# Exploring a novel strategy to enhance efficacy of insect pathogens and disrupt cuticle hardening in insects

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**Final Report**

# Exploring a novel strategy to enhance efficacy of insect pathogens and disrupt cuticle hardening in insects

**HAL Project No: VG08053**

(28 July 2011)

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This final report provides all the experimental procedures and results obtained from the HAL-funded project VG08053 "*Exploring a novel strategy to enhance efficacy of insect pathogens and disrupt cuticle hardening in insects*".

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# Table of contents

## Media Summary

Resistance to chemical pesticides and issues with environmental contamination requires continues research to explore non-chemical alternatives. Microorganisms used as microbial biocontrol agents provide environmentally friendly alternatives with relatively low risk of development of resistance by insect pests.

Parasitic insects inject a variety of components into the body of their host larvae. This ensures successful development of their progeny by modifying their host's immune system. One of the major immune responses to microorganisms that are used for biological control of insect pests is the melanization response which involves formation of a dark-brown precipitate which eliminates the pathogen. In various cases, the failure/inefficiency of microbial control agents can be traced back to the immune resistance of host insects.

Using natural components that can interfere with insect immune responses could potentially be utilized to inhibit insect immune responses and therefore increase the efficacy of microbial control agents. In this project, we studied the mechanisms of action of a venom protein isolated from a parasitoid wasp in inhibition of melanization and its effect in a genetically modified insect and a biopesticide. We found that:

- several serum components from the host bind to the venom protein upon exposure, especially those key enzymes involved in the melanization cascade;
- the intact venom protein is required for its mode of action and none of the functional components of the protein on their own were able to inhibit melanization;
- production of the venom protein in a genetically modified model insect (fruit fly) made it vulnerable to fungal infection due to suppression of it immune system; however, the protein did not have a major effect on the fly's development or on its parasitoid success in parasitism;
- when the venom protein was produced in a microbial control agent (a baculovirus) it significantly enhanced its efficacy as significantly less virus was required to kill the host compared to the wild-type virus; however, it was found that this enhancing effect may not occur in all host-virus interactions

Overall, the outcomes provide evidence that immune suppressors may offer an option in enhancing the efficacy of microbial control agents, either by producing genetically modified insects that are more susceptible to microbial pathogens or genetically modified microbial control agents that have a superior efficacy over wild-type strains. Considering the effect of the venom protein in enhancing the efficacy of baculoviruses, it will be worth constructing genetically modified baculoviruses based on species present in Australia and test them against a variety of insect pests, which requires further R&D funding.

## Technical summary

Melanization is a key component of the insect immune system. Parasitoid wasps introduce a variety of components into the body of their host at the time of parasitization to interfere with the melanization response, as well as other immune components involved in insect immunity. The effects of a wasp venom protein of 50 kDa in size, Vn50, from *Cotesia rubecula* (Braconidae) on the melanization cascade of insects were studied. Firstly, by producing and purifying Vn50 and its derivatives (with truncations in clip and the protease-like domains) by constructing recombinant viruses, we showed that none of the derivatives are as effective as the full-length protein. Therefore, both domains are essential for Vn50 activity. Secondly, we generated a stable line in the model insect, *Drosophila melanogaster*, which ectopically expressed Vn50. Results indicated that Vn50 reduces melanization in the haemolymph of transgenic flies as well as causing accelerated larval development and increased oviposition in the transgenic flies. Furthermore, we examined the impact of Vn50 expression on susceptibility of the transgenic flies to pathogens. Transgenic Vn50 flies challenged with the fungus *Beauveria bassiana* had increased mortality compared to control flies, but no significant change in survival was seen in flies challenged with the pathogenic bacteria, *Serratia marcescens*. Interestingly, mortality induced by the natural pathogen *Drosophila* C virus was significantly delayed in Vn50-expressing flies. The ability to protect against the parasitoid, *Leptopilina boulardi*, was also explored. While no difference in success of parasitism or fitness, in terms of weight and wing length, was seen in parasitoids emerging from the two fly lines, parasitoids emerged slightly earlier from the Vn50-expressing flies and the sex ratio of parasitoids that had emerged tended towards a higher proportion of females. This suggested that the Vn50-expressing flies are a more suitable host. In addition, genetically modified baculoviruses producing the venom protein were significantly more efficient than the wild-type as far less virus inoculum was required to kill the host; although this effect was found to be species-specific. These studies indicate a wider range of potential hosts that may be affected by Vn50 and its potential for manipulation of the immune system in insects to improve the efficacy of microbial control agents.

# 1. Introduction

Insect are very prosperous animals on earth, owing this partly to their very effective immune system which is able to recognize foreign intruders and mount a response to eliminate them. Successful development of endoparasitoids inside the insect host is dependent on a variety of components that accompany the egg upon parasitization to suppress the host immune system. These include venom, ovarian proteins, and virus/virus-like particles that are involved in the regulation of host physiology. Endoparasitoid venoms are rich sources of biomolecules that consist mainly of peptides and proteins, which play key roles in venom metabolism and host-parasitoid interactions. Suppression of the host immune system, either independently or in association with mutualistic viruses, and developmental alterations are the main functions of venom from endoparasitoids.

Melanization is a key component of the insect immune system which involves formation of a solid black-brown precipitate comprising of polymerized phenolic compounds (Söderhäll & Cerenius, 1998). The melanin and the intermediate components produced during melanization are toxic to both host and parasites and must be strictly regulated and localised so that it does not harm the insect (Cerenius & Söderhäll, 2004). Some insect endoparasitoids inject components which target various steps in the melanin formation pathway and the effects of these components and their binding interactions may be used to gain a greater understanding of the metabolic intermediates in the melanization cascade (Asgari, 2011). A venom protein named Vn50 was previously isolated from the venom of the endoparasitoid wasp, *Cotesia rubecula* (Hymenoptera: Braconidae), which showed inhibition of melanization in the host blood (haemolymph), *Pieris rapae* (Asgari *et al.*, 2003a) and then further tested in *Manduca sexta* where it showed a similar property (Zhang *et al.*, 2004). It inhibits activation of a key enzyme (prophenoloxidase) in the host haemolymph that controls melanization (Asgari *et al.*, 2003a; Zhang *et al.*, 2004).

The venom protein Vn50 shows significant structural similarity to a host protein which activates a key enzyme in the melanization process. By binding to this key enzyme, instead of the host protein, Vn50 inhibits activation of the enzyme and therefore no melanization occurs. This project was undertaken to increase our understanding of a key control point in melanin formation by looking into basic insights into insect immunity in general but also testing its potential value in applied pest control. Towards this goal, the following specific aims were followed:

**Aim 1:** Identify insect haemolymph components that interact with Vn50,

**Aim 2:** Establish structure-activity relationships for Vn50, via mutagenesis and in vitro binding,

**Aim 3:** Test the immune suppression capacity of Vn50 and its derivatives in the model system, *Drosophila melanogaster*,

**Aim 4:** Assess the effect of Vn50 on baculovirus efficacy as a pest control agent in non-permissive insects.

## 2. Materials & methods

*2.1. Construction of a recombinant baculovirus expressing Vn50*

To express Vn50 as a secreted protein, we expressed the full-length protein in an insect cell line derived from *Spodoptera frugiperda* (Sf9) using a baculovirus expression system. The protein was expressed in fusion with 6×histidine residues at the N-terminus to provide a tag to facilitate purification of the protein. We used the Bac-to-Bac baculovirus expression system kit manufactured by Invitrogen. Sf9 cells were maintained as monolayers in culture flasks using a serum-free medium (SF900II, Invitrogen) at 27°C and subcultured every week. First, the full-length gene was amplified by polymerase chain reaction (PCR) using a previous Vn50 clone to insert suitable restriction sites at the ends for cloning into the baculovirus transfer plasmid vector. After successful amplification of the gene, it was digested with restriction enzymes, ran on an agarose gel, DNA purified and ligated into the transfer vector pFastBac-HT-a, which was also digested with relevant enzymes (*Bam*HI and *Kpn*I). Clones were screened by colony PCR using specific primers to the vector to confirm the presence of an insert. Following this, purified plasmids containing inserts were reconfirmed by restriction digestion using *Bam*HI and *Kpn*I enzymes and also sequencing of clones in both directions. A special *Escherichia coli* strain (DH10α) containing a baculovirus genomic replicon (Bacmid) was transformed with the transfer vector for 4 hours. This allowed the foreign gene (Vn50) to be transposed into the bacmid (virus genome). After confirmation of transposition by PCR, virus genomic DNA was isolated from positive clones using a method described in the manufacturer's manual (Invitrogen). The purified DNA was then transfected into Sf9 cells using a lipid-droplet based transfection reagent (Cellfectin, Invitrogen). This allows entrance of viral DNA into insect cells that are subsequently translocated into the nucleus where they initiate replication of the virus and production of complete recombinant virions. Three days after transfection, medium containing recombinant viruses was collected from transfected cells and used as an inoculum to infect fresh cells to amplify the virus. Three days after infection of large number of cells, amplified virus was collected and used for confirmation of expression.

*2.2. Western blot analysis to confirm expression of proteins*

To confirm expression of proteins produced by recombinant viruses, Sf9 cells were inoculated with the amplified recombinant virus. Cells and medium were collected at various times after infection and tested for expression. First, the samples were separated on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulolse membrane using Western blotting technique. The transferred proteins were probed with antibodies to Vn50 raised in rabbits that specifically recognises the protein. An anti-rabbit antibody conjugated with alkaline phosphatase (Sigma) and anti-His-tag monoclonal antibodies (Sigma) were used to detect the primary antibody and the fusion protein, respectively. Nitroblue Tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) were used to develop the blots.

*2.3. Large scale production and purification of Vn50*

Large culture flasks containing Sf9 cells were inoculated with the recombinant virus. Medium containing the secreted protein was collected from cells and subjected to purification. Since the protein is fused with six histidine residues at the N-terminus (His-tag), the tag can be used for purification of the protein using Ni-agarose beads (QIAGEN). Therefore, the medium collected from the cells was mixed with Ni-beads for a period of 1 h with shaking. The beads were then

transferred onto a chromatography column. The flow through was collected for testing later. Then, in a stepwise increase of imidazole the protein was eluted from the beads. This chemical separates the protein from the beads. Purification was further tested by Western blotting (2.2) and specific antibodies to the His-tag and Vn50.

*2.4. Haemolymph collection*

For bleeding, several 4$^{th}$ instar *P. rapae* larvae were cooled on ice and their forelegs removed by a fine pair of forceps. Haemolymph was collected in a chilled tube and subsequently mixed in a 1:1 ratio with an anticoagulant buffer (4 mM NaCl, 40 mM KCl, 0.1% polyvinylpyrrolidone, 1.9 mM PIPES, 4.8 mM citric acid monohydrate, 13.6 mM sodium citrate, 5% sucrose, pH 6.8) to prevent darkening and coagulation of the haemolymph. The collected haemolymph was then stored at -80°C for later use in assays.

*2.5. Affinity purification of plasma proteins interacting with Vn50*

Haemolymph collected from *P. rapae* larvae (see above) were mixed in a 1:1 ratio with the binding buffer containing 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol and 0.001% final concentration of phenylthiourea, which inhibits melanization. After centrifugation at 14,000 ×*g* at 4°C for 10 min, the supernatant was incubated with 50 µl of recombinant Vn50 in Ni-NTA binding buffer at 4°C for 90 min and then mixed with calibrated Ni-agarose beads (QIAGEN) for further 60 min at 4°C with gentle shaking. The beads were then transferred onto a chromatography column and washed with 800 µl of Ni-NTA binding buffer three times before eluting with 160 µl of elution buffer (25 mM Tris- HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 100 mM imidazole). Eluted proteins were then analyzed by SDS-PAGE and Western blotting.

The purified Vn50 protein was immobilized on Nickel- beads (Invitrogen) by mixing the beads with the purified protein for 1 h at room temperature. The beads were then washed several times with phosphate buffer saline (PBS). Beads were then resuspended in 0.5 ml of PBS and mixed with serum collected from *P. rapae* larval haemolymph. To obtain cell-free serum from *P. rapae* larvae, several larvae were sacrificed by removing one of the prolegs and collecting the haemolymph in a tube as described above (2.4). The tube was then centrifuged at high speed (12000 rpm) for 1 min to remove cells (haemocyte). After 1 h incubation of the serum with Vn50-covered beads, they were washed extensively with PBS and then with high salt (1M NaCl) to wash unspecifically bound proteins. Beads covered with an irrelevant protein that was prepared as above were used as control. The beads were then run on a 10% SDS-PAGE gel to separate the proteins.

*2.6. Construction of recombinant baculoviruses expressing three forms of Vn50*

Vn50, like other serine proteinase-like proteins, consists of two main domains, clip and proteinase-like domains. To find out which of the two domains is responsible for inhibition of melanization activity of Vn50, we expressed the full-length protein, clip and proteinase-like domains in insect cell culture (Sf9) using a baculovirus expression system in fusion with histidine residues at the N-terminus. We used the Bac-to-Bac baculovirus expression system kit manufactured by Invitrogen. Sf9 cells were maintained as monolayers in culture flasks using a serum-free medium (SF900II, Invitrogen) at 27°C and subcultured every week. First, the three versions of the gene were amplified by polymerase chain reaction (PCR) using a previous Vn50 clone to insert suitable restriction sites at the ends for cloning into the baculovirus transfer plasmid vector. After successful amplification

of the fragments, they were digested with restriction enzymes and ligated into the transfer vector pFastBac-HT-a. Special *E. coli* strain (DH10α) containing a baculovirus genomic replicon (Bacmid) was transformed with the transfer vector for 4 hours. This allows the foreign gene to be transposed into the bacmid (virus genome). After confirmation of transposition by PCR, virus genomic DNA was isolated from positive clones. The purified DNA was then transfected into Sf9 cells using a lipid-droplet based transfection reagent (Cellfectin, Invitrogen). This allows entrance of viral DNA into insect cells that are subsequently translocated into the nucleus where they initiate replication of the virus and production of complete recombinant virions. Three days after transfection, medium containing recombinant viruses was collected from transfected cells and used as an inoculum to infect fresh cells to amplify the virus. Three days after infection of large number of cells, amplified virus was collected and used for confirmation of expression.

