59% and 57% when compared with untreated and water pricked control larvae, respectively ($F_{2,18}=57.63$; $P<0.0001$), but there was no difference in total food consumption between untreated control larvae and water treated control larvae (LSD, $P=0.366$) (Fig. 5B).

Inoculation of L2 larvae with HvAV-3e significantly reduced the weight of pupae that developed ($F_{2,47}=134.9$; $P<0.0001$) and significantly increased the median time that it took for pupae to develop ($H=28.3$; $DF=2$; $P<0.0001$) when compared with untreated and water pricked controls (Table 2).

Table 2: Sub-lethal effects of HvAV-3e on developmental parameters of *C. pavonana* following inoculation of second instar larvae.

Treatment	Mean Pupal weight (mg) (\pm SE) ¹	Median time to pupation (days) ²
Control	47.93 (\pm 0.71)a	9.0a
Water	48.92 (\pm 0.73)a	8.0a
HvAV3e	26.64 (\pm 1.50)b	11.5b

¹Means followed by a different letter are significantly different ($F_{2,47}=134.9$; $P<0.0001$)

²Medians followed by a different letter are significantly different ($H=28.3$; $DF=2$; $P<0.0001$)

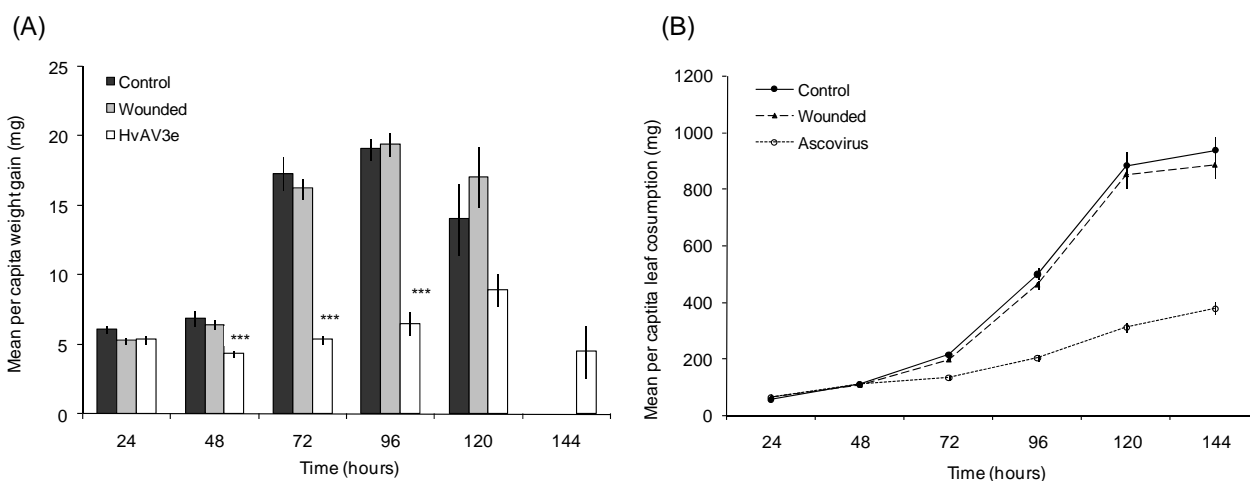


Fig. 5: (A) Mean daily weight gains of control *C. pavonana* larvae and *C. pavonana* larvae after pricking with sterile water (water) or HvAV-3e inoculum. For each 24 h feeding period columns marked by * ($P<0.05$) or *** ($P<0.0001$) are significantly different to other columns within the feeding period. (B) Mean cumulative per capita cabbage leaf consumption by control *C. pavonana* larvae and *C. pavonana* larvae following pricking with water (water) or HvAV-3e inoculum as L2 larvae (***) ($P<0.0001$).

3.5. Effect of larval infection on DBM adult fecundity

When inoculated with isolate-1 as early L4 larvae, only 49% of treated DBM larvae formed pupae while 93% of untreated and 91% of larvae treated with sterile water formed pupae (Table 3). Overall, only 9% of AV inoculated larvae developed to the adult stage and many were deformed (Fig. 2C) when compared with healthy adults (Fig. 2D). In stark contrast, the control groups yielded 83% (untreated control) and 89% (sterile water treated) adults, respectively (Table 3); none of which were deformed. However, there was no significant difference between the weights of pupae in any of the treatment groups ($F_{2,74}=1.257$; $P=0.291$; Table 3). Inoculation of female larvae with AV significantly reduced the fecundity of those individuals which completed development and eclosed as adults when compared with the fecundity of moths which developed from either group of control larvae ($F_{2,27}=5.51$; $P=0.009$; Table 3).

Table 3: Effect of inoculation of L4 DBM larvae with HvAV-3e isolate-1 on development, survival and fecundity of subsequent adults

Treatment	n	Proportion of insects developing to		Mean pupal weight (\pm SE) (mg) ^a	Mean no. eggs per female (\pm SE) ^a
		Pupae	Adults		
Control	70	0.93	0.83	552 (\pm 15)a	183 (\pm 27)a
Sterile water	40	0.91	0.89	539 (\pm 18)a	236 (\pm 18)a
AV-isolate-1	90	0.49	0.09	507 (\pm 23)a	113 (\pm 21)b

^a Within these columns means marked by a different letter are significantly different ($P<0.05$). For pupal weights ($F_{2,74}=1.257$; $P=0.291$) and for egg production ($F_{2,27}=5.51$; $P=0.009$; means separated by LSD, $P<0.05$).

3.6. Effect of larval infection on host selection by *D. semiclausum*

Diadegma semiclausum females did not discriminate between healthy larvae and larvae previously inoculated with isolate-1. Of the 30 parasitoids which responded when given a choice of healthy larvae and larvae inoculated with isolate-1 three days previously, 11 attacked an infected host first while 19 parasitoids attacked a healthy host larva first (χ^2 (df=1)= 0.122; $P>0.05$). Similarly when parasitoids were given a choice of healthy larvae or larvae inoculated with isolate-1 4 or 6 days previously there was no preference for infected or healthy hosts was detected (4 days post inoculation: 30 parasitoids responded, 14 attacked infected larvae first and 16 attacked healthy larvae first (χ^2 (df=1)= 0.067; $P>0.05$); 6 days post inoculation: 30 parasitoids responded, 12 attacked infected larvae first and 18 attacked healthy larvae first (χ^2 (df=1)= 0.600; $P>0.05$).

3.7. Transmission assays

Ascovirus infection was detected by PCR in 8 of the 10 healthy hosts attacked by female *D. semiclausum* parasitoids that had previously attacked an infected host. Ascovirus infection was not detected in control larvae that were attacked by uncontaminated parasitoids (Fig. 6).

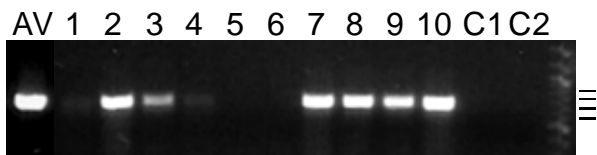


Fig. 6: Transmission of HvAV-3e to DBM L2 larvae by *D. semiclausum*. A pair of primers specific to *orf28* was used for PCR detection. AV, HvAV-3e genomic DNA; 1-10, healthy larvae subsequently attacked by “virus-carrying” wasps and collected 6 days post-attack; C1 & C2, healthy larvae subsequently attacked by “sterile water-carrying” wasps and collected 6 days post-attack. Molecular marker bars shown on the right represent 600, 700, 800 and 900 bps from bottom to top.

3.8. Effect of ascovirus on *D. semiclausum* development

All DBM larvae which were neither inoculated with isolate-1 nor exposed to *D. semiclausum* parasitism successfully completed development to adult moths. All DBM larvae which were inoculated with isolate-1 but not exposed to *D. semiclausum* parasitism completed development to pupae but all died at that stage (Table 4). DBM inoculated with isolate-1 took significantly longer to develop to the pupal stage (median development time= 6.5 days) than healthy control larvae (median development time= 5 days) (Table 4). There were no significant differences between the egg-pupa development times of *D. semiclausum* developing in hosts manually inoculated with isolate-1 prior to parasitism, hosts parasitised immediately after oviposition in an isolate-1 infected host or in healthy control hosts ($H=2.75$ (DF=2); $P=0.253$) (Table 4). Similarly, host infection status had no significant effect on the duration of the pupal period of *D. semiclausum* ($H=5.76$ (DF=2); $P=0.06$), the longevity of adults after eclosion ($H=0.239$ (DF=2); $P=0.888$) or the dry weight of adults ($F_{2,24}=2.96$; $P=0.07$).

Table 4: Development of *D. semiclausum* in healthy and AV-inoculated (isolate-1) DBM hosts.

Treatment	Median <i>D. semiclausum</i> development time (days) ^b			Mean adult dry weight (±SE) ^b
	Egg-Pupa	Pupal period	Adult longevity	
Control	10	8	4	55.3 (±2.5)
Manual AV- inoculation of host	9	8	3	51.7 (±2.8)
Previous oviposition in AV-infected host	9	9	3	61.5 (±3.1)

^a In controls, all 15 AV-inoculated DBM died as pupae and all 15 DBM which were neither inoculated with AV nor exposed to parasitism survived to adulthood. AV-inoculated DBM larvae took significantly longer to reach the pupal stage (median time =6.5 days) than non-inoculated DBM larvae (median time= 5 days) $Z= 2.245$; $P=0.025$).

^b For *D. semiclausum*, host infection status had no significant effect on the development time from egg- pupa ($H=2.75$ (DF=2); $P=0.253$), the duration of the pupal period ($H=5.76$ (DF=2); $P=0.06$), the longevity of adults after eclosion ($H=0.239$ (DF=2); $P=0.888$) or the dry weight of adults ($F_{2,24}=2.96$; $P=0.07$).