### *2.7. Confirmation of expression of Vn50 derivatives*

To confirm that the recombinant viruses in fact express the proteins, Sf9 cells were inoculated with the amplified recombinant viruses. Cells and media were collected at various times after infection and tested for expression. First, the samples were separated on a 10% SDS-PAGE and then transferred onto a nitrocellulolse membrane using Western blotting technique. The transferred proteins were probed with antibodies to Vn50 that specifically recognises the protein.

### *2.8. Large scale production and purification of Vn50 derivatives*

Large culture flasks containing Sf9 cells were inoculated with the recombinant virus. Medium containing the secreted protein was collected from cells and subjected to purification. Since the protein is fused with six histidine residues at the N-terminus (His-tag), the tag can be used for purification of the protein using Ni-agarose beads (QIAGEN). Therefore, the medium collected from the cells was mixed with Ni-beads for a period of 1 h with shaking. The beads were then transferred onto a chromatography column. The flow through was collected to be tested later. Then, in a stepwise increase of imidazole the protein was eluted from the beads. This chemical separates the protein from the beads.

### *2.9. Melanization inhibition assays*

The expressed proteins were used in melanization assays to find out which of the two domains are responsible for inhibition of melanization in the haemolymph. *Pieris rapae* 4$^{th}$ instar larvae were bled into ice cold PBS buffer and haemocytes were removed from the serum by high speed centrifugation for 1 min. After 10 min, L-DOPA (L-3,4-dihydroxyphenylalanine; substrate for phenoloxidase enzyme) was added to PBS to 20 mM final concentration and vortexed to dissolve. In each well of a 96-well plate 10 µL of haemolymph was mixed with 90 µL of 20 mM L-DOPA by pipetting. Absorbance at 485 nm was then measured every 10 min for 300 min in a plate reader.

### *2.10. Generation of genetically modified Drosophila melanogaster flies expressing Vn50*

Full-length Vn50 gene was amplified using polymerase chain reaction (PCR) with GCGCGGTACCTCAGCATGAAGTGGAAAATATTTAG as the forward and GCGCTCTAGATTAGTCAGCAAATGCTTC as the reverse primer containing restriction sites for direct cloning into the transfer vector (underlined nucleotides). Vn50 was cloned into the pUASp transposable vector at the *Kpn*I and *Xba*I sites and confirmed using a restriction digest and sequencing in both directions. To remove endonucleases and extract plasmid from bacterial culture

EndoFree Plasmid Maxi Kit (QIAGEN) was used according to the manufacturer's instructions. pUASp and pUChsneo Δ2-3 were mixed and diluted in injection buffer (0.1mM sodium phosphate buffer, 5 mM KCl) to a final concentration of 250 ng/μL and 50 ng/μL, respectively. Seven hundred and ninety seven eggs of the $W^{1118}$ strain of *D. melanogaster* (pre-treated with tetracycline to remove *Wolbachia*) were microinjected in the posterior end within 1 hr of being laid using a pulled glass needle attached to a Narishige microinjector and after 24 hrs they were heat shocked at 37°C for 1 hr. Eclosing adults were mated with $W^{1118}$ males and virgin females. One mating resulted in red-eyed progeny. These were mated with 2nd and 3rd chromosome balancer lines (*CyO/Gla* and *TM3/TM6B*, respectively) to determine the position of insertion of UAS-Vn50 which allowed production, through mating, of a fly line homozygous for UAS-Vn50 (Vn50-Vn50). This line was crossed with a da-GAL (P[Gal4-da.G32]UH1; Bloomington Stock Centre) line to enable ubiquitous expression of Vn50 in the cells of the progeny.

### *2.11. Confirmation of expression of Vn50 in D. melanogaster flies*

Confirmation of transposition of UAS-Vn50 into the flies' genome and, consequently, expression of Vn50 was first carried out using reverse transcription PCR (RT-PCR). The primers used were the forward primer described above and the internal reverse primer GCGCGGTACCTTATCTAAAACCTACACCAT. RNA samples extracted from flies were treated with DNase I (Promega) prior to cDNA synthesis to ensure removal of contaminating DNA. Samples were run on a 1% agarose gel and visualized under UV light. In addition, expression of Vn50 gene was confirmed using Northern blotting. Complementary DNA to Vn50 gene labeled with [$^{32}$P]-dCTPs was used as a probe. Hybridizations and washings were carried out under stringent conditions (65°C).

### *2.12. Treatments of flies with microorganisms*

#### *2.12.1. Treatment with Serratia*

An overnight suspension of *Serratia* in lysogeny broth (LB) was centrifuged and resuspended to a final O.D. of 0.5. Each 4-8 day old fly was first anaesthetized with $CO_2$ and then stabbed in the side of the abdomen with a thin needle which had been dipped in the suspension. Care was taken to be as gentle as possible while ensuring that the needle pierced the exoskeleton. Control flies were stabbed with medium only. Flies that did not survive treatment were removed after the first day and mortality was recorded everyday afterwards. Twenty flies were used for each treatment. Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

#### *2.12.2. Treatment with Beauveria*

Treatment of flies with *Beauveria* also involved anaesthetizing 4-8 day old flies with $CO_2$ but on a piece of filter paper to soak up excess liquid. 0.5 μL of a $1\times10^{10}$ spores/mL of *Beauveria* (Strain GHA, BotaniGard) in sunflower oil was pipetted onto the abdomen of each fly. Control flies were treated with sunflower oil only. Flies that did not survive treatment were removed after the first day and mortality was recorded everyday afterwards. Twenty flies were used for each treatment. Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

*2.12.3. Treatment with Drosophila C virus*

*Drosophila* 6 to 7 days old were infected with DCV or mock infected by micro-injection of virus or PBS as previously described (Osborne *et al.*, 2009). For each fly line assayed, two groups of 15 flies were injected with virus and one group of 15 flies was injected with PBS. Mortality that occurred within one day of injection was deemed to be due to injury. Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

*2.13. Larval development*

Three day old virgin flies were mated for two days and then placed in plastic bottles with molasses lids. These had some yeast paste (yeast and water) placed on the middle of the plate and were stored upside-down. After 24 hrs, eggs were collected and placed, 20 each, in fly food vials. The number of Vn50-expressing and control pupae formed and adults eclosing were recorded daily.

*2.14. Oviposition*

Ten four day old, Vn50-expressing male flies and ten females were placed in each fly food vial. These were left for three days before they were removed from the vials. The same was carried out for control flies. The number of progeny from these flies was recorded as the total number of pupae that formed in these vials.

*2.15. Leptoplina boulardi parasitization assays*

*L. boulardi* is a parasitoid wasp that specifically parasitizes *Drosophila* larvae. To control the number of eggs given to parasitoids in each assay, flies of both lines (Vn50 and control) were given molasses plates with a bit of yeast paste to lay on over an 18 hr period. Eggs were then collected and placed in vials with fly food with four replicates of 20 eggs each. Two 2 day old, mated *L. boulardi* females were placed into each vial and left to parasitize the *Drosophila* eggs for 48 hrs. Pupal formation and any *Drosophila* eclosion were recorded daily. *Leptopilina* eclosion was also recorded daily. Wasps were sexed and then placed in Eppendorf tubes and stored at -20 ºC. Success of parasitism was monitored to ensure that there was no difference in parasitisation levels between the host lines. Sex ratio was measured by the proportion of *Leptopilina* that eclosed, in each vial, that were female.

*2.16. Fitness measurements*

The fitness of the parasitoids that emerged from the Vn50 and control fly lines in the assays was determined by measuring weight and wingspan of males and females. For weighing of *Leptopilina*, Eppendorf tubes containing the parasitoids from the assays were removed from -20 ºC and allowed to defrost. Before weighing each wasp, they were lightly dabbed with paper towel to remove any condensation. Eight male and female parasitoids from Vn50+ and Vn50– lines were weighed on a microweighter. The same number of parasitoids was used for wing measurements. Whole wings were removed by forceps, aligned on microscope slides and attached with tape. Microscope photos were taken of all wings and the pixel distance between the base and tip of the wing was measured. A picture of a micrometer slide at the same magnification was used to convert these distances into mm.

*2.17. Construction of polyhedrin positive recombinant baculoviruses*

Specific primers to Vn50 and Green fluorescent protein (GFP) genes were designed to amplify and

clone the corresponding genes into a transfer vector (pAcPDIE1) which allowed cloning of both genes. GFP gene was used as a reporter gene so that infection can be traced. Therefore, two recombinant viruses were produced, one with Vn50+GFP (rec-Vn50+GFP BV) and the other only GFP (rec-GFP BV) as control. The transfer vectors together with the linearized virus genome (BaculoGOLD) were transfected into insect cells. After 72 h, insect culture media containing viruses were collected and recombinant viruses were plaque purified, amplified and analysed for the expression of Vn50 and GFP using Western blotting.

*2.17.1. Insect Bioassays*

After confirming expression of Vn50, BV polyhedra (virus containing inclusion bodies) were produced in large numbers in insect cells, purified and quantified using a haemocytometer. Three different lepidopteran insects were tested in bioassays using droplet feeding method. These were *Crocidolomia pavonana, Plutella xylostella* and *Spodoptora littoralis*. $2^{nd}$ instar larvae were used for bioassays. The larvae were starved for at least 2 h before they were used in the bioassay experiment. Viral inoculum was prepared by adding sucrose (5% w/v) and blue food dye (50 µL dye/2mL) to purified polyhedra. 50 µL of viral inoculum was prepared with concentrations of $10^3$, $10^4$, $10^5$ polyhedra respectively. Once the larvae were starved, droplets of the viral inoculum were added to the parafilm followed by the placement of the larvae at the center of the petri-dish. Controls were also set up that contained 5% sucrose and the food dye only. Larvae that had ingested the viral inoculum and control could be recognized by the blue colour dye seen in the larvae. They were immediately transferred to another petri-dish that had non-contaminated leaf diet and placed at 27˚C. The larvae were monitored every 24 hr for mortality until all larvae died or until they started to pupate. The mortalities observed within the first 24 hr were excluded from the study. The corrected percent mortality was calculated using Abbott's formula.

## 3. Results

*3.1. Construction of a recombinant baculovirus expressing Vn50*

To identify serum components from the host larva, *Pieris rapae*, that interact with Vn50, we expressed the full-length protein in insect cell culture (Sf9) using a baculovirus expression system in fusion with histidine residues to facilitate purification of the protein. To confirm that the recombinant virus in fact expresses the protein, Sf9 cells were inoculated with the amplified recombinant virus. Cells and medium were collected at various times after infection and tested for expression using Western blotting using antibodies specific to Vn50. The results showed that the protein is not only produced in cells from 48 hours after infection but also secreted into the medium (**Fig. 1**) which facilitated its purification.

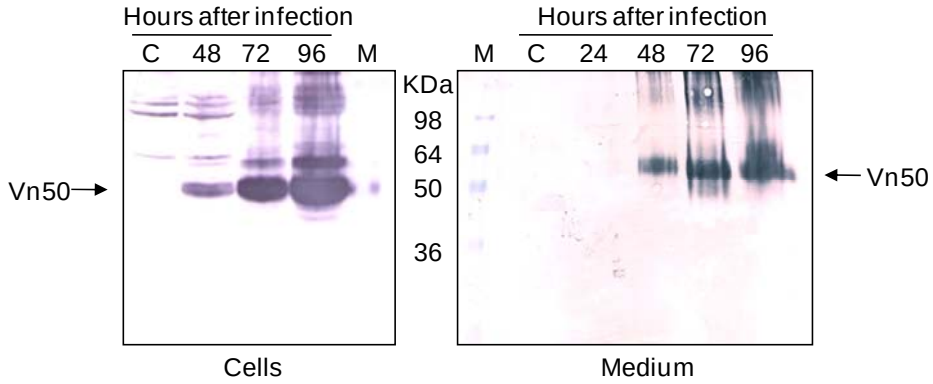


**Fig. 1:** Expression of recombinant Vn50 in insect cells using baculovirus expression system. Expression of the protein was confirmed by Western blotting in which specific antibodies to Vn50 was used as a probe. The protein was detected from 48 hours after infection both in Sf9 cells and medium collected from the cells indicating that the protein is secreted. C: control expressing an irrelevant protein.

*3.2. Large scale production and purification of Vn50*

Large culture flasks containing Sf9 cells were inoculated with the recombinant virus. Medium containing the secreted protein was collected from cells and subjected to purification. Using 10, 50, 100, 250 and 500 mM of imidazole, we found that the best concentration is 100 mM (**Fig. 2**). The purified protein was used for the next step.

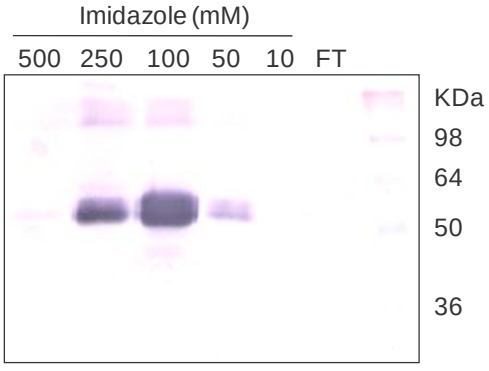


**Fig. 2:** Production and purification of Vn50 expressed by baculovirus in Sf9 cells. Medium was collected from baculovirus infected cells at 72 hours after infection and subjected to purification using agarose beads. The recombinant protein was eluted by increasing the concentration of imidazole. 100 mM imidazole was found the best condition for protein elution. FT: flow through.

*3.3. Identification of haemolymph proteins that bind to Vn50*

To identify haemolymph (serum) proteins that bind to Vn50, the purified protein was immobilized on Ni- beads by mixing the beads with the purified protein. After stringent washing of the beads, they were mixed with haemolymph isolated from *P. rapae* larvae. After 1 h incubation of the serum with Vn50 covered beads, they were washed extensively with buffer and then with high salt (1M NaCl) to wash unspecifically bound proteins. Proteins bound to the beads were then separated on a 10% SDS-PAGE gel (**Fig. 3**). Those proteins that were unique to the beads covered with Vn50 were identified as haemolymph proteins interacting specifically with Vn50 (**Fig. 3**; shown by arrows).