3.9. Effect of ascovirus on DBM and immature *D. semiclausum* under semi-field conditions

Neither inoculation of female *D. semiclausum* with ascovirus prior to release into cages nor provision of a DBM larva heavily infected with ascovirus to act as a reservoir of inoculum for foraging parasitoids affected the outcomes for DBM larvae in the field cage (Table 2). In light of other studies which indicate that simultaneous ascovirus infection within a host larva does not affect parasitoid survival or development, this result is not surprising.

Table 5: *D. semiclausum* parasitism rates, fate of DBM larvae and host and parasitoid development in transmission in an ascovirus transmission study conducted under semi-field conditions.

Treatment	Proportion of DBM larvae ^a		Median development time (d)		Mean adult dry weight <i>D. semiclausum</i> (mg) ^b
	parasitised	killed	<i>D. semiclausum</i>	DBM	
Control	0.13 (±0.11)	0.18 (±0.08)	12	10	457 (±28)
DBM infected larva on plant	0.08 (±0.06)	0.13 (±0.03)	12	10	465 (±57)
Inoculated <i>D. semiclausum</i>	0.35 (±0.10)	0.15 (±0.03)	12	10	456 (±15)

^a There was no significant difference between parasitism rates ($F_{2,6}=2.21$; $P=0.19$) or the rates of DBM larval mortality ($F_{2,6}=0.02$; $P=0.984$) in the different treatments. ^b There was no significant difference between the dry weight of adult parasitoids from the different treatments ($F_{2,26}=0.26$; $P=0.98$)

4. Discussion

This study shows that HvAV-3e can infect non-noctuid lepidopteran hosts, DBM and CCC, but that the resulting disease symptoms are atypical of those described from diseased noctuids. Disease progression was slower in DBM and CCC than in conventional hosts. In noctuids, by 6 days p.i. the host hemolymph is typically teeming with liberated viral vesicles ($\sim 10^8/\text{mL}$) causing the milky white colour characteristic of ascovirus infection (Federici, 1983a; Marti & Hamm, 1987). This was not observed in DBM and CCC, and 6 days p.i. there were no or little circulating, liberated vesicles although vesicles were forming in enlarged cells. The lack of obvious pathology demonstrated in these non-noctuid hosts could explain why ascoviruses have never been reported in these hosts before and could in part be due to DBM and CCC being only semi-permissive hosts for the ascovirus.

Host insects are commonly co-infected by a mixture of virus genotypes (Ennos, 1992; Frank, 2000; Hudson & Carner, 1981; Lopez-Ferber *et al.*, 2003). In the case of baculoviruses, it has been shown that co-infection by a mixed population of viral genotypes contributes to the fitness of the virus population; the mortality caused by mixed genotype infections being higher than that of individual genotype infections (Lopez-Ferber *et al.*, 2003). This genetic heterogeneity might be the result of selection pressures imposed on the virus when replicating within a host or the result of replication in a range of hosts when it is transmitted from one host to another. In the latter scenario, a new host may support replication of certain variants that are not favoured by previous host(s). Although the genotypic composition of HvAV-3e has not been explored, we assume that the isolated HvAV-3e is a virus strain and not an isogenic virus clone.

The different isolates of HvAV-3e had different pathogenicities against DBM (Table 1). Although DBM was readily infected by the three isolates of HvAV-3e tested, disease manifestation was not typical of that described in conventional noctuid hosts (Federici, 1983b; Hamm *et al.*, 1985b). In DBM, infection slowed larval development (Fig. 4B and Table 4) but was more likely to cause death in the pupal stage or lead to the eclosion of a deformed adult (Table 3). In infected noctuid hosts, arrested larval development typically precedes death in that stage and precludes pupal development. In addition, cell pathology was similar to what is observed in infected cell lines, Sf9 and HzFB (Asgari, 2006). DBM also showed a marked reduction in food consumption when infected with AV (Fig. 3A). Similarly, infected *C. pavonana* larvae continued to grow and moult and most developed into pupae. However, all larvae infected as 2nd instars that subsequently progressed to pupation died in the pupal stage (Fig. 3). The observation that infected *C. pavonana* larvae consumed less food than healthy individuals (Fig. 5) is also consistent with ascovirus infection in noctuids (Federici & Govindarajan, 1990a; Hamm *et al.*, 1985a; Newton, 2004).

Ascovirus infected DBM adults were 39-52 % less fecund than healthy controls (Table 3) and it is likely that under field conditions the extensive deformities suffered by many adults as a result of infection (Fig. 2C) would result in their complete inability to reproduce. Although not investigated here, it is also likely that flight ability and adult lifespan would be compromised in infected DBM, both of which are factors in an individual's ability to mate and reproduce.

The study also showed that HvAV-3e is transmissible to healthy DBM larvae by *D. semiclausum* following oviposition in heavily infected hosts, although with variable transmission outcomes (Fig. 6). It is interesting that AV was not readily detected from *D. semiclausum* by PCR even when they

had transmitted the disease to healthy hosts. This is probably attributable to the fact that DBM is a semi-permissive host to the virus. As a result, the number of viral progeny produced is likely to be low and the number of virions acquired by the parasitoid ovipositor limited. In addition, we tested the wasps after subsequent oviposition in healthy larvae which is likely to remove some of the viral particles from the ovipositor. A previous study showed that the length of the parasitoid ovipositor is an important determinant of the efficiency with which parasitoids are able to transmit AV infection in subsequent oviposition attempts (Tillman *et al.*, 2004). The longer the ovipositor, the more efficient the vector, due to the attachment of more virions to longer ovipositors. *Diadegma semiclausum* has relatively a short ovipositor and this might contribute to acquisition of few virions by the ovipositor during oviposition into an infected host. These could then be rapidly lost in subsequent oviposition attempts. Further, when the hemolymph from infected larvae was viewed by light microscopy neither the characteristic circulating vesicles nor the enlarged vesiculated cells were observed (Fig. 2A). Rather, the cells took on an appearance similar to that observed in a fat body cell line from *Helicoverpa zea* (Asgari, 2006). This pathology may prevent virions from effectively adhering to the ovipositor.

Although *D. semiclausum* is capable of transmitting the AV to healthy hosts that it parasitises, host infection appears to have little impact on the developing parasitoid (Table 4). Healthy parasitoids readily developed within host larvae which were manually inoculated with doses of isolate-1 which killed non parasitised hosts, thus transmission of the AV by infected parasitoids is unlikely to have a detrimental effect on parasitoid populations. These results were corroborated in a semi-field experiment (Table 5) which demonstrated that the availability of a source of ascovirus inoculum in caged foraging arenas or oviposition by *D. semiclausum* into DBM hosts heavily infected by ascovirus had no detrimental impact on the development or survival of *D. semiclausum* within the exposed DBM population (Table 5).

In conclusion, the research carried out in this project has shown that the investigated hosts, CCC and DBM, were susceptible to the virus. Although the symptoms were not as severe or obvious in these novel hosts, the overall effects were the same as have been previously described for noctuid hosts. This suggests that AVs may be much more widespread in lepidopteran populations than previously thought. In noctuids, it has been shown that AVs are readily transmitted by mechanical means, including parasitoid wasps during oviposition, while they are poorly transmitted by ingestion (Govindarajan & Federici, 1990). DBM has many parasitoids associated with it and so it might reasonably be expected that they too could transmit AV from infected to healthy hosts. In addition, the fact that the AV does not seem to have detrimental effects on the development of the parasitoid in semi-permissive hosts such as DBM or CCC, in contrast to noctuid hosts, these may provide alternative hosts that facilitate maintenance of AV populations in the field.

5. Technology transfer

One paper was published and one recently submitted to an international peer reviewed journal (see the list below). One article was also published in Ausveg (Volume 4.5-2009) and the *Brassica* IPM newsletter each

Smede, M., Furlong, M. J. & Asgari, S. (2008). Effects of *Heliothis virescens* ascovirus (HvAV-3e) on a novel host, *Crociodolomia pavonana* (Lepidoptera: Crambidae) *J Invertebr Pathol* **99**, 281-285.

Furlong, M. J. & Asgari, S. (2009). Effects of an ascovirus (HvAV-3e) on diamondback moth, *Plutella xylostella*, and evidence for virus transmission by a larval parasitoid. *Journal of Invertebrate Pathology* **submitted**.

6. Recommendations - scientific and industry

Scientific recommendations. We showed for the first time that Lepidoptera species outside the Noctuidae family, DBM and CCC, can be infected by ascoviruses. The virus causes mortality in both species at larval stage or inhibits emergence of adults even if the infected larvae develop into pupae. The virus is also transmitted by parasitoid wasps. Interestingly, the virus does not have detrimental effects on the developing endoparasitoid. However, since DBM and CCC are not the habitual hosts of the virus, HvAV-3e does not replicate readily in the hosts. This poses problems in establishment and transmission of the virus under field conditions. The challenge is to develop ways to deliver the virus to larvae in the field and perhaps adapt the virus into the new host to increase their pathogenicity. However, if such efficient viruses can be produced, its effect on the developing parasitoid(s) should be re-assessed as rapid replication of the virus in the host may have detrimental consequences for the developing larval parasitoid.

Industry recommendations. The research indicates that it is unlikely that the ascovirus can be efficiently transmitted and established in the field. This is mainly due to weak replication of the virus in DBM and CCC despite having a significant impact on the hosts under laboratory conditions. As a consequence, the number of viruses replicated in the hosts is not high enough to lead to establishment of epizootics in the field. However, prior to decisions on the introduction of the virus to DBM field populations, controlled field experiments should be carried out to find out how the virus performs in the field and to determine the impact of the virus on the host and parasitoid populations. The virus causes significant mortalities in major noctuid pest populations (eg. *Helicoverpa armigera*) and if introduced into DBM populations and established it may contribute to natural control of the pest.