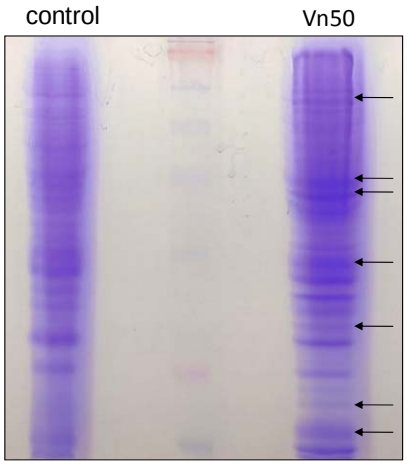


**Fig. 3:** Identification of haemolymph proteins binding to Vn50. Control beads covered with an irrelevant protein and Vn50 covered beads were separately incubated with haemolymph collected from *P. rapae* larvae. After extensive washing, the beads were run on SDS-PAGE gel to separate the proteins that bind to the beads. Unique bands to Vn50 beads were identified as haemolymph proteins interacting with Vn50 indicated by arrows.

To identify the proteins, eluted proteins were separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane using a mini-gel Western blot apparatus (BioRad). To identify the bound proteins to Vn50, specific antibodies to proteins that are suspected to interact with the protein were used as probes. Four important proteins were found to specifically interact with Vn50, the pro-phenoloxidase and its active form, phenoloxidase, and the pro-phenoloxidase activating proteinases and its inactive form (**Fig. 4**). These two enzymes are key enzymes involved in the melanization defence response in insects. Pro-phenoloxidase activating proteinases cleave pro-phenoloxidase into active phenoloxidase that starts oxidization of phenolic compounds in the haemolymph leading to the formation of dark melanin. This also sheds light on the possible function of the protein, which is inhibition of phenoloxidase activity, by binding to the inactive form of the enzyme, pro-phenoloxidase, and its activating enzyme, thereby, blocking their activation.

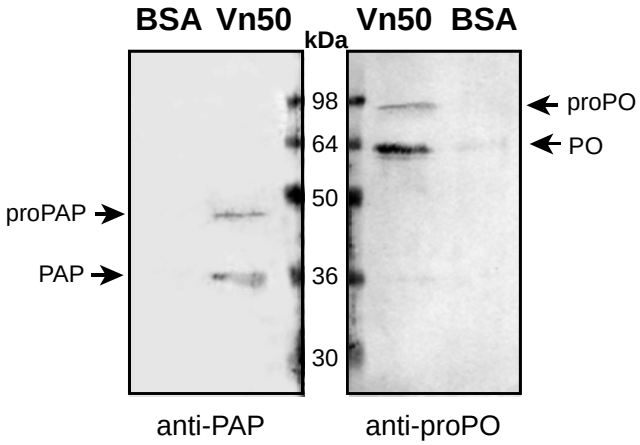


**Fig. 4:** Interaction of Vn50 with host haemolymph components. Cell-free haemolymph from *P. rapae* larvae was incubated with beads coated with bovine serum albumin (BSA, control) or recombinant Vn50 and washed with buffers and salt. Proteins bound to the beads were then analysed by SDS-PAGE and Western blotting, which were probed with PAP-1 (anti-PAP) or proPO (anti-proPO) antibodies. Both antibodies recognized proteins bound only to the beads that were coated with Vn50. Their sizes are consistent with those of precursor and cleaved forms of PAP and PO.

### *3.4. Production of the three forms of Vn50 using baculovirus expression system*

Vn50, like other serine proteinase-like proteins, consists of two main domains, clip and proteinase-like domains. To find out which of the two domains is responsible for inhibition of melanization activity of Vn50, we expressed the full-length protein, clip and proteinase-like domains in insect cell culture (Sf9) using a baculovirus expression system in fusion with histidine residues at the N-terminus. To confirm that the recombinant viruses express the proteins, Sf9 cells were inoculated with the amplified recombinant viruses. Cells and media were collected at various times after infection and tested for expression using Western blotting. The results showed that the protein is not only produced in cells from 48 hours after infection but also secreted into the medium (**Fig. 5**) which facilitated its purification.

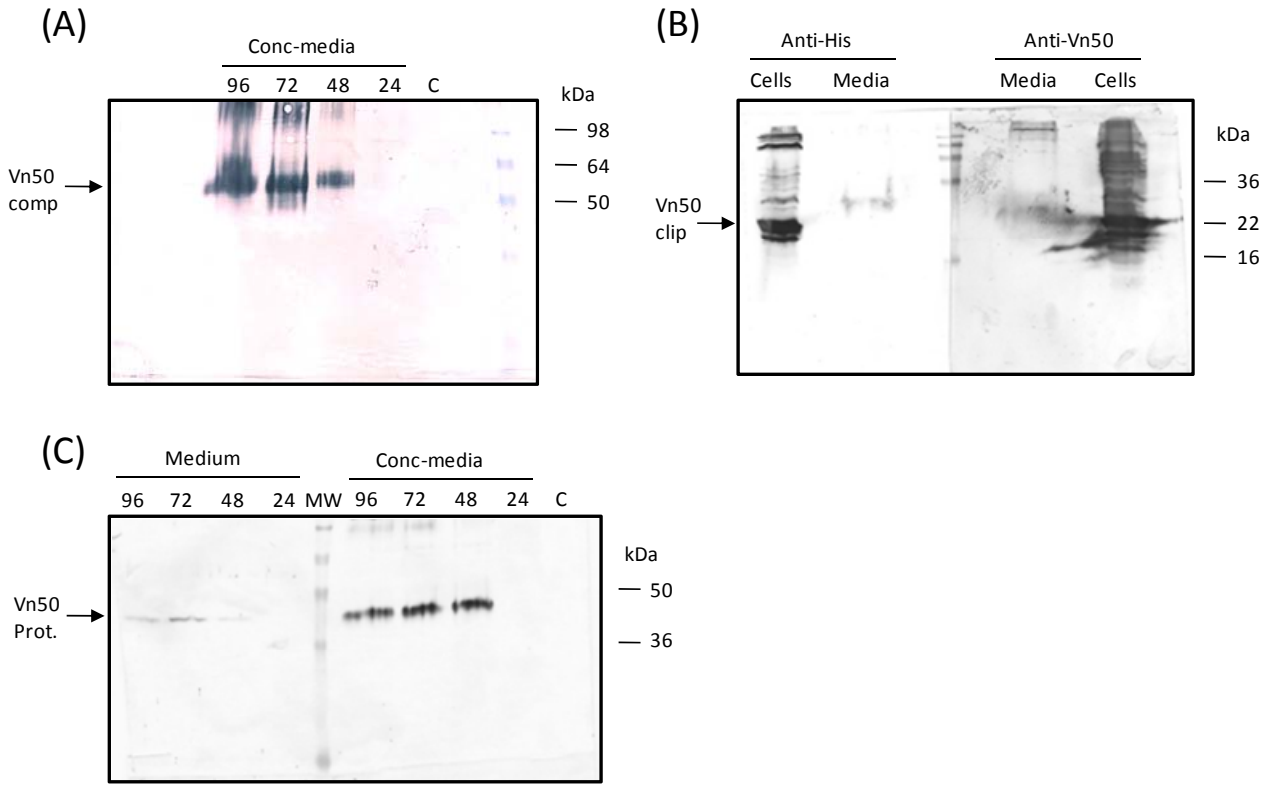


**Fig. 5:** Western blot analysis of cells and media from insect cells infected with recombinant baculoviruses expressing **(A)** the full-length Vn50 (Vn50 comp), **(B)** the clip domain (Vn50 clip), and **(C)** the proteinase-like domain (Vn50 prot.). Cells were analysed at various times after infection (24, 48, 72 and 96 h). The expressed and identified proteins are shown by arrows.

### *3.5. Large scale production and purification of Vn50 derivatives*

Large culture flasks containing Sf9 cells were inoculated with the recombinant virus. Medium containing the secreted protein was collected from cells and subjected to purification. Since the protein is fused with six histidine residues at the N-terminus (His-tag), the tag could be used for purification of the protein using Ni-agarose beads (QIAGEN). Using 10, 50, 100, 250 and 500 mM of imidazole, we found the best concentration for each Vn50 derivative (**Fig. 6**). The purified proteins were used for the next step.

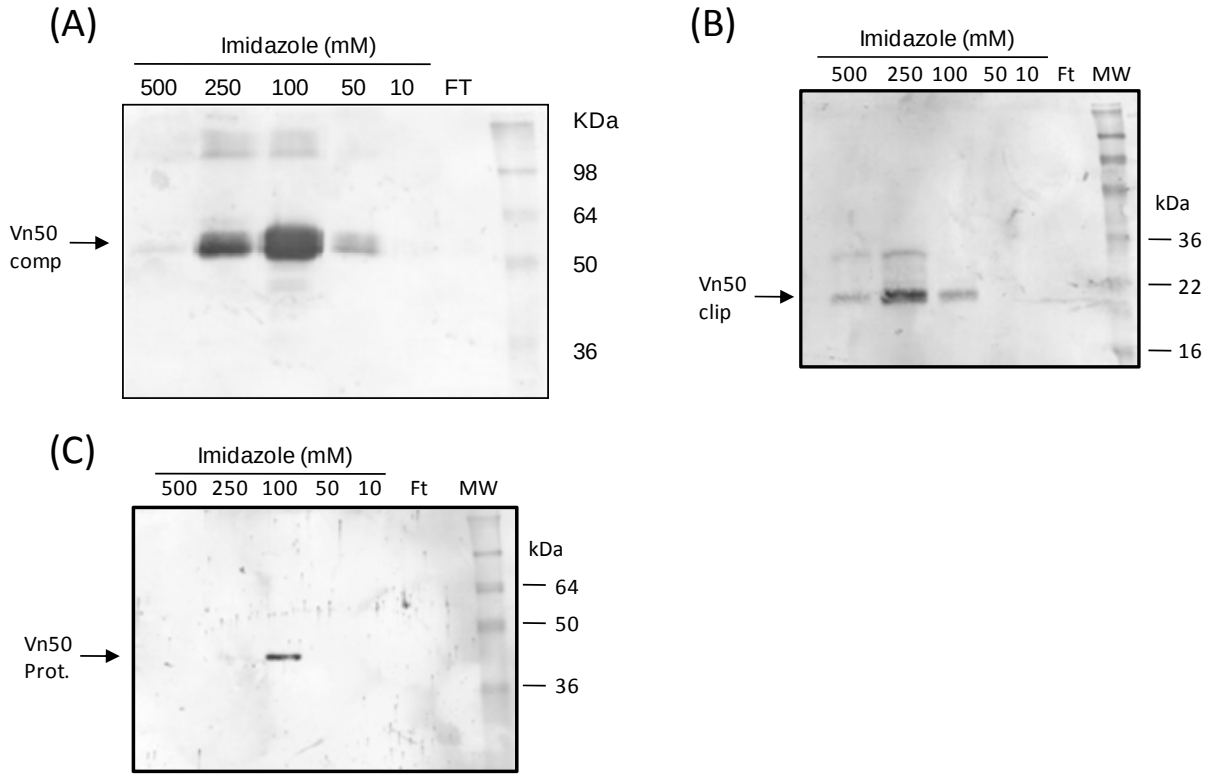


**Fig. 6:** Western blot analysis of purified Vn50 derivatives from cells infected with recombinant baculoviruses expressing **(A)** the full-length Vn50 (Vn50 comp), **(B)** the clip domain (Vn50 clip), and **(C)** the proteinase-like domain (Vn50 prot.). Different concentrations of Imidazole were used to find the best concentration for large scale purification. The expressed and identified proteins are shown by arrows.

*3.6. Melanization inhibition assays*

The expressed proteins were used in melanization assays to find out which of the two domains are responsible for inhibition of melanization in the haemolymph. The results showed that the clip and the protease-like domains on their own did not reduce melanization but the complete protein containing both the clip domain and the protease-like domain is more efficient in inhibition of melanization (**Fig. 7**).

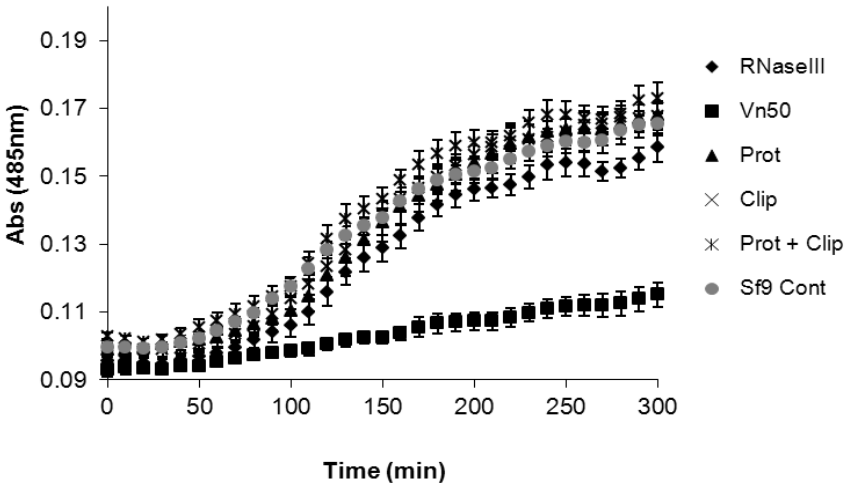


**Fig. 7:** Melanization assay of haemolymph in the presence of Vn50, Vn50 clip (Clip), Vn50 protease-like (Prot), mixture of Clip and Prot (Prot+Clip) and two controls (Sf9 Cont) and RNaseIII protein as an irrelevant protein. Increase in absorbancy is an indication of an increase in melanization.

*3.7. Generation of a Vn50-expressing Drosophila line*

To test the effect of Vn50 on the immune system of insects *in vivo*, we used *D. melanogaster* as a model system to insert the full-length gene coding for Vn50 into the fly genome. Genetically modified *D. melanogaster* flies were generated that produce the wasp venom protein.

Following injection of embryos and emergence of adults, they were mated with $W^{1118}$ flies, twelve fly lines were created from these, with six determined to contain UAS-Vn50 on the 3rd chromosome and one on the 2nd chromosome. The other five did not stably keep red eyes and were discarded. The seven successful lines were used in Northern blotting and RT-PCR to confirm Vn50 expression and to give an indication of which lines had the greatest Vn50 expression with the best line shown in Figures 8A and 8B. When the gel was visualized, the 380 bp clip domain was seen as a distinct band present only in the Vn50 expressing *Drosophila* sample (Vn50+) and in insect cells (Sf9) expressing a recombinant Vn50 using a baculovirus expression system as a control (**Fig. 8A**). The band was not found in the driver line control sample (Vn50-). Further, the samples were analyzed by Northern hybridization which only displayed the expression of Vn50 gene in the transformed line (**Fig. 8B**). This demonstrated successful introduction of Vn50 gene into the genome and its ectopic expression.

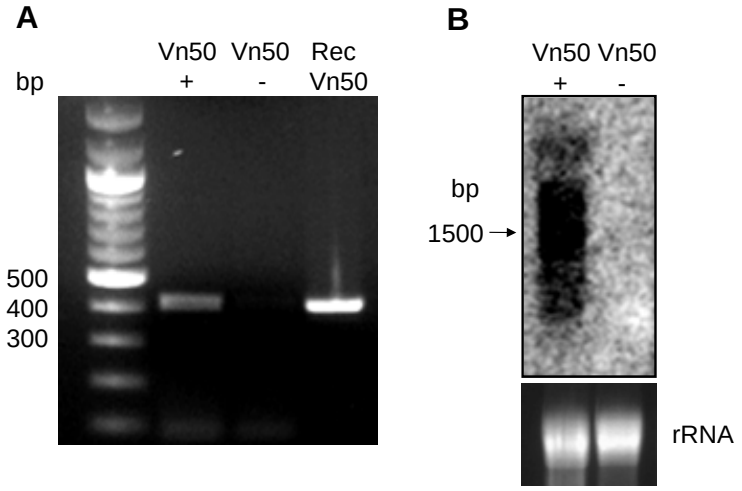


**Fig. 8:** Confirmation of expression of Vn50 in transformed flies. **A)** RT-PCR analysis using RNA extracted from whole flies and Sf9 cells infected with a Vn50 recombinant baculovirus (Rec Vn50) as a positive control and RNA from da-GAL flies as negative control. bp, base pairs. **B)** Northern blot analysis of RNA using a Vn50 cDNA as a probe. rRNA is shown for equal loading. The black signal shows extensive expression of Vn50 in transformed flies. bp, base pairs.

*3.7.1. Inhibition of melanin formation*

To find out if expression of Vn50 has any effect on melanin formation in transformed flies, melanization assays were carried out in the presence of a phenoloxidase substrate, L-DOPA. The haemolymph of both male and female Vn50-expressing flies showed a decrease in absorption at 485 nm in the presence of L-DOPA (**Fig. 9**). However, the difference in females was statistically significant ($p < 0.05$) whereas in males it was not ($p > 0.1$). This suggested that Vn50 expressed in transformed flies is functional and had an inhibitory effect on melanization of *D. melanogaster* haemolymph.

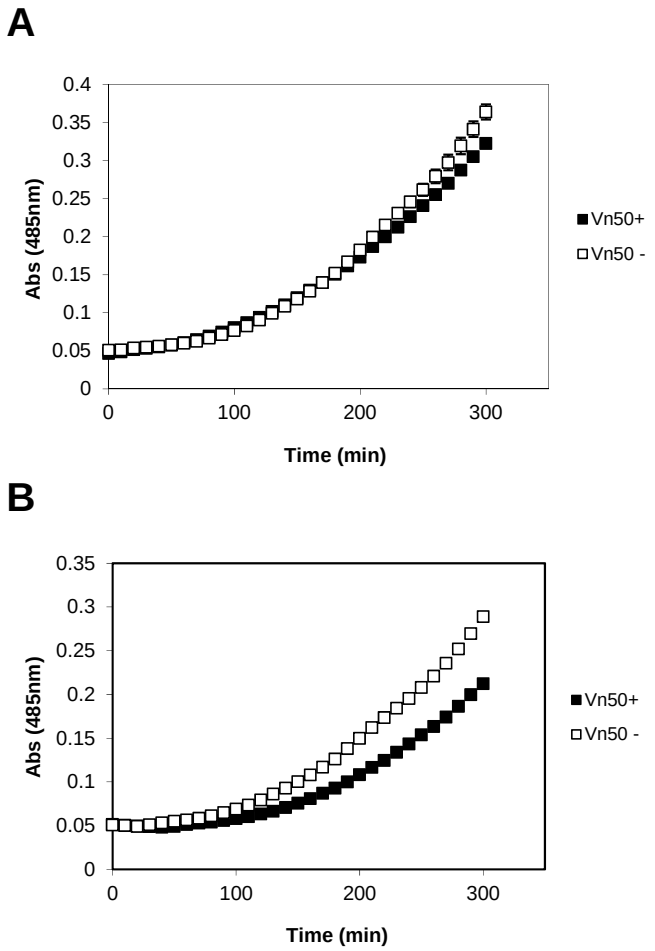


**Fig. 9:** Vn50 reduces melanization in transformed female flies. Absorbance readings over 300 min for male (A) and female (B) phenoloxidase assays using L-DOPA as substrate. Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard deviations of averages from four replicates.

*3.7.2. Treatment with Serratia*

When *Drosophila* male and female flies were stabbed with an optical density (O.D.) = 0.5 suspension of *Serratia* in culture medium, very little difference was observed between Vn50-expressing and control flies with respect to mortality (**Fig. 10**). The two lines showed no difference in survival after being treated with the pathogenic bacteria both in males ($p > 0.5$) and females ($p > 0.1$). The data demonstrates that the expression of Vn50 in this GAL4-UAS system did not translate into any significant difference in survival when treated with *Serratia*.

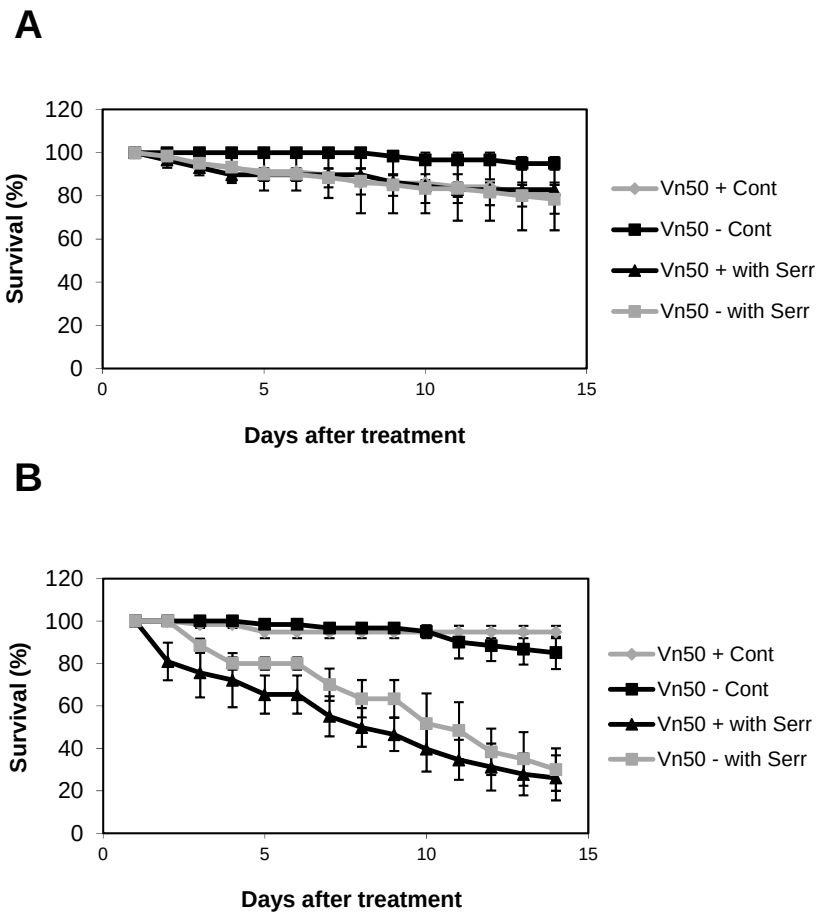


**Fig. 10:** Survival of male **(A)** and female **(B)** *Drosophila* after being treated with *Serratia* (with Serr) or just medium (Cont). Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

### *3.7.3. Treatment with Beauveria*

When adult flies were topically treated with *Beauveria* (0.5 μL of a $1\times10^{10}$ spores/mL in canola oil), survival results were in agreement with those of the phenoloxidase (PO) activity assays (**Fig. 11**). The small difference in melanization between the Vn50 and control line male flies did not convert into a significant difference in survivability against *Beauveria* (**Fig. 11A**; $p > 0.1$). However, female flies, which ectopically expressed Vn50, suffered with reduced survival when treated with the fungus compared to control females (**Fig. 11B**; $p < 0.005$). This showed that the difference in melanization levels caused by the introduction of Vn50 resulted in a reduction in immune function against this pathogenic fungus. There was no significant difference between Vn50 and control flies treated with oil in males and females (**Fig. 11A & B**; $p > 0.1$).

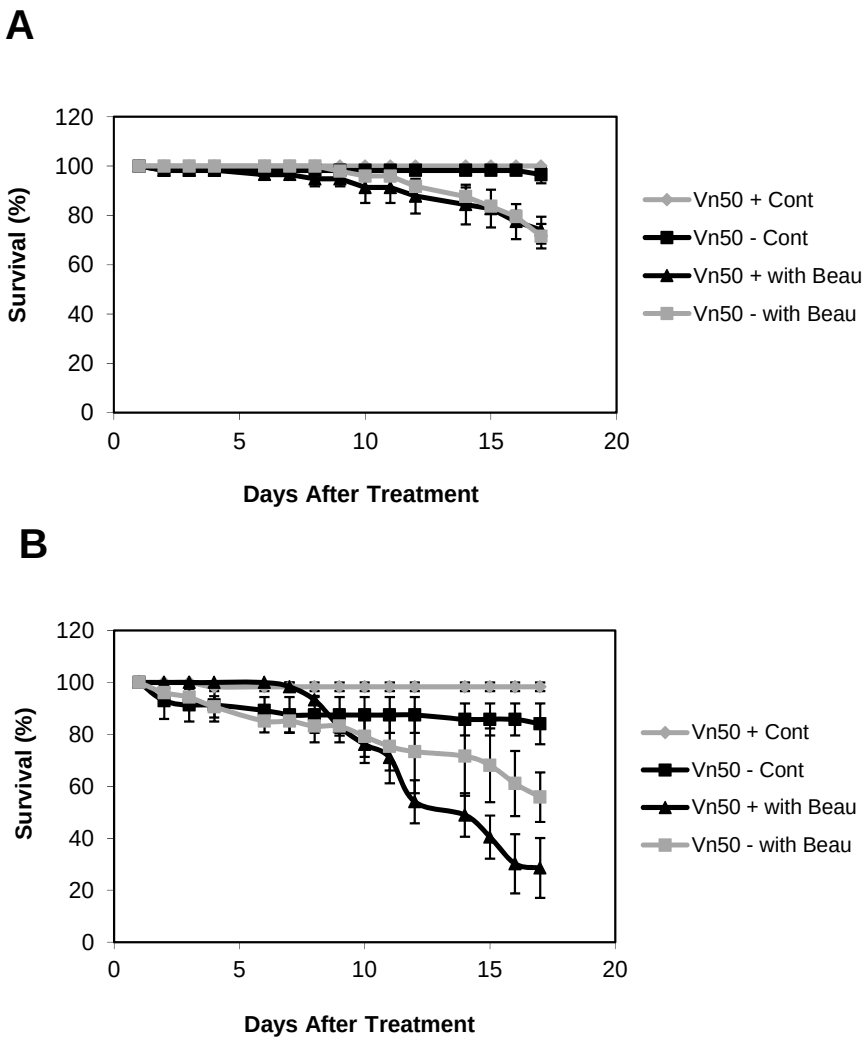


**Fig. 11:** Survival of male **(A)** and female **(B)** *Drosophila* after being treated with *Beauveria* (with Beau) or oil (Cont). Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard error of the mean from four replicates.

*3.7.4. Treatment with Drosophila C virus (DCV)*

Vn50 flies challenged with DCV showed a delay in virus-induced mortality. For both Vn50 and control flies the mortality in PBS injected controls was negligible. All DCV injected male control flies died by 4 days post infection (**Fig. 12A**), whereas mortality of male Vn50 flies challenged with DCV was significantly delayed ($p < 0.0001$) not reaching 100% until 7 days post infection. Similarly there was a significant delay in the mortality in female Vn50 flies compared to control flies **(Fig. 12B**, $p < 0.0001$). These results indicate that expression of Vn50 in *D. melanogaster* mediates a significant decrease in DCV induced mortality.

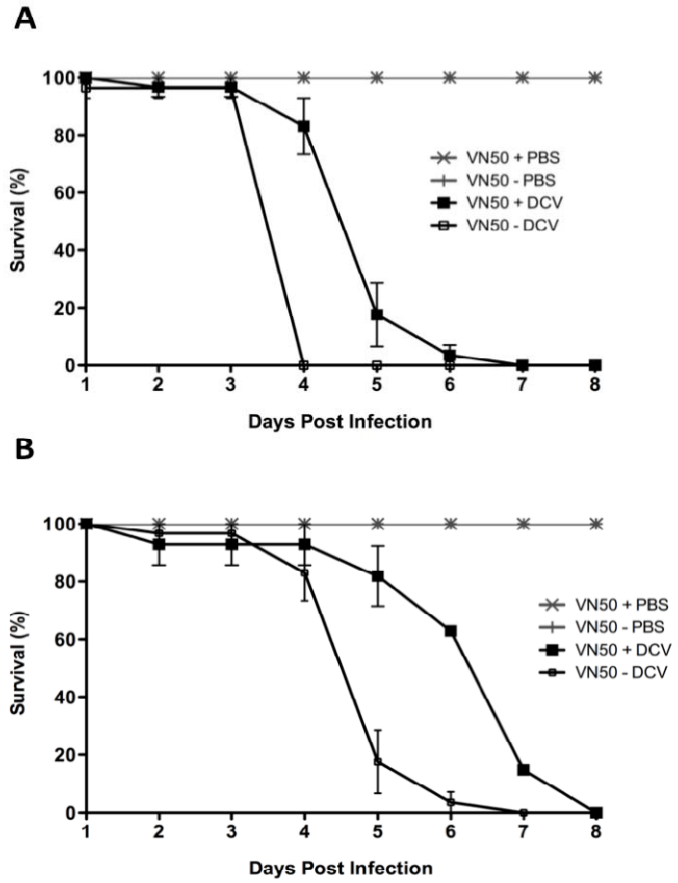


**Fig. 12:** Survival of *Drosophila* adults after being treated with *Drosophila* C virus. Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Survival of flies mock-infected with buffer also shown. **(A)** males, **(B)** females. Error bars indicate standard error of the mean from two replicates.