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Effects of *Heliothis virescens* ascovirus (HvAV-3e) on a novel host, *Crocidolomia pavonana* (Lepidoptera: Crambidae)

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ABSTRACT

Ascoviruses are double-stranded DNA viruses which cause fatal disease in lepidopteran host larvae. They induce a unique pathology, causing cleavage of host cells into virion-containing vesicles. With the single exception of *Diadromus pulchellus* ascovirus, all ascoviruses have been exclusively reported from the Noctuidae. To investigate whether *Heliothis virescens* AV (HvAV-3e) has a broader host range at the family level, larvae of *Crocidolomia pavonana* F. (Lepidoptera: Crambidae), a major pest of brassica crops in tropical and sub-tropical regions of the Old World and Australasia, were inoculated with HvAV-3e. Larvae were readily infected by the ascovirus and feeding, growth and survival were significantly affected. However, the milky white discolouration of the haemolymph which is characteristic of ascovirus infection in noctuid hosts was not apparent. In further contrast to infected noctuid host larvae that do not develop to the pupal stage, a significant proportion of infected *C. pavonana* larvae pupated but all were killed at this stage. Thus, *C. pavonana* appears to be a semi-permissive host of the ascovirus, the presence of such hosts in the field might be an explanation for the conundrum for the ascovirus–noctuid–wasp relationship, helping explain the persistence of the ascovirus.

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1. Introduction

Ascoviruses are large, enveloped double-stranded DNA viruses which are principally transmitted to their lepidopteran hosts by parasitoid wasps during oviposition (Federici, 1983; Federici et al., 1990). They cause a chronic, ultimately fatal infection in host larvae (Federici, 1983; Hamm et al., 1985) and typical symptoms include loss of appetite and stunted growth (Federici, 1983; Govindarajan and Federici, 1990). Ascoviruses exhibit a unique pathology. Following invasion by the ascovirus, host cells undergo nuclear and cellular hypertrophy and genome fragmentation, before invagination of the plasma membrane leads to the formation of many intracellular vesicles (Federici, 1983; Hamm et al., 1985). The process of vesicle formation resembles apoptosis and ascoviruses were the first viruses shown to encode caspases (apoptosis activating enzymes) (Bideshi et al., 2005). However, subsequent studies have suggested that variations from this process might be involved (Asgari, 2006). Eventually the infected cell ruptures releasing the vesicles into the haemolymph. The virus-containing vesicles disseminate throughout the haemolymph, giving it a distinctive milky white colour. The

appearance of milky haemolymph has been the diagnostic symptom of ascovirus infection since their discovery.

Based on host range and hybridisation studies, five species of ascovirus are currently recognised. Four of these species, *Spodoptera frugiperda* ascovirus (SfAV-1), *Trichoplusia ni* ascovirus (TnAV-2), *Heliothis virescens* ascovirus (HvAV-3) and *Spodoptera exigua* ascovirus (SeAV-5) are very closely related phylogenetically and have only been reported to infect members of the Noctuidae. The fifth species, *Diadromus pulchellus* ascovirus (DpAV-4), infects the leek-moth (*Acrolepiopsis assectella*, Lepidoptera: Plutellidae) and is more distantly related to the other four species. It is also the only ascovirus species known to have a mutualistic relationship with its parasitoid vector, *D. pulchellus*; the other species are all pathogenic to their vectors (Stasiak et al., 2005).

The ascovirus species isolated from noctuid hosts have different host ranges and tissue tropisms (Federici and Govindarajan, 1990; Hamm et al., 1986). None of these species have been isolated from non-noctuid hosts in the field. To investigate whether HvAV-3e has a broader host range at the family level, larvae of *Crocidolomia pavonana* F. (Lepidoptera: Crambidae), a major pest of brassica crops in tropical and sub-tropical regions of Africa, southern and south-east Asia and the Asia-Pacific were inoculated with HvAV-3e in the laboratory. Studies investigated the pathogenicity of HvAV-3e to *C. pavonana* and examined the effects of infection on larvae, pupae and adult insects.

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2. Materials and methods

2.1. Ascovirus

A strain of *H. virescens* ascovirus (HvAV-3e) was isolated from *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) larvae in south-east Queensland, Australia (Asgari et al., 2007). Laboratory stocks of HvAV-3e were amplified in *Spodoptera litura* F. (Lepidoptera: Noctuidae) larvae. The inoculum used for subsequent infections was produced by collecting the haemolymph from *S. litura* larvae 6–7 days post ascovirus infection. To collect haemolymph, larvae were surface sterilized in 70% ethanol and dried. One of the prolegs was cut using a pair of fine forceps and the haemolymph was collected in an Eppendorf tube (1.5 mL) and then stored at -80°C until used in experiments.

2.2. Insects

A laboratory culture of *C. pavonana* was established from larvae collected in a cabbage crop in the Lockyer Valley in south-east Queensland, Australia and maintained in an incubator under controlled conditions ($25 \pm 2^{\circ}\text{C}$; L:D 12:12 h). Larvae were reared in ventilated plastic boxes ($15 \times 20 \times 30$ cm) lined with tissue paper and supplied with fresh glasshouse grown common cabbage plants (*Brassica oleracea* var Warrior) daily. Waste leaf material was removed daily and tissue paper was replaced as required. Pupae, which formed between layers of tissue paper, were collected within 1–2 days of formation and transferred to Petri dishes (9 cm diameter) to develop. Just before eclosion (5–6 days at 25°C), approximately 50 pupae were placed in the bottom of a Petri dish (9 cm diameter) and transferred to the base of an oviposition cage ($50 \text{ cm} \times 50 \text{ cm} \times 50 \text{ cm}$) containing a potted cabbage plant for oviposition and 10% honey solution as a food source. Once oviposition commenced, potted plants on which egg masses were laid were removed from the cage and replaced with fresh plants. Egg masses were allowed to hatch *in situ* and neonate larvae fed on the plant until they reached the 2nd instar (L2), at which point the cabbage was cut off below the first leaf and transferred to a ventilated container ($15 \times 20 \times 30$ cm) lined with tissue paper and fed on fresh cabbage leaves as described above.

2.3. Susceptibility of *C. pavonana* to ascovirus

Approximately 40 L2 *C. pavonana* larvae were selected without bias from an infested cabbage plant. The larvae were then equally divided between two Petri dishes (9 cm diameter) and anaesthetised on ice for 2–5 min. Larvae in one group were then inoculated with HvAV-3e by pricking the dorsal surface of each larva with a UV-sterilised minuten pin that had been dipped into a $30 \mu\text{L}$ aliquot of ascovirus inoculum. Following inoculation larvae were arranged in batches of 5, placed into Petri dishes (9 cm diameter) and supplied with fresh cabbage leaf. Larvae in the remaining group served as controls and were pricked with a UV-sterilised minuten pin previously dipped into a $30 \mu\text{L}$ aliquot of sterile milliQ water before being transferred in batches of 5 to Petri dishes (9 cm diameter) and supplied with fresh cabbage leaf. Treated and control larvae were incubated ($25 \pm 2^{\circ}\text{C}$; L:D 12:12 h) for 6 days. On the seventh day post inoculation fine forceps were used to remove a proleg from each larva and a single drop of haemolymph was collected into $10 \mu\text{L}$ of 10% PBS in glycerol on a microscope slide. Cells were allowed to settle for 10 min at room temperature before examination by light microscopy. Samples containing typical ascovirus cell cleavage and vesicle formation were considered infected. The experiment was

conducted on three separate occasions; data from the three replicates were pooled for presentation.

The effect of ascovirus infection on the fate of L2 larvae was also investigated. Second instar *C. pavonana* larvae were selected from infested cabbage plants. Batches of larvae were anaesthetised on ice and then 25–50 insects were inoculated with ascovirus or pricked with a sterile minuten pin as described above. An additional group of 15 L2 larvae, which were anaesthetised but not exposed to ascovirus or wounding, were set up as an untreated control. Batches of 5 larvae from each of the controls and the ascovirus treatment were reared in a Petri dish (9 cm diameter) containing a moistened filter paper and fresh cabbage leaves. Larvae were incubated ($25 \pm 2^{\circ}\text{C}$; L:D 12:12 h) and monitored daily until death or the emergence of adult moths.

2.4. Effects of ascovirus infection on *C. pavonana*

Second instar larvae were selected and inoculated with ascovirus, pricked with a sterile minuten pin or prepared as untreated controls as described previously. Larvae inoculated with ascovirus and larvae from each of the controls were randomly divided into groups of three and weighed on a microbalance (Sartorius BP 210 D). Each weighed batch of larvae was then transferred to a Petri dish (9 cm diameter) containing approximately 5 mL of set 2% tap water agar and a weighed cabbage leaf disc (5 cm diameter) cut from the centre portion of a fresh cabbage leaf. Seven such dishes were prepared for each treatment. All dishes were then transferred to a sealed 10 L container and incubated ($23 \pm 2^{\circ}\text{C}$; L:D 12:12 h). Each batch of larvae was weighed daily and any frass was removed from the Petri dish. Leaf discs were also weighed daily and replaced with fresh, pre-weighed leaf discs. When larvae reached the non-feeding pre-pupal phase, weighing ceased so as not to disrupt developing pupae in pupal chambers. Once pupae formed they were removed from the pupal chamber on the day of formation and weighed. Insects continued to be monitored every day until death or eclosion of adults.

2.5. Restriction fragment length polymorphism (RFLP) analysis

The isolated HvAV-3e is a virus strain and not an isogenic virus clone. To test whether or not only certain ascovirus genotypes replicated within *C. pavonana*, isolated genomic DNA from the original inoculum and from virus amplified in *C. pavonana* larvae, was analysed by RFLP. Thirty *C. pavonana* L2 larvae were inoculated with HvAV-3e as previously described. Seven days after inoculation, the larvae were bled as above and haemolymph containing viral vesicles was collected. In separate extractions, total genomic DNA was isolated from the collected haemolymph and a sample of the original inoculum using a QIAamp DNA mini kit (Qiagen). The genomic DNA extracted from both samples was then compared following digestion with the BamHI restriction enzyme and separation on a 0.7% agarose gel.

2.6. Statistical analysis

All statistics were conducted using the Statview for Windows version 5 (SAS Institute Inc., 1998). Where appropriate, data were analysed by ANOVA. All proportional data were subject to arcsine transformation before being subjected to ANOVA. When ANOVA indicated a significant difference between treatment means, individual treatment means were compared by Fisher's LSD. Medians were compared using the Kruskal–Wallis test.

3. Results

3.1. Susceptibility of *C. pavonana* to ascovirus

Crociodolomia pavonana larvae were readily infected by HvAV-3e and enlarged vesiculated cells were detected in the haemolymph smears prepared from 94% ($n = 66$) of HvAV-3e inoculated larvae (Fig. 1A). The pathology was not observed in any haemolymph smears prepared from larvae pricked with sterile water ($n = 59$) (Fig. 1B).

When L2 larvae were inoculated with HvAV-3e ($n = 50$) all were killed; 6% as larvae and the remaining 94% as pupae (Fig. 2). For untreated control larvae ($n = 15$) and larvae pricked with sterile water ($n = 25$) mortality rates were only 20% and 28%, respectively, although 7% and 12% of adults emerging from the control and water-pricked treatments were deformed.

3.2. Effects of ascovirus infection on *C. pavonana*

In the 24 h period immediately after pricking with sterile water or inoculating with HvAV-3e, the treatments had no significant impact on the weight gain of *C. pavonana* larvae when compared with untreated controls ($F_{2,18} = 2.55$; $P = 0.106$; Fig. 3A). On the second, third and fourth days after inoculation with HvAV-3e daily weight gains of larvae were significantly reduced when compared with untreated and water-pricked controls (24–48 h, $F_{2,18} = 10.94$, $P = 0.0008$; 48–72 h, $F_{2,18} = 61.40$, $P < 0.0001$; 72–96 h, $F_{2,18} = 80.04$, $P < 0.0001$; Fig. 3A). On the fifth day after inoculation with HvAV-3e, inoculated larvae gained significantly less weight than water-pricked controls (96–120 h, $F_{2,18} = 4.10$, $P = 0.0349$; Fig. 3A) but there was no significant difference between the weight gain of HvAV-3e inoculated larvae and untreated controls (LSD $P = 0.095$; Fig. 3A). Similarly, inoculation with HvAV-3e reduced total food consumption by larvae by 59% and 57% when compared with untreated and water-pricked control larvae, respectively ($F_{2,18} = 57.63$; $P < 0.0001$), but there was no difference in total food consumption between untreated control larvae and water treated control larvae (LSD, $P = 0.366$) (Fig. 3B).

Inoculation of L2 larvae with HvAV-3e significantly reduced the weight of pupae that developed ($F_{2,47} = 134.9$; $P < 0.0001$) and significantly increased the median time that it took for pupae to develop ($H = 28.3$; $DF = 2$; $P < 0.0001$) when compared with untreated and water-pricked controls (Table 1).

3.3. RFLP analysis

To see if the difference in response is due to strain selection or not we analysed the ascovirus DNA before and after amplification in *C. pavonana*. The restriction patterns for the original inoculum (Fig. 4, lane 1) and the ascovirus following amplification in *C. pavo-*

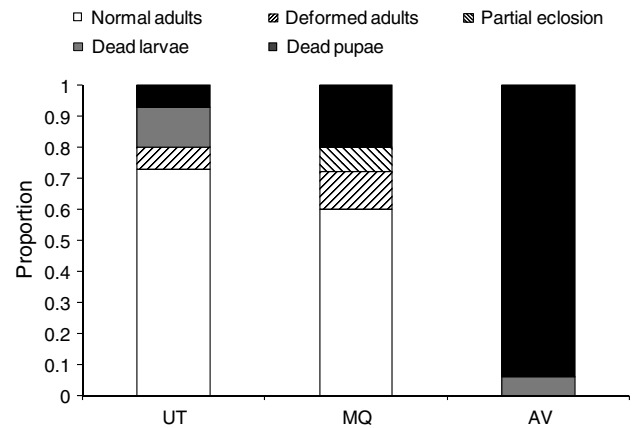


Fig. 2. Fate of untreated *C. pavonana* larvae and *C. pavonana* larvae pricked with water or inoculated with HvAV-3e as L2 larvae.

nana (Fig. 4, lane 2) were identical suggesting selection did not take place.

4. Discussion

The study shows that HvAV-3e can infect a non-noctuid lepidopteran host but that the resulting disease symptoms are atypical of those described from diseased noctuids. Disease progression was slower in *C. pavonana* than in conventional hosts. In noctuids, by 6 days p.i. the host haemolymph is typically teeming with liberated viral vesicles ($\sim 10^8$ /mL) causing the milky white colour characteristic of ascovirus infection (Federici, 1983; Marti and Hamm, 1987). This was not observed in *C. pavonana* and 6 days p.i. there were no or little circulating, liberated vesicles although vesicles were forming in enlarged cells. The lack of obvious pathology demonstrated in *C. pavonana* could explain why ascoviruses have never been reported in the host before and could in part be due to *C. pavonana* being only a semi-permissive host for the ascovirus. However, a lack of liberated vesicles in HvAV-3e infected insect cell lines, Sf9 and HzFB, from *S. frugiperda* and *Helicoverpa zea*, respectively, has also been reported previously (Asgari, 2006) and so the pathology in *C. pavonana* may be a characteristic of HvAV-3e in certain hosts rather than the host response *per se*.

Host insects are commonly co-infected by a mixture of virus genotypes (Ennos, 1992; Frank, 2000; Hudson and Carner, 1981; Lopez-Ferber et al., 2003). In the case of baculoviruses, it has been shown that co-infection by a mixed population of viral genotypes contributes to the fitness of the virus population; the mortality caused by mixed genotype infections being higher than that of individual genotype infections (Lopez-Ferber et al., 2003). This genetic heterogeneity might be the result of selection pressures

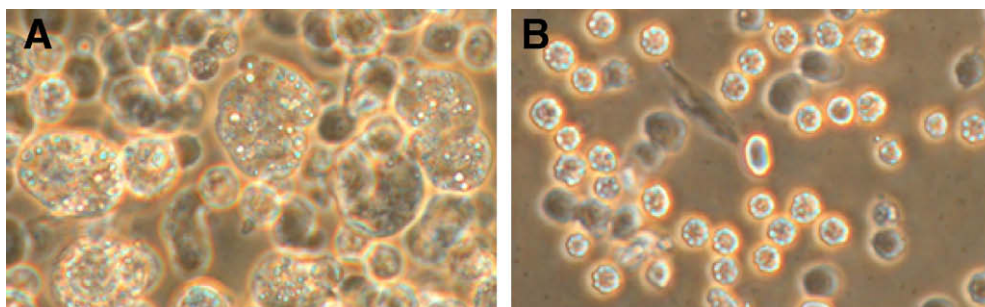


Fig. 1. Haemolymph smears from *C. pavonana* larvae: (A) inoculated with HvAV-3e for 6 days and (B) uninfected (pricked with sterile water). 40× magnification.

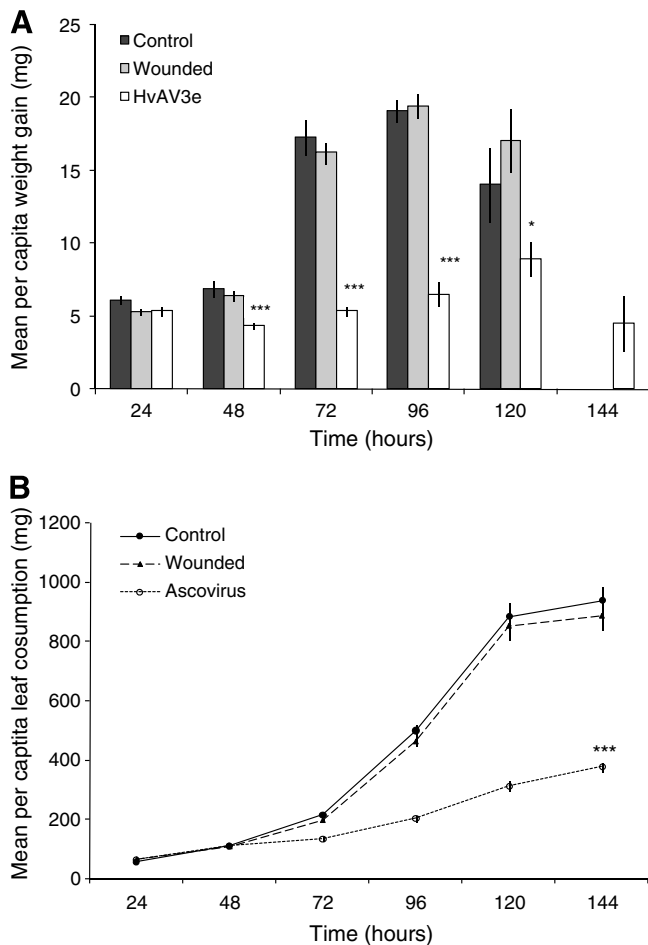


Fig. 3. (A) Mean daily weight gains of control *C. pavonana* larvae after pricking with sterile water (water) or HvAV-3e inoculum. For each 24 h feeding period columns marked by (^{*} $P < 0.05$) or (^{***} $P < 0.0001$) are significantly different to other columns within the feeding period. (B) Mean cumulative per capita cabbage leaf consumption by control *C. pavonana* larvae and *C. pavonana* larvae following pricking with water (water) or HvAV-3e inoculum as L2 larvae (^{***} = $P < 0.0001$).