### *3.7.5. Larval development*

In density controlled vials, pupal formation of Vn50-expressing flies began 5 days after egg laying and peaked on day 6 whereas control flies only began having pupae formed on day 6, peaking on the 7th day (**Fig. 13A**). This converted into a one day delay of adult eclosion with Vn50 flies beginning to eclose on day 10 and peaking on day 11 and control flies beginning to eclose on day 11 and peaking on day 12 (**Fig. 13B**).

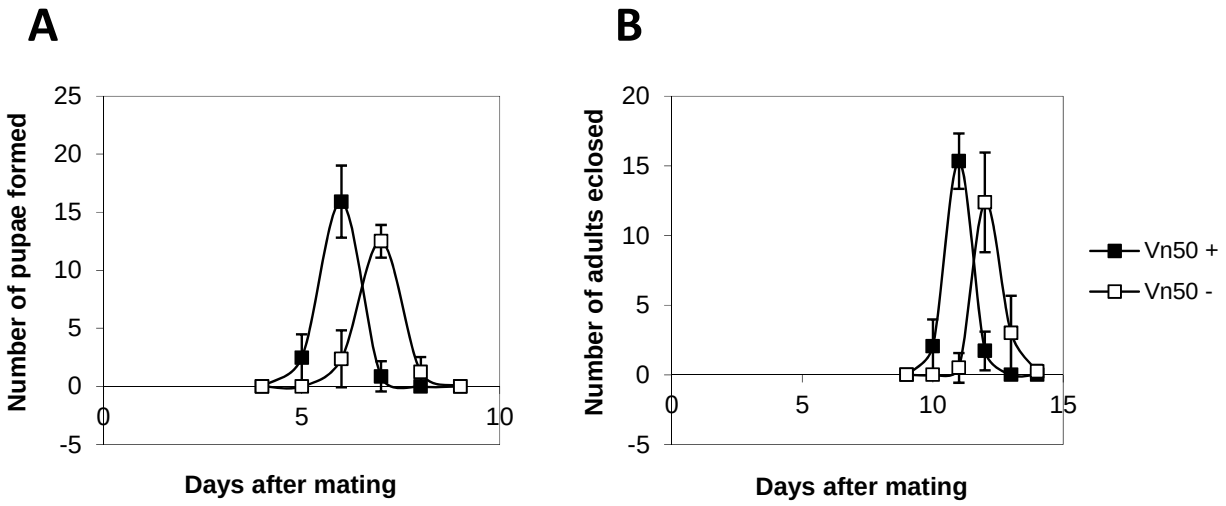


**Fig. 13:** Daily pupal formation **(A)** and adult eclosion **(B)**. Vn50+ represents the Vn50-expressing flies and Vn50– represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

*3.7.6. Oviposition*

The total number of progeny produced from 3 days of egg-laying was significantly greater for the Vn50 flies than the control flies (**Fig. 14**). The average number of progeny produced from ten Vn50 females was 209 compared to 146 for control flies.

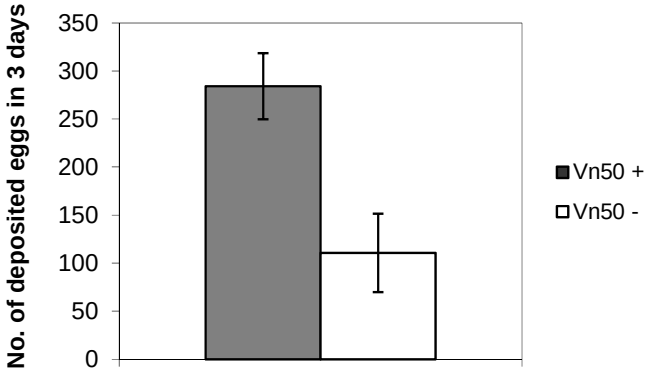


**Fig. 14:** Number of progeny produced from 3 days of egg-laying. Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

*3.7.7. Parasitism success*

As in the wasp's host, *P. rapae*, Vn50 decreased melanization, which affected immune function as well as development of the transformed flies. We challenged the Vn50-expressing *Drosophila* and a control line with the parasitoid, *Leptopilina boulardi* and recorded parasitoid success, fitness in terms of weight and wing length, development time and sex-ratio. Once all *Leptopilina* wasps emerged in all treatments and replicates, parasitism success was determined by total *Leptopilina* eclosed as a proportion of the total number of eggs that were available for parasitism. Average

parasitism success in Vn50-expressing flies was determined to be 0.8125 compared to 0.6875 in the control line. This difference was found not to be statistically significant (P = 0.0565).

*3.7.7.1. Effects of Vn50 expression on parasitoid sex ratio*

*Leptopilina* were sexed as they were collected and the number of females in each vial was divided by the total number of emerged parasitoids to determine the sex ratio. The average sex ratio for parasitoids which emerged from Vn50-expressing *Drosophila* was 0.7076 compared to 0.5012 yielded from control line flies (**Fig. 15**). This increase in the proportion of female parasitoids which emerged from the Vn50-expressing *Drosophila* was statistically significant (P = 0.0463).

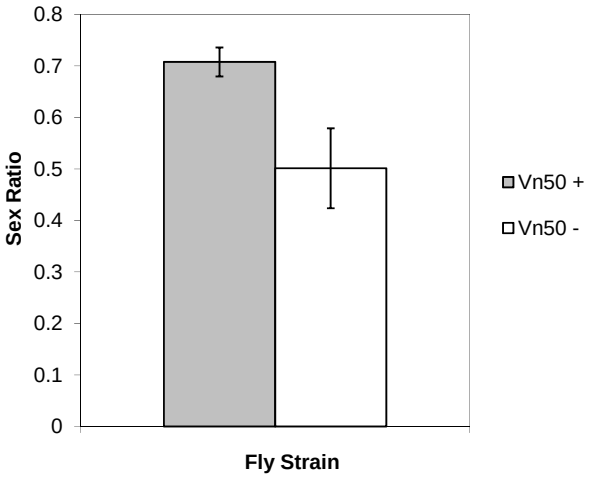


**Fig. 15:** Sex ratio of parasitoids emerging from Vn50+ and Vn50- hosts. Error bars indicate standard error of the mean from four replicates.

*3.7.7.2. Effects of Vn50 expression in flies on parasitoid development and fitness*

Eclosion of parasitoids peaked at day 22 from both Vn50-expressing and control line (**Fig. 16**), while male *Leptopilina* peaked at 21 days in both the control line and the Vn50 test (**Fig. 17A**). However, female eclosion was different, with female parasitoid eclosion peaking at 22 days, a day earlier than control line parasitoids (**Fig. 17B**).

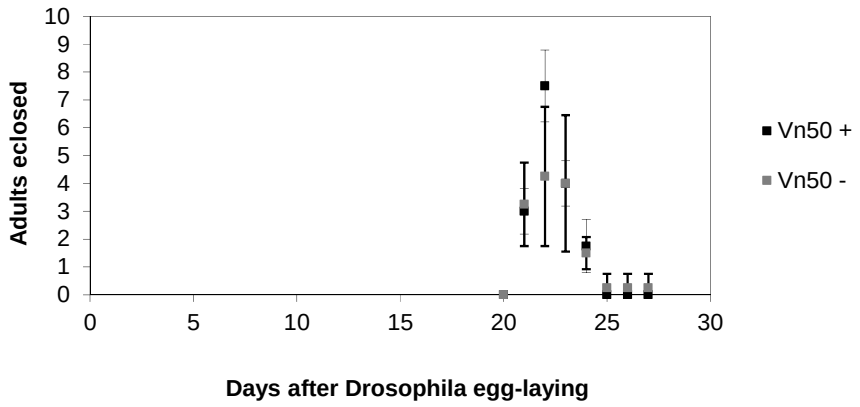


**Fig. 16:** Total daily eclosion of parasitoids from Vn50+ and Vn50- hosts. Error bars indicate standard error of the mean from four replicates.

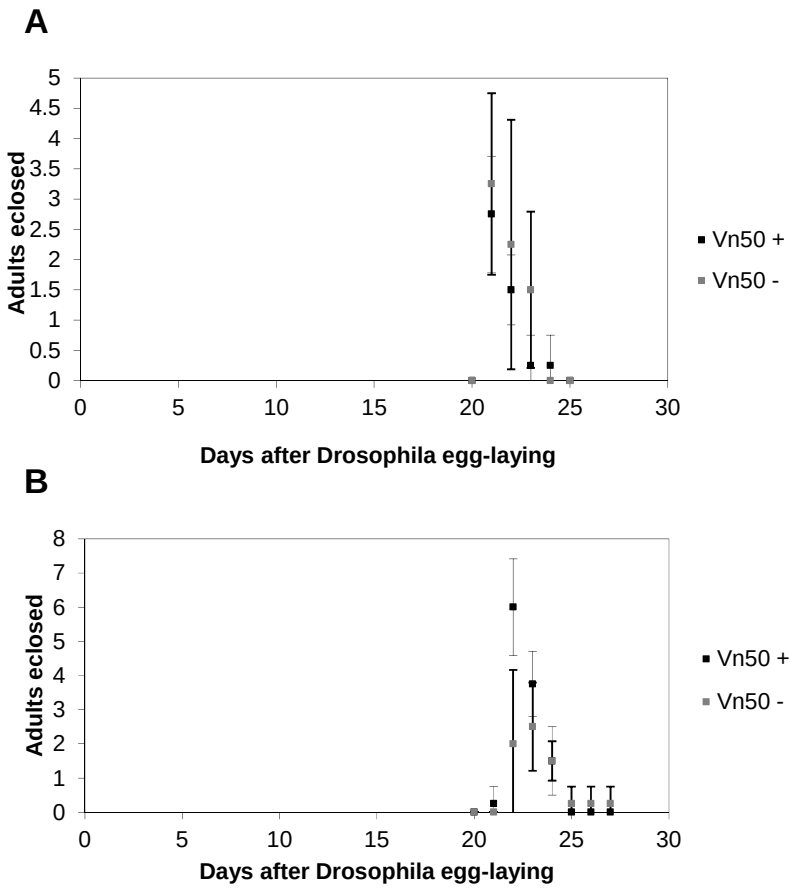


**Fig. 17:** Daily eclosion of male **(A)** and female **(B)** parasitoids from Vn50+ and Vn50- hosts. Error bars indicate standard error of the mean from four replicates.

Adult parasitoid fitness was assessed by measuring the weight and wing length of eclosed parasitoids. No significant difference in parasitoid weight was seen between the males or females of the Vn50 or control line (**Fig. 18**). The average weight of males which eclosed from Vn50-expressing flies was 0.2044 mg (+/- 0.0304) compared to 0.1854 mg (+/- 0.0314) in control line parasitoids. Female Vn50 line parasitoids had an average weight of 0.2833 mg (+/- 0.0245) and control line parasitoids 0.2888 mg (+/-0.0310).

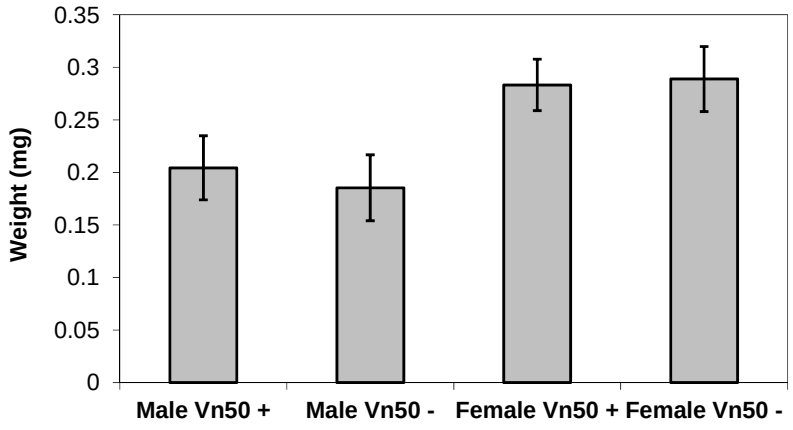


**Fig. 18:** Weight of male and female parasitoids emerged from Vn50+ and Vn50- hosts. Error bars indicate standard error of the mean from eight replicates.

When wings were fixed to a microscope and digital photographs taken, a computer program was used to measure the pixel difference between the base and tip of the wing. These pixel distances were converted into millimetres. Similarly, the control and Vn50 line parasitoids displayed no differences in size (**Fig. 19**). The wing length of male parasitoids from the Vn50 line flies was 1.3621 mm (+/- 0.0537) compared to 1.3528 mm (+/- 0.0292) in the control. Female parasitoids eclosing from Vn50 line flies had an average wing length of 1.4246 mm (+/- 0.0397) and control line parasitoids 1.4317 mm (+/-0.0276).

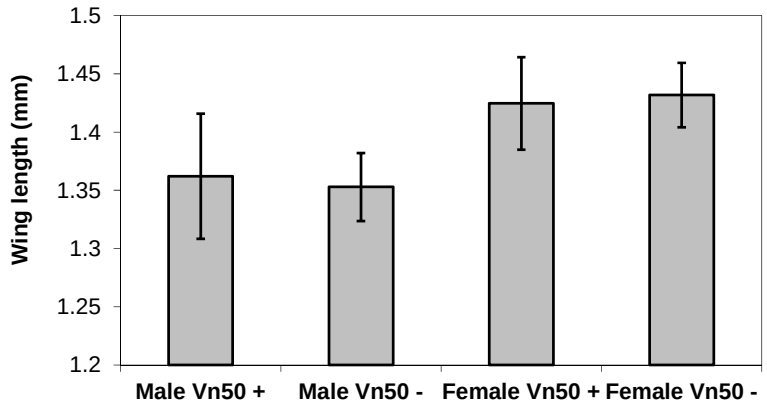


**Fig. 19:** Wing length of male and female parasitoids emerged from Vn50+ and Vn50- hosts. Error bars indicate standard error of the mean from eight replicates.