Table 1
Sub-lethal effects of HvAV-3e on developmental parameters of *C. pavonana* following inoculation of second instar larvae

Treatment	Mean pupal weight ^A (mg) (\pm SE)	Median time to pupation ^B (days)
Control	47.93 (\pm 0.71)a	9.0a
Water	48.92 (\pm 0.73)a	8.0a
HvAV-3e	26.64 (\pm 1.50)b	11.5b

^A Means followed by a different letter are significantly different ($F_{2,47} = 134.9$; $P < 0.0001$).

^B Medians followed by a different letter are significantly different ($H = 28.3$; $DF = 2$; $P < 0.0001$).

imposed on the virus when replicating within a host or the result of replication in a range of hosts when it is transmitted from one host to another. In the latter scenario, a new host may support replication of certain variants that are not favoured by previous host(s). Although the genotypic composition of HvAV-3e has not been explored, we assume that the isolated HvAV-3e is a virus strain and not an isogenic virus clone. Therefore, we compared virus recovered from *C. pavonana* larvae 7 days following inoculation with original inoculum to ensure that the same genotypes replicated in *C. pavonana*. The RFLP analysis demonstrated that the virus was the same before and after replication in *C. pavonana*.

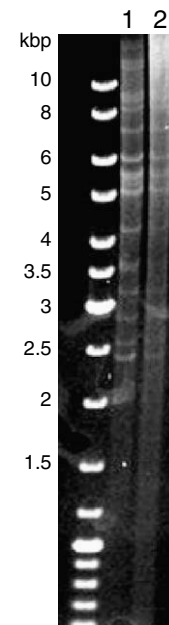


Fig. 4. RFLP analysis of HvAV-3e inoculum (1) and the virus recovered from *C. pavonana* L2 larvae 7 days after inoculation (2). Isolated genomic DNA (1 μ g) was digested with BamHI and analysed on a 0.7% agarose gel. Both samples produced the same restriction pattern.

In contrast to the arrested larval development observed in infected noctuid hosts (Federici, 1983; Govindarajan and Federici, 1990; Hamm et al., 1985), infected *C. pavonana* larvae continued to grow and moult and most developed into pupae. However, all larvae infected as 2nd instars that subsequently progressed to pupation died in the pupal stage (Fig. 2). The observation that infected *C. pavonana* larvae consumed less food than healthy individuals (Fig. 3) is also consistent with ascovirus infection in noctuids (Federici and Govindarajan, 1990; Hamm et al., 1985; Newton, 2004).

Larval parasitoids of *C. pavonana* are rarely reared from field collected material through much of its geographic distribution (Sastrosiwajo and Setiawati, 1992; Furlong, unpublished data). Parasitoids can be very effective vectors of ascoviruses (Tilman et al., 2004) and it is possible that the virus is also transmissible by larval parasitoids of *C. pavonana* where they occur. This leads to the possibility that many other lepidopterans are susceptible to ascovirus infection and the current view that ascoviruses are mainly restricted to noctuids is simply due to a lack of exhaustive surveys in other Lepidoptera. Compounding this could be the current finding that the principal diagnostic symptom of ascovirus infection (milky white haemolymph) is not observed in at least *C. pavonana*. The conundrum for the ascovirus–noctuid–wasp relationship is that the virus is spread by parasitoids yet the virus directly inhibits the wasp population. So how does the virus reach such high infection rates? Perhaps there are many hosts that support virus replication but the “typical” symptoms are only observed in noctuids. Extending this idea, the maintenance of a reservoir of infection in non-noctuid hosts which is transmitted to seasonally occurring noctuids by generalist parasitoids could help to explain how ascovirus returns so rapidly to noctuid populations.

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Effects of an ascovirus (HvAV-3e) on diamondback moth, *Plutella xylostella*, and evidence for virus transmission by a larval parasitoid

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ABSTRACT

Ascoviruses (AVs) are pathogenic to lepidopteran larvae, and most commonly attack species in the Noctuidae. The unique pathology includes cleavage of host cells into virion-containing vesicles which leads to the milky white colouration of the hemolymph as opposed to the clear hemolymph of healthy larvae. Recently, we showed that a *Heliothis virescens* AV (HvAV-3e) isolate is able to induce disease in *Crociodolomia pavonana* F. (Lepidoptera: Crambidae), affecting feeding, growth and survival of infected larvae. In this study, we investigated the effect of different variants of HvAV-3e on diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) larvae, another non-noctuid host. In hemolymph inoculation bioassays fourth instar larvae showed a significant dose response to each of the HvAV-3e variants and significant differences between the virulence of the three variants were detected. Both second and fourth instars were readily infected with the virus and infected individuals demonstrated significant reductions in food consumption and growth. The majority of infected individuals died at the larval or pupal stage and individuals which developed into adults were usually deformed, less fecund than non-infected controls and died shortly after emergence. In transmission studies, *Diadegma semiclausum* (Hymenoptera: Ichneumonidae), a key parasitoid of diamondback moth, infected healthy host larvae during oviposition following previous attack of HvAV-3e infected hosts. In choice tests *D. semiclausum* did not discriminate between infected individuals but host infection had no detectable impact on the development of immature *D. semiclausum* or on subsequent adults.

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1. Introduction

Ascoviruses are large, dsDNA viruses that infect Lepidoptera (Federici, 1983). They cause a chronic, fatal infection in the larvae of host species in which typical symptoms of infection are loss of appetite, stunted growth and a milky hemolymph (Federici, 1983; Hamm et al., 1985). Once the virus enters a cell, the cell enlarges and the plasma membrane invaginates forming many intracellular vesicles. The milky appearance is due to the liberation of these virion-containing vesicles that are released from lysing cells (Federici, 1983; Federici and Govindarajan, 1990; Federici et al., 1990). Ascoviruses have a relatively low transmissibility via ingestion (Govindarajan and Federici, 1990) and the primary mode of transmission is by parasitoid wasps during oviposition. To date, there are 5 recognised species of ascovirus: *Spodoptera frugiperda* ascovirus (SfAV-1), *Trichoplusia ni* ascovirus (TnAV-2), *Heliothis virescens* ascovirus (HvAV-3), *Diadromus pulchellus* ascovirus (DpAV-4) and *Spodoptera exigua* ascovirus (SeAV-5) (Federici et al., 2005). Four of these species (SfAV-1, TnAV-2, HvAV-3 and

SeAV-5) are closely related phylogenetically and have been predominantly reported from the Noctuidae. The fifth species, DpAV-4, is the only AV named after its parasitoid vector, *D. pulchellus*, and infects pupae of its host *Acrolepiopsis assectella* (Lepidoptera: Plutellidae) (Bigot et al., 1997).

Recently, it was reported that HvAV-3e causes a lethal infection in *Crociodolomia pavonana* (Lepidoptera: Crambidae), although the disease pathology was subtly different to that in noctuids (Smede et al., 2008). *C. pavonana* showed a marked reduction in appetite and stunted growth following infection, but most infected larvae developed to the pupal stage before succumbing to the disease. In contrast, infected noctuids tend to lag healthy hosts in the larval stage and death finally occurs up to 30 days post-infection (Federici and Govindarajan, 1990). The other key difference noted was that *C. pavonana* did not develop the characteristic milky hemolymph seen in noctuids. However, enlarged vesicle-containing cells could still be seen by light microscopy (Smede et al., 2008).

In this study we sought to determine if DBM is susceptible to HvAV-3e, examined the effects of infection on the pest and investigated whether *D. semiclausum* could transmit the virus between infected and healthy individuals.

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2. Methods and materials

2.1. Insects

Plutella xylostella was reared on leaves of glasshouse grown potted cabbage plants (*Brassica oleracea* var. *capitata* cv Warrior) in a controlled temperature cabinet (23 ± 2 °C, photoperiod of 12:12 [L:D] h, 60% RH). Adults were fed on aqueous honey solution [10% (wt/vol)] and supplied with fresh potted cabbage plants for oviposition daily.

Diadegma semiclausum was reared on *P. xylostella* and adults were fed with aqueous honey solution [10% (wt:vol)] as a food source.

2.2. Virus

Three variants of *H. virescens* ascovirus (HvAV-3e) were isolated from *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) collected from cotton farms in southeast Queensland; these are referred to as variants 1–3. Variant-1 was the original isolate previously reported (Asgari et al., 2007).

Laboratory stocks of each HvAV-3e variant were amplified in *Spodoptera litura* F. (Lepidoptera: Noctuidae) larvae. The inoculum used for subsequent infections was produced by collecting the hemolymph from *S. litura* larvae 6–7 days post AV infection. To collect hemolymph, larvae were surface sterilized in 70% ethanol and dried. One of the prolegs was cut using a pair of fine forceps and the hemolymph was collected in an Eppendorf tube (1.5 ml) and then stored at -80 °C until used in experiments.

2.3. Ascovirus DNA isolation and RFLP analysis

Virus DNA was isolated as previously described (Smede et al., 2008). A restriction fragment length polymorphism (RFLP) analysis was conducted on each of the three variants of HvAV-3e. Four μ g of viral genomic DNA was used in single enzyme digests with either *Bam*HI or *Hind*III restriction enzymes. The digests were run on an 8% agarose gel overnight containing ethidium bromide and viewed under UV light.

2.4. Ascovirus dose response assays

The concentration of virus-containing vesicles in stock solutions of each of the three AV variants was determined by making a 1000-fold dilution of viral stock in sterile water and then counting the virus-containing vesicles (vcv) in the solution in a hemocytometer (variant-1 = 3.3×10^8 vcv ml⁻¹; variant-2 = 2.7×10^8 vcv ml⁻¹; variant-3 = 1.0×10^9 vcv ml⁻¹). Five 10-fold dilutions of the viral stock of each variant were then made in sterile water and used in the assays. Fourth instar (L4) larvae were anaesthetised on a cooling table (surface temperature -2 °C) and then individuals were inoculated with one test concentration by pricking the larva along its dorsal surface with a UV-sterilised minuten pin that had been dipped into the inoculum. For variants 1 and 2, 60 insects were treated with each test concentration and a further 60 were treated with sterile water in the same manner to serve as a control. For variant 3, 30 insects were treated with each test concentration and sterile water as the control. Treated and control larvae were reared in groups of 10 in Petri dishes containing a moistened filter paper and incubated (25 ± 2 °C; L:D 12:12 h). Larvae were supplied with fresh cabbage leaf as required and monitored daily until death or the eclosion of adult moths.

2.5. Effect of ascovirus infection on larval feeding and growth

One hundred and fifty 2nd instar (L2) DBM were selected without bias from a rearing box. They were randomly split into groups of five larvae and each group was then weighed. Fifty L2 were inoculated with HvAV-3e variant-1 as described above, 50 were treated with sterile water and the remaining 50 were left untreated. Each group of five larvae was then placed in a Petri dish containing 5 ml of 2% (w/v) tap-water agar and a weighed freshly cut common cabbage leaf disc (2 cm diameter). Each day each group of five larvae and the leaf disc upon which they had been feeding was weighed. Larvae were then returned to the Petri dish and supplied with a fresh, weighed leaf disc. The experiment was terminated when larvae ceased feeding and entered the pre-pupal stage.

2.6. Effect of larval infection on adult fecundity

One hundred female and 100 male DBM L4s (sex determined by the presence or absence of visible male gonads) were selected from a rearing box. Sexed larvae were then separated into single sex cohorts of 10 insects and held in Petri dishes lined with a moistened filter paper. From these cohorts 20 female L4s and 50 male L4s were left untreated, 20 of each sex were treated with sterile water and 60 female L4s and 30 male L4s were inoculated with variant-1 as described above. Larvae were supplied with a portion of fresh common cabbage leaf and incubated at 25 ± 2 °C; L:D 12:12 h. Insects were monitored daily, mortality was recorded and larvae were supplied with fresh leaves as required. As soon as larvae developed to the pupal stage they were removed from the Petri dish, weighed and transferred singly to Petri dishes. Upon emergence female insects from each treatment were paired with a male insect from the control treatment in a clean Petri dish and supplied with a small ball of cotton wool saturated with aqueous honey solution [10% (wt:vol)] as a food source and a common cabbage leaf disc (4.5 cm diameter) as an oviposition substrate. After 24 h the leaf disc was removed and replaced with a fresh leaf disc; the number of eggs laid upon the first leaf disc was then counted under a binocular microscope. This process was repeated daily until the death of each female moth when the total number of eggs laid by each during its lifetime was calculated.

2.7. Effect of larval infection on host selection by *D. semiclausum*

The preference of *D. semiclausum* female wasps for AV-inoculated or non-infected healthy larvae was investigated in choice tests. One hundred and fifty L2 DBM larvae were inoculated with HvAV-3e variant-1 by pricking with an inoculated minuten pin as described above. Single larvae were then transferred to single Petri dishes containing a small (2.0 cm diameter) cabbage leaf disc and incubated (20 °C; L:D 12:12 h). One hundred and fifty untreated L2 larvae were also transferred singly to Petri dishes containing a small (2.0 cm) cabbage leaf disc and incubated under the same conditions. After 3 days, 50 inoculated larvae and 50 untreated larvae were randomly selected from each group. The abdomen of each untreated larva was then delicately marked with a fine-pointed red coloured permanent marker pen so that they could be readily identified. Fifty Petri dishes were lined with a filter paper and furnished with a cabbage leaf disc (3.0 cm diameter). A single AV-treated larva and a marked untreated larva were then introduced into five of the dishes and they were arranged on a white plastic tray on the laboratory bench and larvae were allowed to establish feeding sites (approximately 10 min after introduction). A single mated *D. semiclausum* female (1–3 days post eclosion) was then introduced into each dish and observed by two experimenters for a maximum of 10 min. The first larva to be attacked by each *D. semiclausum* in each dish was recorded; dishes

in which the parasitoid did not attack a larva within 10 min were discarded. Each parasitoid was used only once and all were discarded at the end of the experiment. The following day, four days after inoculation of the larvae, the experiment was repeated with another 50 of the inoculated and 50 of control larvae and a fresh batch of parasitoids. Six days after the inoculation of larvae the experiment was conducted for a third time using the remaining larvae and a fresh batch of parasitoids.

2.8. Transmission assay

Approximately 30 L2 DBM larvae were inoculated with HvAV-3e variant-1 and 30 control larvae were treated with sterile water as previously described. Larvae were transferred in groups of five to Petri dishes containing a portion of cabbage leaf and a moistened filter paper and incubated (20 °C; L:D 12:12 h) for 6 days. Ten surviving inoculated larvae (infection confirmed by examination under a light microscope on completion of the experiment) and 10 surviving control larvae were selected and then transferred singly to Petri dishes which contained a portion of cabbage leaf. A single newly emerged mated *D. semiclausum* female was then introduced to each dish and observed until the provided host was attacked. The parasitoid was then immediately removed and placed in a second oviposition arena containing three healthy L2 DBM larvae. The parasitoid was again observed until one of the healthy hosts was attacked and then both the parasitoid and the freshly attacked host were isolated. The attacked host larva was reared for 6 days (20 °C; L:D 12:12 h) and then stored at –80 °C prior to DNA analysis to confirm the presence or absence of AV infection. Total DNA extraction was performed for each insect as described previously (Glatz et al., 2003) and then PCR was performed using primers specific to *orf28* of HvAV-3e. PCR conditions were 95 °C for 5 min initial denaturation followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by the final extension at 72 °C for 5 min.

2.9. Effect of ascovirus on *D. semiclausum* development

To provide AV-infected hosts, 20 L2 DBM were inoculated with variant-1 as previously described and then incubated at 20 °C (L:D 12:12 h) for 6 days. Five days after these insects were inoculated, a further 40 L2 DBM larvae were inoculated with variant-1 in the same manner. These larvae, together with a further 45 untreated L2s, were then transferred singly to Petri dishes containing 5 ml of 2% (w/v) tap-water agar and a freshly cut common cabbage leaf disc (2 cm diameter) and incubated at 20 °C. After 24 h larvae were examined, any larvae which had died as a result of inoculation were discarded. Fifteen of the larvae inoculated 6 days previously were selected and transferred singly to Petri dishes containing a moistened filter paper and a common cabbage leaf disc (2 cm diameter). Larvae were allowed 5 min to settle and then a single female *D. semiclausum* (1–3 days post eclosion) was introduced into each dish. Each parasitoid was observed carefully and as soon as it attacked the infected host larva it was removed and transferred to one of the Petri dishes containing an untreated L2. Each parasitoid was again watched carefully until it attacked the L2 and then it was removed and discarded. Next, a single fresh female *D. semiclausum* (1–3 days post eclosion) was introduced into each of the Petri dishes containing an L2 that was inoculated with variant-1 24 h previously; each parasitoid was observed carefully until it attacked the host. Finally, a single fresh female *D. semiclausum* (1–3 days post eclosion) was introduced into each of the Petri dishes containing a healthy untreated L2 and observed until it attacked the host whereupon it was removed from the dish and discarded. All DBM L2s attacked (15 healthy larvae attacked by a parasitoid immediately after oviposition into an infected host, 15 healthy larvae at-

tacked by a fresh uncontaminated parasitoid and 15 larvae manually inoculated with variant-1 24 h previously and then attacked by a fresh uncontaminated parasitoid), 15 manually inoculated larvae not exposed to *D. semiclausum* and 15 larvae which were neither inoculated nor exposed to *D. semiclausum* were incubated at 20 °C. Larvae were examined twice daily and supplied with fresh cabbage leaf discs if required. For each individual, mortality, the time to parasitoid cocoon development, the duration of the parasitoid pupal period and the longevity and dry weight of adults was recorded.

2.10. Statistical analysis

Dose response data were subject of logit analysis in Poloplus (LeOra software, 2002). All other statistical tests were conducted in Statview (SAS Institute Inc., 1998). Where appropriate, treatments were compared by ANOVA and means separated by LSD. When data were not normally distributed they were compared by the Mann–Whitney *U*-test when there were two treatment groups and by the Kruskal–Wallis test when there were three treatment groups. Choice data were compared by the χ^2 -test.

3. Results

3.1. Restriction fragment length polymorphism (RFLP) analysis

The RFLPs produced unique restriction profiles for all three HvAV-3e variants with each enzyme (Fig. 1), indicating that each had a distinct genetic profile. Sequencing of DNA polymerase (*orf1*) and thymidine kinase (*orf117*) genes indicated that these three isolates are indeed variants of HvAV-3e described previously (Asgari et al., 2007).