*3.8. Improving efficacy of baculoviruses by genetic modification*

Several reports indicate that semi-permissiveness of certain hosts towards baculovirus infections is due to immune resistance exhibited by the host especially by melanizing the foci of secondary infection (Rivkin *et al.*, 2006; Washburn *et al.*, 2000). As shown above, Vn50 proved to be a general inhibitor of melanin formation, and that Vn50 enhances the efficacy of an insect pathogenic fungus, *Beauveria bassiana*. Therefore, we attempted to find out if the venom protein enhances the efficacy of a commonly used insecticidal baculovirus (BV) *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) by improving time of death and dosage required to kill host insects. To achieve this, recombinant (genetically modified) baculoviruses were constructed by introducing the gene coding for Vn50 into the virus genome.

Two recombinant baculoviruses (BVs) were constructed one expressing Vn50 (recVn50) and the other one only expressing a reporter gene (GFP; recGFP) as control. Expression of Vn50 was confirmed by Western blotting using specific antibodies to the protein. We found that the insect cells infected with the rec-Vn50+GFP BV produce the Vn50 protein and secret it into the medium (**Fig. 20, lane 5**), but the protein was not detected in the controls; non-infected cells or those infected with the rec-GFP BV.

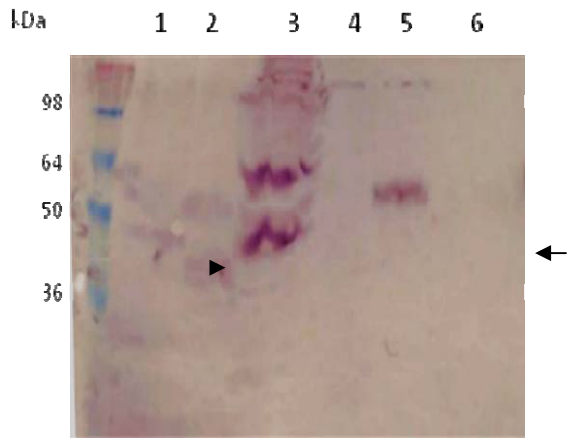


**Fig. 20:** Northern blot analysis of cells infected with recombinant Vn50 and GFP only viruses. Proteins were run on a gel, transferred onto a membrane and probed with a specific antibody to Vn50. Expression of Vn50 was detected in cells (lane 2, arrowhead) and medium collected from the cells (lane 5, arrow) but not in other lanes. (1) Cells infected with rec-GFP BV, (2) cells infected with rec-Vn50+GFP BV, (3) non-infected cells showing two bands but they are not the right size for Vn50, (4) medium from cells infected with rec-GFP BV, (5) medium from cells infected with rec-Vn50+GFP BV, (6) medium collected from control non-infected cells. The numbers on the left are protein molecular weight markers shown in kDa.

After confirming expression of Vn50, BV polyhedra (virus containing inclusion bodies) were produced in large numbers in insect cells, purified. Three different lepidopteran insects were tested in bioassays using droplet feeding method. These were *Crocidolomia pavonana, Plutella xylostella* and *Spodoptora littoralis*. *C. pavonana* treated with recombinant baculoviruses succumbed to infection. The number of deaths as a result of infection with recGFP and recVn50 in *C. pavonana* was not statistically significantly different. We also obtained similar results in *P. xylostella*. However, the recombinant virus caused significantly more mortality in *S. littoralis* larvae as shown in **Table 1**. This indicated that Vn50 may function in a species-specific manner to enhance the efficacy of BV infection.

**Table 1.** *Spodoptera littoralis* neonates droplet assay using rec-Vn50 and the wild-type virus. PIB: polyhedra inclusion bodies.

| **Virus (PIB)** Wild-type | **Mortality (%)** | **Virus (PIB)** Rec-Vn50 | **Mortality (%)** |
|---|---|---|---|
| 1000 | 3.3 | 1000 | 34.6* |
| 5000 | 17.6 | 5000 | 67.7* |
| 10000 | 28.1 | 10000 | 73.1* |

# 4. Discussion

Microbes and parasites that infect insects all must interact with the host's immune system in order to thrive (Beck & Habicht, 1996). The key response against larger objects, such as parasitoids, involves the initial recognition by the host's recognition molecules, followed by encapsulation of the foreign body by haemocytes and finally the deposition of melanin on the intruder (Carton & Nappi, 1997). As well as venom proteins, parasitoids may inject other components, such as polydnaviruses, into the host during oviposition (Andrew *et al.*, 2006; Asgari, 2011). These maternal factors can have a range of effects, from regulation of developmental factors such as juvenile hormone (Dong *et al.*, 1996) to castration of the host (Jones, 1996) to more passive forms of evasion of the host's immune cells (Reineke *et al.*, 2006).

In this project, we were interested in the melanization response and the way in which a parasitoid venom protein (Vn50) influences this enzymatic cascade in the host. The prophenoloxidase (proPO)-activating system is a terminal component of a proposed strictly regulated serine proteinase cascade in which each enzyme activates the next via limited proteolysis (Cerenius & Söderhäll, 2004). The importance of melanization in combating pathogens, especially parasitoids, has been demonstrated but in some systems phenoloxidase (PO) activity has been proven to be non-essential (Cerenius *et al.*, 2008). PO has been shown to be important in pigmentation and inactivation of molecules that regulate this pathway can result in pigmentation defects and widespread melanization of tissues (Scherfer *et al.*, 2008; Tang *et al.*, 2006).

As well as in the encapsulation and exclusion of parasites, PO has also been shown to be important in clotting and wound-healing (Christensen *et al.*, 2005; Dushay, 2009). In some species, PO is required for soft clot formation but in others, PO was not essential. Using PO-deficient mutants which lack crystal cell differentiation, it was shown that in both normal and mutant flies, bacteria were caught within the clot, but that dead bacteria were found in both clots during melanization. Though wound-healing may be important to defend against bacteria, PO is not the key antibacterial agent. Other studies have also shown that the Toll/Dif and Imd/Relish pathways are more important for immune activity against bacteria (Hedengren-Olcott *et al.*, 2004; Nehme *et al.*, 2007).

The GAL4-UAS system in *D. melanogaster* is a well-established tool which gives the ability to investigate the effects of over-expression or ectopic gene expression in a comprehensively studied organism (Duffy, 2002; Spradling, 1986). Using this system, we were able to ubiquitously express the wasp venom protein, Vn50, in the model insect *D. melanogaster*. This allowed for the further study into the venom protein's effects on immune function against common pathogens and parasitoids as well as secondary effects on development and fecundity. The expression of Vn50 in *D. melanogaster* reduced melanization in the host. This agreed with past studies which showed that purified Vn50 expressed by recombinant bacteria inhibited the activation of proPO in the haemolymph of the natural host, *Pieris rapae* (Asgari *et al.*, 2003b), as well as in the model lepidopteran system, *Manduca sexta* (Zhang *et al.*, 2004). The protein sequence of Vn50 suggests that Vn50 inhibits proPO by competing with serine protease homologues (SPHs) for binding but lacks the residues essential for cleavage. It was also important to use an insect system to express this venom protein to obtain a more natural protein including correct glycosylation. Our results show that Vn50 has a similar inhibitory effect in *Drosophila*, by suppressing melanization confirming that Vn50 can have an inhibitory effect in a much wider range of insects.

The expression of Vn50 in *Drosophila* had a significantly larger effect on reducing melanization in females than in males. Male and female insects have been shown to have marked differences in immune investment strategy (Winterhalter & Fedorka, 2009). PO activity has been shown to be higher in female *D. melanogaster* than in males (Kraaijeveld *et al.*, 2008). This could mean that it can be easier to see significant differences caused by inhibitors in female *Drosophila*. This increased investment in female flies has been shown to correlate with stronger immune responses

(Kraaijeveld *et al.*, 2008). Female flies challenged with the larval parasitoid, *Asobara tabida*, encapsulated a higher proportion of parasitoid eggs than male flies. Also, females infected with the microsporidian, *Tubulinosema kingi*, had a higher survival rate than males (Kraaijeveld *et al.*, 2008).

We also investigated the consequences that this reduction in PO activity may have on *Drosophila*'s immune function against parasites. Vn50-expressing female flies showed increased mortality against the pathogenic fungus, *Beauveria bassiana* compared to control line flies but males showed no difference. This agrees with the melanization data, with females having less PO activity converting to a lesser ability to defend against the pathogenic fungus. A strong link between antifungal recognition proteins and the proPO cascade has been established in past studies (Gillespie *et al.*, 1997; Wilson *et al.*, 2001) and the mode of infection through the cuticle would also enhance the importance of PO in defence against this fungus.

No difference was seen between Vn50-expressing flies and control line flies when challenged with the entomopathogenic bacteria, *Serratia marsescens*. This is understandable because other components of the immune system, such as the Toll- and IMD-mediated humoral response, are the main components necessary for immunity against this Gram-negative pathogen and not the melanization cascade. When injected with *Drosophila* C virus (DCV), the opposite to the fungus response was seen. Vn50-expressing flies showed delayed mortality when infected with DCV. This could possibly be due to a shift in investment of resources from PO into other components of the immune system.

The immune function of the Vn50-expressing flies was further investigated with regard to their response to an endoparasitoid, *Leptopilina boulardi*. Eggs laid by these flies were collected and the first instar larvae were introduced to mated, female *L. boulardi*. The success of these endoparasitoids was then recorded, with respect to parasitism success, development time, size of eclosed parasitoid and sex ratio. As expected, there was no difference in parasitism success in the Vn50 line (Rizki & Rizki, 1990; Ruuhola *et al.*, 2010). No difference was observed in the size of parasitoids emerging. As size is often related to fitness, it was important to determine whether the Vn50 flies had different weights or wing lengths (Ellers *et al.*, 2001; Jervis *et al.*, 2008; Lykouressis *et al.*, 2009). This led us to the conclusion that expression of Vn50 in the *Drosophila* host does not affect the fitness of parasitoids. Surprisingly, we saw a difference in sex ratio of *Leptopilina* emerging from the two fly lines with Vn50-expressing flies yielding a higher ratio of female parasitoids. Past studies which examined parasitoid egg-laying in hosts of varying suitability showed that females allocate a higher number of female eggs to more suitable hosts (Cheng *et al.*, 2010; Ghimire & Phillips, 2010). The significantly higher ratio of female parasitoids emerging from the Vn50-expressing *Drosophila* line suggests that the parasitoids are somehow able to determine that these are more suitable hosts and that this is independent of host size.

Secondary effects other than on immune function were seen in the Vn50-expressing flies. These flies appeared to have faster larval development and greater oviposition. This could be due to the PO pathway also affecting dopamine levels which is known to have a wide range of effects on many other insect physiological functions (Gruntenko *et al.*, 2003; Hiruma & Riddiford, 2009; Hodgetts & O'Keefe, 2006). However, we did not observe any effects on cuticle tanning or hardening. Dopamine levels are closely related to juvenile hormone levels especially during larval development cycles. When dopamine levels are low, juvenile hormone is degraded, which leads to moulting of the larvae (Noguchi & Hayakawa, 1996). This could explain our observation of faster pupation and eclosion in Vn50 flies. The lower levels of dopamine could be causing the precocious moulting. This might be further investigated by sampling larvae at different times to determine at which larval instar they are. Vn50 expression within *Drosophila* also caused higher fertility with more eggs being laid than by control females. The increased oviposition rate can also be explained by looking at the implications of high dopamine levels in young females. When dopamine levels

were increased using treatment with ecdysone, egg-laying and fecundity in young females was reduced (Gruntenko *et al.*, 2003). This trend is in agreement with the egg-laying we observed in the Vn50-expressing fly line.

In conclusion, we investigated the effects that a parasitoid venom protein has on the melanization cascade of insect hosts. The full-length of the protein was found to be required for the inhibitory effect of the protein. In the case of introduction of Vn50 into *D. melanogaster*, this had a direct effect on the host's PO cascade. It was seen that the modification of this component of the host's defence system had significant implications on the immune function of the hosts, making them more vulnerable to entomopathogenic fungi. In the case of genetically modified baculoviruses, it was shown that the viruses have a significant advantage over the wild-type virus in a species-specific manner by requiring significantly less virus occlusion bodies to cause mortality in insects.

## 5. Technology transfer

One paper was published in Insect Molecular Biology (see Appendix) and another one is in preparation for submission to another peer reviewed journal (Insects). Updates were also provided throughout the life of the project as summaries for publication in the HAL annual reports.

## 6. Recommendations - scientific and industry

**Scientific recommendations**. The demonstrated link between suppression of melanization and Vn50 in non-host insects (*Manduca sexta* and *Drosophila melanogaster*) suggests that the protein has this activity in a wider range of insects. The fact that none of the main domains in the protein (clip and protease-like) on their own inhibited melanization suggests that if the protein is to be used for any applications (transgenic insect or microbial control agent), the full-length protein should be produced. Although Vn50 suppressed the immune system of genetically modified *D. melanogaster*, it had no negative impact on parasitoid wasps developing in the transgenic fly larvae. The enhancement of the effect of an entomopathogenic fungus against insects expressing Vn50 or enhancement of the efficacy of genetically modified baculoviruses producing Vn50 demonstrated that the protein has the potential to be utilized for further applications to enhance the efficacy of microbial control agents.