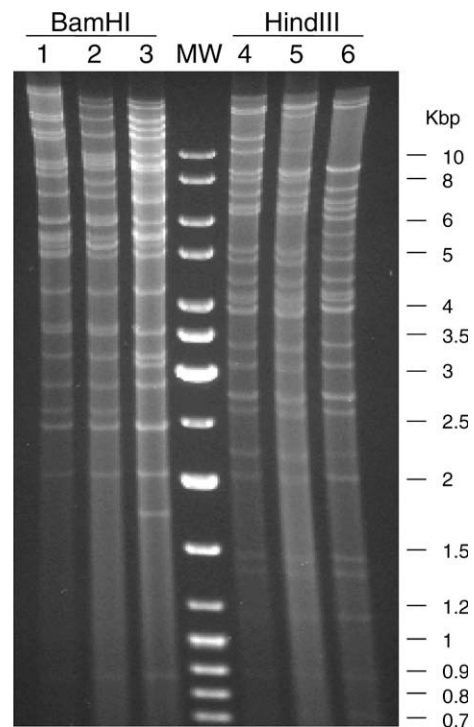


Fig. 1. RFLP of 3 different isolates of HvAV-3e. Lanes 1 and 4: variant-1 (original isolate), lanes 2 and 5: variant-2, lanes 3 and 6: variant-3. Lanes 1–3 were digested with *Bam*HI. Lanes 4–6 were digested with *Hind*III. MW is GeneRuler molecular mass marker.

3.2. Ascovirus dose response assays

DBM larvae were readily infected by HvAV-3e and enlarged vesiculated cells were detected in hemolymph smears (Fig. 2A). The pathology was not observed in any hemolymph smears prepared from control larvae pricked with sterile water (Fig. 2B). The dose–response assays revealed that DBM is susceptible to HvAV-3e, but that the virulence of each variant to DBM varied significantly (Table 1). Variant-1 was the most virulent of the three variants and comparison of LC_{50} values indicated that it was approximately 261-times as virulent as variant-2 and 31-times as virulent as variant-3.

3.3. Effect of ascovirus infection on larval feeding and growth

Control larvae and larvae treated with sterile water completed development and ceased feeding by day 6, whereas larvae inoculated with variant-1 did not cease feeding and pupated at day 8 after inoculation at the earliest. Despite this longer development period the total amount of leaf material eaten by larvae inoculated at L2 was significantly lower than that consumed by control larvae and larvae treated with sterile water ($F_{2,17} = 4.76$; $P < 0.023$) (Fig. 3A). There was no significant difference between the amount of leaf material consumed by control and sterile water treated larvae (LSD, $P > 0.05$; Fig. 3A). Similarly, by day 6 after treatment larvae inoculated with variant-1 weighed significantly less than larvae treated with sterile water or larvae which were left untreated ($F_{2,17} = 9.70$; $P = 0.0025$; Fig. 3B). There was no significant

difference between the weights of control and sterile water treated larvae (LSD, $P > 0.05$; Fig. 3B).

3.4. Effect of larval infection on adult fecundity

When inoculated with variant-1 as early L4 larvae, only 49% of treated larvae formed pupae while 93% of untreated and 91% of larvae treated with sterile water formed pupae (Table 2). Overall, only 9% of AV-inoculated larvae developed to the adult stage and many were deformed (Fig. 2C) when compared with healthy adults (Fig. 2D). In stark contrast, the control groups yielded 83% (untreated control) and 89% (sterile water treated) adults, respectively (Table 2); none of which were deformed. However, there was no significant difference between the weights of pupae in any of the treatment groups ($F_{2,74} = 1.257$; $P = 0.291$; Table 2). Inoculation of female larvae with AV significantly reduced the fecundity of those individuals which completed development and emerged as adults when compared with the fecundity of moths which developed from either group of control larvae ($F_{2,27} = 5.51$; $P = 0.009$; Table 2).

3.5. Effect of larval infection on host selection by *D. semiclausum*

D. semiclausum females did not discriminate between healthy larvae and larvae previously inoculated with variant-1. Of the 30 parasitoids which responded when given a choice of healthy larvae and larvae inoculated with variant-1 three days previously, 11 attacked an infected host first while 19 parasitoids attacked a

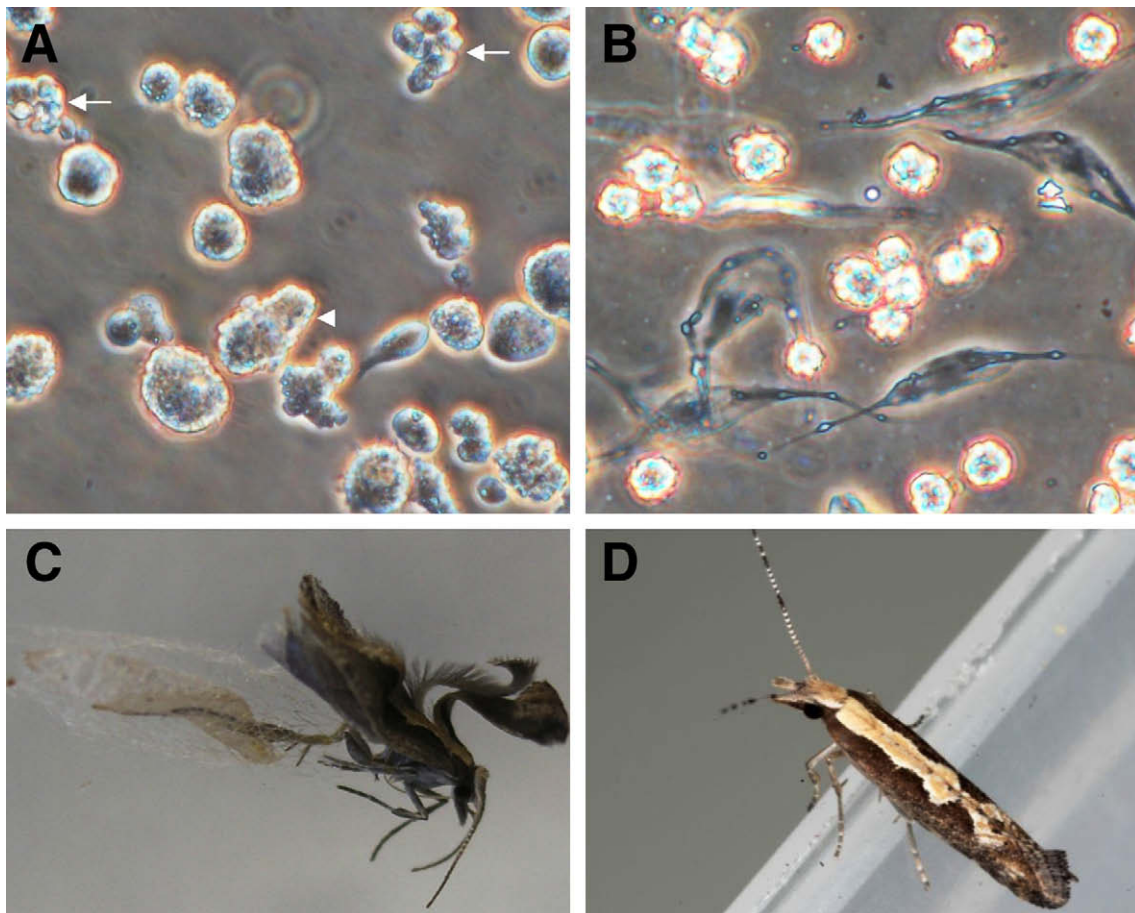
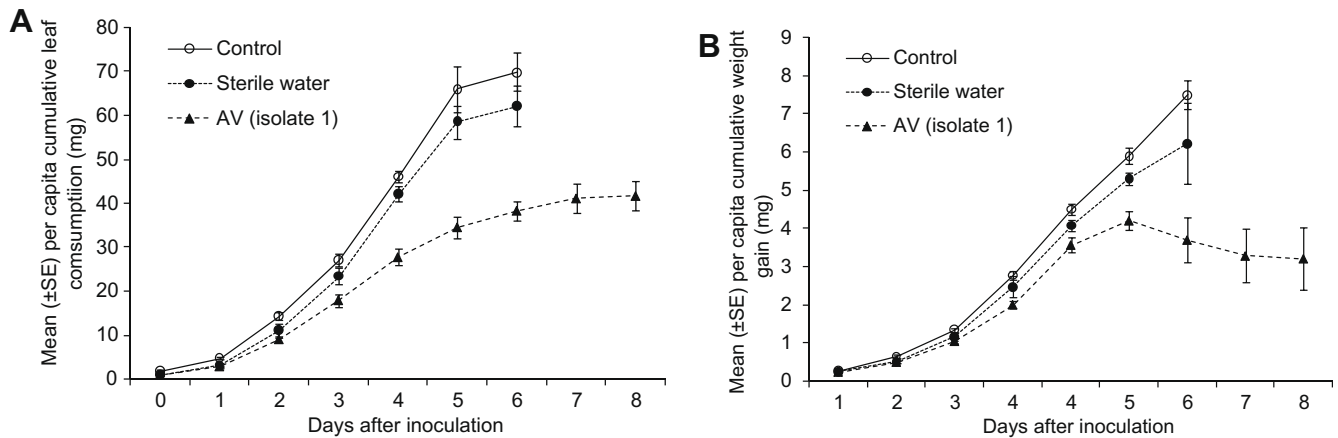


Fig. 2. Hemolymph smears from DBM larvae: (A) inoculated with HvAV-3e variant-1 and incubated for 6 days; and (B) non-infected (pricked with sterile water). 40 × magnification. DBM adults developing from (C) L4 larvae infected with HvAV-3e variant-1 and (B) control (water).

Table 1

Dose response of L4 DBM larvae to three variants of HvAV-3e following manual inoculation with a minuten pin.

Variant ^a	n	LC ₅₀ (95% confidence interval)	Slope (±SE) ^b	Heterogeneity (df)
1	360	8.38 × 10 ⁵ (1.71 × 10 ⁵ –2.66 × 10 ⁶)	0.60 (±0.12)	0.151 (28)
2	360	2.19 × 10 ⁸ (1.05 × 10 ⁸ –6.86 × 10 ⁸)	1.64 (±0.42)	0.195 (28)
3	180	2.62 × 10 ⁷ (4.75 × 10 ⁶ –1.48 × 10 ⁹)	0.93 (±0.20)	1.825 (13)

^a Regression lines for the dose response of *P. xylostella* larvae to each AV variant were significantly different from each other (χ^2 (df = 4) = 85.84; $P < 0.001$).^b Slopes significantly different (χ^2 (df = 2) = 10.03; $P = 0.006$).**Fig. 3.** Effect of HvAV-3e variant-1 on DBM larvae. (A) cumulative *per capita* food consumption prior to pupation. (B) cumulative *per capita* weight gain of DBM larvae following inoculation.**Table 2**

Effect of inoculation of L4 DBM larvae with HvAV-3e variant-1 on development, survival and fecundity of subsequent adults.

Treatment	n	Proportion of insects developing to		Mean pupal weight (±SE) (mg) ^a	Mean no. eggs per female (±SE) ^a
		Pupae	Adults		
Control	70	0.93	0.83	552 (±15)a	183 (±27)a
Sterile water	40	0.91	0.89	539 (±18)a	236 (±18)a
AV-variant-1	90	0.49	0.09	507 (±23)a	113 (±21)b

^a Within these columns means marked by a different letter are significantly different ($P < 0.05$). For pupal weights ($F_{2,74} = 1.257$; $P = 0.291$) and for egg production ($F_{2,27} = 5.51$; $P = 0.009$; means separated by LSD, $P < 0.05$).

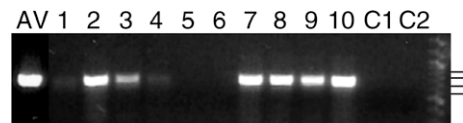
healthy host larva first (χ^2 (df = 1) = 0.122; $P > 0.05$). Similarly, when parasitoids were given a choice of healthy larvae or larvae inoculated with variant-1 4 or 6 days previously there was no preference for infected or healthy hosts detected (4 days post inoculation: 30 parasitoids responded, 14 attacked infected larvae first and 16 attacked healthy larvae first (χ^2 (df = 1) = 0.067; $P > 0.05$); 6 days post inoculation: 30 parasitoids responded, 12 attacked infected larvae first and 18 attacked healthy larvae first (χ^2 (df = 1) = 0.600; $P > 0.05$).

3.6. Transmission assays

AV infection was detected by PCR in 8 of the 10 healthy hosts attacked by female *D. semiclausum* parasitoids that had previously attacked an infected host. AV infection was not detected in control larvae that were attacked by uncontaminated parasitoids (Fig. 4).

3.7. Effect of ascovirus on *D. semiclausum* development

All DBM larvae which were neither inoculated with variant-1 nor exposed to *D. semiclausum* parasitism successfully completed development to adult moths. All DBM larvae which were inoculated with variant-1 but not exposed to *D. semiclausum* parasitism completed development to pupae but all died at that stage (Ta-

**Fig. 4.** Transmission of HvAV-3e to DBM L2 larvae by *D. semiclausum*. A pair of primers specific to *orf28* was used for PCR detection. AV, HvAV-3e genomic DNA; 1–10, healthy larvae subsequently attacked by “virus-carrying” wasps and collected 6 days post-attack; C1 and C2, healthy larvae subsequently attacked by “sterile water-carrying” wasps and collected 6 days post-attack. Molecular marker bars shown on the right represent 600, 700, 800 and 900 bps from bottom to top.

ble 3). DBM inoculated with variant-1 took significantly longer to develop to the pupal stage (median development time = 6.5 days) than healthy control larvae (median development time = 5 days) (Table 3). There were no significant differences between the egg-pupa development times of *D. semiclausum* developing in hosts manually inoculated with variant-1 prior to parasitism, hosts parasitised immediately after oviposition in a variant-1 infected host or in healthy control hosts ($H = 2.75$ (DF = 2); $P = 0.253$ (Table 3)). Similarly, host infection status had no significant effect on the duration of the pupal period of *D. semiclausum* ($H = 5.76$ (DF = 2); $P = 0.06$), the longevity of adults after eclosion ($H = 0.239$ (DF = 2); $P = 0.888$) or the dry weight of adults ($F_{2,24} = 2.96$; $P = 0.07$).

Table 3
Development of *Diadegma semiclausum* in healthy and AV-inoculated (variant-1) DBM hosts.

Treatment	Median <i>D. semiclausum</i> development time (days) ^b			Mean adult dry weight (±SE) ^b
	Egg-pupa	Pupal period	Adult longevity	
Control ^a	10	8	4	55.3 (±2.5)
Manual AV- inoculation of host	9	8	3	51.7 (±2.8)
Previous oviposition in AV-infected host	9	9	3	61.5 (±3.1)

^a In other controls, all 15 AV-inoculated DBM died as pupae and all 15 DBM which were neither inoculated with AV nor exposed to parasitism survived to adulthood. AV-inoculated DBM larvae took significantly longer to reach the pupal stage (median time = 6.5 days) than non-inoculated DBM larvae (median time = 5 days) $Z = 2.245$; $P = 0.025$.

^b For *D. semiclausum*, host infection status had no significant effect on the development time from egg-pupa ($H = 2.75$ (DF = 2); $P = 0.253$), the duration of the pupal period ($H = 5.76$ (DF = 2); $P = 0.06$), the longevity of adults after eclosion ($H = 0.239$ (DF = 2); $P = 0.888$) or the dry weight of adults ($F_{2,24} = 2.96$; $P = 0.07$).

4. Discussion

The different variants of HvAV-3e had different virulences against DBM (Table 1). Although DBM was readily infected by the three variants of HvAV-3e tested, disease symptomology was not typical of that described in noctuid hosts (Federici, 1983; Hamm et al., 1985). In DBM, infection slowed larval development (Fig. 3B and Table 3) but was more likely to cause death in the pupal stage or lead to the eclosion of a deformed adult (Table 2). In infected noctuid hosts, arrested larval development typically precedes death in that stage and precludes pupal development. In addition, cell symptomology was similar to what is observed in infected cell lines, Sf9 and HzFB (Asgari, 2006). DBM also showed a marked reduction in food consumption when infected with AV (Fig. 3A). This is consistent with other hosts described and the overall disease profile of HvAV-3e in DBM was similar to that reported in another novel host, *C. pavonana* (Smede et al., 2008).

Ascovirus infected DBM adults were 39–52% less fecund than healthy controls (Table 2) and it is likely that under field conditions the extensive deformities suffered by many adults as a result of infection (Fig. 2C) would result in their complete inability to reproduce. Although not investigated here, it is also likely that flight ability and adult lifespan would be compromised in infected DBM, both of which are factors in an individual's ability to mate and reproduce.

The study also showed that HvAV-3e is transmissible to healthy DBM larvae by *D. semiclausum* following oviposition in heavily infected hosts, although with variable transmission outcomes (Fig. 4). It is interesting that AV was not readily detected from *D. semiclausum* by PCR even when they had transmitted the disease to healthy hosts (data not shown). This is probably attributable to the fact that DBM is a semi-permissive host to the virus. As a result, the number of viral progeny produced is likely to be low and the number of virions acquired by the parasitoid ovipositor limited. In addition, we tested the wasps after subsequent oviposition in healthy larvae. This is likely to remove some of the viral particles from the ovipositor. A previous study showed that the length of the parasitoid ovipositor is an important determinant of the efficiency with which parasitoids are able to transmit AV infection in subsequent oviposition attempts (Tillman et al., 2004). The longer the ovipositor, the more efficient the vector, due to the attachment of more virions to longer ovipositors. *D. semiclausum* has relatively a short ovipositor and this might contribute to acquisition of few virions by the ovipositor during oviposition into an infected host. The small number of acquired virions could then be rapidly lost in subsequent oviposition attempts. Further, when the hemolymph from infected larvae was viewed by light microscopy neither the characteristic circulating vesicles nor the enlarged vesiculated cells were observed (Fig. 2A). Rather, the cells took on an appearance similar to that observed in a fat body cell line from *Helicoverpa zea* (Asgari, 2006). Consequently, in the absence of production of large numbers of vesicles in DBM, only a small number may adhere to the ovipositor.

Although *D. semiclausum* is capable of transmitting the AV to healthy hosts that it parasitises, host infection appears to have little impact on the developing parasitoid (Table 3). Healthy parasitoids readily developed within host larvae which were manually inoculated with doses of variant-1 which killed non parasitised hosts, thus transmission of the AV by infected parasitoids is unlikely to have a detrimental effect on parasitoid populations.

This is now the second detailed study on the effects of HvAV-3e on a non-noctuid host. Both studies have shown that the investigated hosts, *C. pavonana* and DBM, were susceptible to the virus. Although the symptoms were not as severe or obvious in these novel hosts, the overall effects were the same as have been previously described for noctuid hosts. This suggests that AVs may be much more widespread in lepidopteran populations than previously thought. In noctuids it has been shown that AVs are readily transmitted by mechanical means, including parasitoid wasps during oviposition, while they are poorly transmitted by ingestion (Govindarajan and Federici, 1990). DBM has many parasitoids associated with it and so it might reasonably be expected that they too could transmit AV from infected to healthy hosts. In addition, the fact that the AV does not seem to have detrimental effects on the development of the parasitoid in semi-permissive hosts such as DBM or *C. pavonana* suggests that these may provide alternative hosts that facilitate maintenance of AV populations in the field.

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