**Industry recommendations**. The research indicates that the venom protein studied (Vn50) interferes with the immune system of insects making them more vulnerable to infection by pathogens. This is because the venom protein blocks one of the important immune responses of insects and therefore pathogens can infect and reproduce more efficiently causing higher mortality in insect pests. Although the effect of Vn50 on other hosts, including plants and animals, has not been investigated, the absence of a similar melanization process in plants and vertebrates suggests that Vn50 may not have any negative effects on these organisms. In addition, baculoviruses are highly selective for insect pests, therefore, they can be safely used without disturbing biological control. Further, several studies have shown that recombinant viruses generally produce fewer progeny compared to wild-type baculoviruses because they kill the host faster; therefore, they are rapidly out-competed in the ecosystem. In other words, they do not persist in nature. Based on the literature, they can also be effectively used with crops expressing *Bacillus thuringiensis* toxins.

The results from this research also showed that the protein has the potential to increase the efficacy of microbial control agents so that less number of those is needed to be sprayed in the field. Although technology to genetically modify different types of pests to produce this protein is limited to few model systems, the most practical application of this protein and other proteins that suppress insect immune responses is in the production of genetically modified baculoviruses that are native to Australia to produce this protein with superior efficacy over wild-type strains. This requires further research into generating these genetically modified viruses and testing them against a range

of insect pests native to Australia. Subject to regulatory approval, genetically modified pathogens expressing Vn50 protein could be commercially produced and utilized in pest control. Currently, many different strains of baculoviruses are commercially produced around the world, including Australia, and used in pest control. Production of genetically modified baculoviruses in large quantities that express Vn50 is possible using facilities available for production of wild-type viruses.

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# Ectopic expression of an endoparasitic wasp venom protein in *Drosophila melanogaster* affects immune function, larval development and oviposition

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## Abstract

**Endoparasitic hymenoptera inject maternal factors into the host, along with their eggs, to subvert the host immune system. The venom protein, Vn50, previously characterized from the wasp *Cotesia rubecula* inhibits prophenoloxidase activation in its host *Pieris rapae* and in another lepidopteran, *Manduca sexta*. We generated a stable line in the model insect, *Drosophila melanogaster*, which ectopically expresses Vn50. Results indicated that Vn50 expression accelerates larval development, increases oviposition and reduces melanization in the haemolymph of the transgenic flies. Since melanization is known to be an important facet of the insect immune response, we examined the impact of Vn50 expression on susceptibility to pathogens. Transgenic Vn50 flies challenged with the fungus *Beauveria bassiana* had increased mortality compared with control flies, but there was no significant change in survival in flies challenged with the pathogenic bacteria, *Serratia marcescens*. Interestingly, mortality induced by the natural pathogen *Drosophila* C virus was significantly delayed in Vn50 expressing flies. This indicates a wider range of potential hosts that may be affected by Vn50 and its potential for manipulation of immune system in insects.**

**Keywords: *Drosophila melanogaster*, Vn50, prophenoloxidase, melanization, immunity, serine protease homologue.**

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## Introduction

A major component of the invertebrate immune system is the production of melanin which is deposited on foreign bodies in the haemolymph to exclude and kill them (Carton & Nappi, 1997). The process is stringently controlled by a cascade of proteinase enzymes, each activating the next. A key enzyme in this pathway is prophenoloxidase (proPO), which catalyses many steps towards the end of the melanization cascade (Söderhäll & Cerenius, 1998). Activation of proPO requires a proPO activating enzyme (PAP) and a serine proteinase homologue (SPH) cofactor in order to be proteolytically cleaved into active phenoloxidase (PO) (Yu *et al*., 2003).

Endoparasitic hymenoptera inject maternal factors along with their eggs in order to evade or inhibit the host's immune system while they develop (Pennacchio & Strand, 2006). These include calyx fluid proteins, virus and virus-like particles and venom components (Asgari, 2006). A 50 kDa venom protein (Vn50) was isolated from the venom sac of the solitary endoparasitoid *Cotesia rubecula* (Hym: Braconidae) which is injected along with its eggs and other maternal factors, including polydnaviruses (PDVs), into its lepidopteran host, *Pieris rapae* (Asgari *et al*., 2003). Vn50 was shown to bind to proPAP, PAP, and proPO reducing melanization in *P. rapae* haemolymph. The protein shows significant sequence similarity to serine protease homologue proteins (SPHs) that have been shown to facilitate activation of proPO into PO upon immune activation following cleavage into proteinase-like and clip domains (Jiang & Kanost, 2000). However, Vn50 is not cleaved into the two domains and remains stable in the host haemolymph (Zhang *et al*., 2004). Further investigation demonstrated that Vn50 also had the same effect in the model lepidopteran organism, *Manduca sexta*, inhibiting activation of proPO to PO. *In vitro* enzyme assays showed that addition of Vn50 to proPO activation assays containing purified proPO, proPAP and SPHs inhibited activation of proPO to PO in a concentration-dependent manner, suggesting that Vn50 competes with

SPHs for binding to proPO and, since Vn50 is not cleaved into the two domains, proPO is not activated into PO (Zhang *et al*., 2004).

The production of transformed *Drosophila melanogaster* flies, which ectopically express a gene of interest, is a well established method that can aid in elucidating the key functions of a protein (Spradling, 1986; van Roessel & Brand, 2000). The GAL4/UAS system has been described as 'a fly geneticist's Swiss army knife' (Duffy, 2002) as it allows the testing of the effects of a protein within this model system. The two components of this system are kept in separate fly lines. GAL4, a yeast transcriptional activator, is the 'driver'. UAS is the Upstream Activating Sequence; a tandem array of five optimized GAL4 binding sites referred to as the 'responder' (Brand & Perrimon, 1993). Rørth (1998) described the assembly of the UASp transposable vector. This UAS vector is modified for expression in the germline, with the basal promoter replaced due to poor activity of the heat shock promoter in germline tissue. The reporter gene used in the UASp has the misleading name 'white' but confers a red eye phenotype (white+). A helper plasmid coding for a transposase must be injected along with the transposable vector to supply the temporary ability to insert the UAS construct into the fly's genome.

Here, we describe generation of *D. melanogaster* transformed flies that ectopically express Vn50 using the GAL4/UAS system. We analysed the effect of Vn50 on immune status of the flies by measuring melanization and mortality following application of three pathogens (a bacterium, a fungus and a virus) that can cause mortality in *Drosophila*. Our results showed that expression of the protein reduced melanization and made them more susceptible to *Beauveria bassiana* fungal infection but not to the bacterium *Serratia marcescens*. Conversely, Vn50 causes increased immunity against *Drosophila* C virus (DCV). Also examined, were the secondary effects that Vn50 impart on the flies' development, resulting in a shorter larval duration time and an increased number of eggs oviposited by Vn50-expressing flies. This is postulated to be attibutable to a decrease in L-3,4-dihydroxyphenylalanine (L-DOPA) concentration indirectly affecting juvenile hormone (JH) levels.

## Results

### *Generation of a Vn50-expressing* Drosophila *line*

Following injection of embryos and emergence of adults, the adults were mated with $W^{1118}$ flies, twelve fly lines were created from these, with six determined to contain UAS-Vn50 on the 3rd chromosome and one on the 2nd chromosome. The other five did not stably keep red eyes and were discarded. The seven successful lines were used in

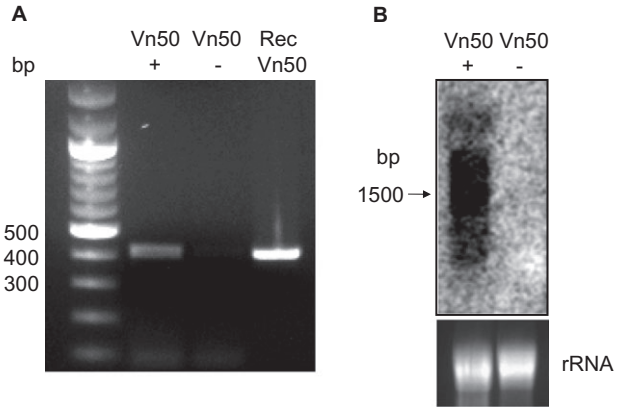


**Figure 1.** Confirmation of expression of Vn50 in transformed flies. (A) reverse transcription (RT)-PCR analysis using RNA extracted from whole flies and Sf9 cells infected with a Vn50 recombinant baculovirus (Rec Vn50) as a positive control and RNA from da-GAL flies as negative control. (B) Northern blot analysis of the samples using a Vn50 cDNA as a probe. rRNA is shown for equal loading.

Northern blotting and reverse transcription (RT)-PCR to confirm Vn50 expression and to give an indication of which lines had the greatest Vn50 expression with the best line shown in Fig. 1A, B. When the gel was visualized, the 380 bp clip domain was seen as a distinct band present only in the Vn50-expressing *Drosophila* sample (Vn50+) and in insect cells (Sf9) expressing a recombinant Vn50, using a baculovirus expression system as a control (Fig. 1A). The band was not found in the driver line control sample (Vn50–). Further, the samples were analysed by Northern hybridization which only displayed the expression of Vn50 gene in the transformed line (Fig. 1B). This demonstrated successful introduction of Vn50 gene into the genome and its ectopic expression. Unfortunately, our specific antibody to Vn50 cross-reacted with an endogenous SPH from *Drosophila* which shows a significant amino acid similarity to Vn50.

### *Inhibition of melanin formation*

To find out if expression of Vn50 has any effect on melanin formation in transformed flies, melanization assays were carried out in the presence of a PO substrate, L-DOPA. The haemolymph of both male and female Vn50-expressing flies showed a decrease in absorption at 485 nm in the presence of L-DOPA (Fig. 2A). However, the difference in females was statistically significant ($P < 0.05$) whereas in males it was not ($P > 0.1$). This suggested that Vn50 expressed in transformed flies is functional and had an inhibitory effect on melanization of *D. melanogaster* haemolymph.

### *Treatment with* Serratia

When *Drosophila* male and female flies were stabbed with an optical density (O.D.) = 0.5 suspension of *Serratia* in

 

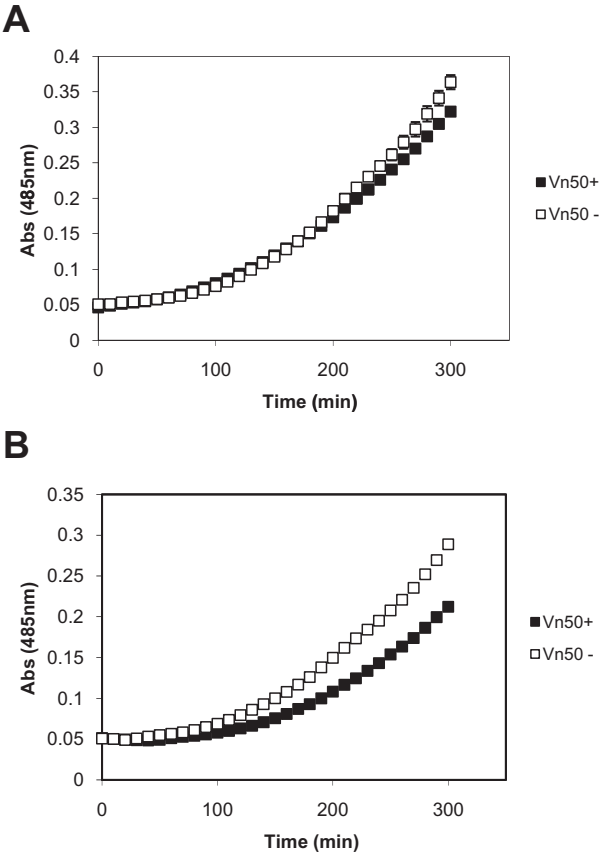


**Figure 2.** Vn50 reduces melanization in transformed female flies. Absorbance readings over 300 min for male (A) and female (B) phenoloxidase assays using L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard deviations of averages from four replicates.

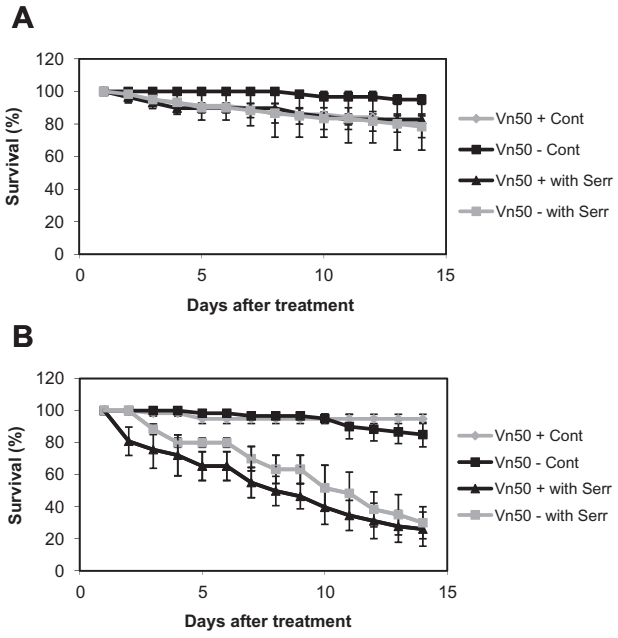


**Figure 3.** Survival of male (A) and female (B) *Drosophila* after being treated with *Serratia* (with Serr) or lysogeny broth (Cont). Vn50+ represents the Vn50-expressing flies and Vn50- represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

lysogeny broth (LB), very little difference was observed between Vn50-expressing and control flies with respect to mortality (Fig. 3). The two lines showed no difference in survival after being treated with the pathogenic bacteria in either males ($P > 0.5$) or females ($P > 0.1$). The data demonstrates that the expression of Vn50 in this GAL4-UAS system did not translate into any significant difference in survival when treated with *Serratia*.

### *Treatment with* Beauveria

When adult flies were topically treated with *Beauveria* (0.5 μL of a $1 \times 10^{10}$ spores/ml in canola oil), survival results were in agreement with those of the PO activity assays (Fig. 4). The small difference in melanization between the Vn50 and control line male flies did not convert into a significant difference in survivability against *Beauveria* (Fig. 4A; $P > 0.1$). However, female flies that ectopically expressed Vn50 suffered reduced survival when treated with the fungus compared with control females (Fig. 4B; $P < 0.005$). This showed that the differ-

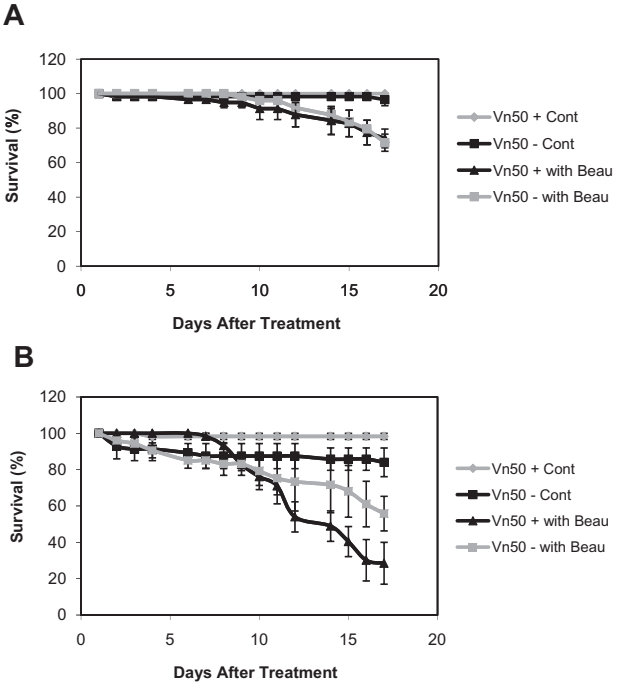


**Figure 4.** Survival of male (A) and female (B) *Drosophila* after being treated with *Beauveria* (with Beau) or oil (Cont). Vn50+ represents the Vn50-expressing flies and Vn50– represents the da-GAL flies used as control. Error bars indicate standard error of the mean from four replicates.

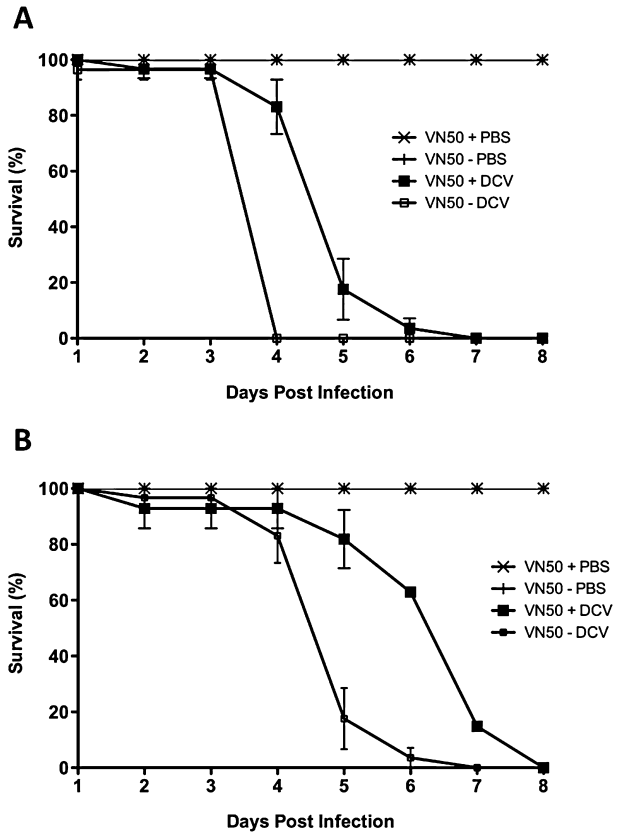


**Figure 5.** Survival of *Drosophila* adults after being treated with *Drosophila* C virus. Vn50+ represents the Vn50-expressing flies and Vn50– represents the da-GAL flies used as control. Survival of flies mock infected with PBS also shown. Error bars indicate standard error of the mean from two replicates.

ence in melanization levels caused by the introduction of Vn50 resulted in a reduction in immune function against this pathogenic fungus. There was no significant difference between Vn50 and control flies treated with oil in males and females (Fig. 4A, B; $P > 0.1$).

*Treatment with Drosophila C virus (DCV)*

Vn50 flies challenged with DCV showed a delay in virus-induced mortality. For both Vn50 and control flies the mortality in phosphate-buffered saline (PBS)-injected

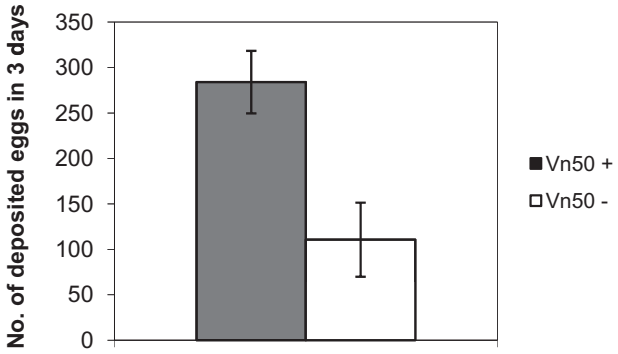


**Figure 7.** Number of progeny produced from three days of egg laying. Vn50+ represents the Vn50-expressing flies and Vn50– represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

controls was negligible. All DCV-injected male control flies died within 4 days of infection (Fig. 5A), whereas mortality of male Vn50 flies challenged with DCV was significantly delayed ($P < 0.0001$), not reaching 100% until 7 days post infection. Similarly there was a significant delay in the mortality of female Vn50 flies compared with control flies (Fig. 5B, $P < 0.0001$). These results indicate that expression of Vn50 in *D. melanogaster* mediates a significant decrease in DCV-induced mortality.

*Larval development*

In density-controlled vials, pupal formation of Vn50-expressing flies began 5 days after egg laying and peaked on day 6 whereas control flies only began having pupae formed on day 6, peaking on the 7th day (Fig. 6A). This converted into a one day delay of adult eclosion with Vn50 flies beginning to eclose on day 10 and peaking on day 11 and control flies beginning to eclose on day 11 and peaking on day 12 (Fig. 6B).

*Oviposition*

The total number of progeny produced from three days of egg laying was significantly greater for the Vn50 flies than the control flies (Fig. 7). The average number of progeny

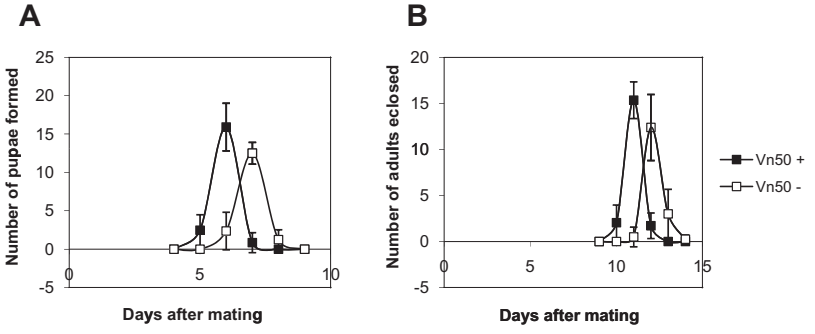


**Figure 6.** Daily pupal formation (A) and adult eclosion (B). Vn50+ represents the Vn50-expressing flies and Vn50– represents the da-GAL flies used as control. Error bars indicate standard error of the mean from three replicates.

 

produced from 10 Vn50 females was 209 compared with 146 for control flies.

## Discussion

Maternal factors introduced by endoparasitoid wasps into their hosts at oviposition are essential to subvert the host's immune system. A concerted action of these components that impact on cellular and humoral components of the host immune system appears to be required to allow successful parasitism (Pennacchio & Strand, 2006). The major immune response towards parasitoid eggs is encapsulation, usually followed by melanization of the egg surface and the capsule (Schmidt *et al*., 2001). Protein products produced by polydnaviruses, that are also injected together with eggs into the host, are the main immunosuppressive factors from ichneumonid and braconid female wasps (Webb & Strand, 2005). In addition to polydnaviruses, components of venom from endoparasitoid wasps have also been implicated in facilitating immune suppression (Asgari, 2006; Colinet *et al*., 2009). One of the targets of these venom proteins includes steps leading to the activation of proPO involved in the melanization cascade. The venom protein Vn50 from the wasp *C. rubecula* was previously shown to inhibit the activation of proPO in the haemolymph of both *P. rapae* and *M. sexta* (Asgari *et al*., 2003; Zhang *et al*. 2004). Vn50, which is structurally very similar to SPHs, is not cleaved in the host into the clip and proteinase-like domains (Zhang *et al*., 2004), the cleavage being essential for promoting proPO activation medicated by SPHs (Jiang & Kanost, 2000). *In vitro* proPO activation assays indicated that Vn50 competes with SPHs for binding to proPO. The mechanism by which SPHs mediate activation of proPO is not understood, but it is postulated that SPHs may bring PPO into a correct position for proteolysis by PAP or cause conformational changes in PPO to moderate its cleavage by PAP (Yu *et al*., 2003).

In this study, a transformed *D. melanogaster* fly line was produced which ectopically expressed Vn50 throughout its tissues. Expression of this venom protein was confirmed using both Northern blotting and RT-PCR. Asgari *et al*. (2003) demonstrated that native Vn50 or the recombinant protein produced by *Escherichia coli* significantly reduced PO activity in *P. rapae* haemolymph. Our data shows that Vn50 produced within the *D. melanogaster* system exhibits the same results, reducing melanization response, indicating that the protein affects a wider range of insects.

The difference in melanization response was markedly larger in female transformed flies. The complexity of the differences in expression of the components of the immune system in females and males and their resource investment towards reproduction and immunity implies that they may be differentially affected by Vn50 which may have caused the decreased effect observed in male flies. This also translated into increased susceptibility of Vn50-expressing female flies to fungal infection by *Beauveria*. However, this effect was not found in male transformed flies, consistent with the melanization response assay. The results from treatment of the female *Drosophila* with the fungus *B. bassiana* demonstrates the effect that Vn50 may have on suppressing the immune system of the host through reduction of melanization. The venom protein reduces the level of PO activity in the host and, consequently, reduces the host's ability to melanize and destroy pathogens. Vn50-expressing females were not able to resist the fungal pathogen as well as control flies and, therefore, suffered a higher level of mortality than control flies. On the other hand, expression of Vn50 did not have any effect on survivability of transformed flies against bacterial infection. This could be due to the fact that phagocytosis and antibacterial peptides, not melanization, are the major immune responses towards invading bacteria (Lemaitre & Hoffmann, 2007). In addition, it has been shown that in a mutant *D. melanogaster* fly line (CG3066), in which activation of proPO is inhibited, flies exhibit variable resistance to a number of bacterial species compared with wild-type, ranging from significantly less resistance to equally susceptible, compared with the wild-type (Ayres & Schneider, 2008).

Interestingly, response of flies to virus infection was affected by Vn50, although in reverse fashion. Expression of the venom protein delayed DCV-induced mortality. This was unexpected since melanization of virus-infected cells in non-permissive hosts has been reported to limit baculovirus infection (Washburn *et al*., 1996; Rivkin *et al*., 2006). However, this response has not been reported in *D. melanogaster* infected with DCV which suggests that responses to RNA versus DNA viruses might be different. Interestingly, *Drosophila* flies harbouring the endosymbiotic bacterium, *Wolbachia*, are protected from DCV-induced mortality, consistent with the delays in mortality we observed in Vn50-expressing flies (Hedges *et al*., 2008; Teixeira *et al*., 2008). Our preliminary data indicates that presence of *Wolbachia* in flies also reduces melanization (unpublished data). Although the mechanism of *Wolbachia*-mediated protection is currently unknown, it has been shown that the bacterium slows down accumulation of the virus, and virus-induced mortality is also delayed (Hedges *et al*., 2008; Teixeira *et al*., 2008). Further investigations showed that the delayed mortality might be attributable to *Wolbachia* conferring tolerance to virus infection rather than affecting virus accumulation in flies since some strains do not affect virus accumulation but confer protection (Osborne *et al*., 2009). Therefore, reduction in melanization, which is also observed in *P. rapae* parasitized by *C. rubecula* from which Vn50 is

derived, may not have a negative effect on infection of host cells by *C. rubecula* polydnaviruses.

Considering that PO plays an important role in oxidation of tyrosine to DOPA, which can be decarboxylated to dopamine by dopa decarboxylase (Huang *et al*., 2005; Nappi & Christensen, 2005), inhibition of PO by Vn50 should lead to a reduction in DOPA and consequently, to less dopamine available. This decrease in dopamine has the potential to affect many physiological functions as it has been shown to be very important in JH degradation, neurotransmission, stress responses, ovarian development and oviposition, among others (Gruntenko *et al*., 2003; Hodgetts & O'Keefe, 2006; Hiruma & Riddiford, 2009). Rauschenbach *et al*. (2006) demonstrated that treating flies with DOPA delayed oviposition and decreased the fecundity of flies. The Vn50-expressing flies followed this correlation with inhibition of PO activation possibly leading to a subsequent decrease in DOPA/dopamine production causing increased oviposition. The retardation of larval growth and delay in pupation caused by elevated dopamine levels after parasitization has also been demonstrated (Noguchi & Hayakawa, 1996). The shortened larval period shown in our experiments could also be attributable to lowered levels of dopamine.

In conclusion, a UAS-Vn50 line was produced that allowed the production of Vn50-expressing progeny after mating with a GAL4 driver line. Vn50 expression was confirmed in these flies and PO assays showed that the females had suffered from reduced melanization. Bioassays showed that this reduction in melanization did not translate into any significant difference in immunity to *S. marcescens* but did cause greater mortality after treatment with *B. bassiana*. Interestingly, expression of Vn50 protected flies from DCV infection similar to the delay seen in *Wolbachia*-infected flies. Expression of the protein also caused further secondary effects on development, with the flies having accelerated oviposition and larval development. The effects of Vn50 on *D. melanogaster* indicate that a wider range of insects could be affected by the protein and the importance of PO enzyme in various physiological processes in insects.

## Experimental procedures

### *Generation of transformed* Drosophila *lines*

Vn50 was amplified using PCR with GCGCGGTACCTCAGCATGAAGTGGAAAATATTTAG as the forward and GCGCTCTAGATTAGTCAGCAAATGCTTC as the reverse primer containing restriction sites for direct cloning into the transfer vector (underlined nucleotides). Vn50 was cloned into the pUASp transposable vector at the KpnI and XbaI sites and confirmed using a restriction digest and sequencing in both directions. EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) was used to remove endonucleases and extract plasmid from bacterial culture. pUASp and pUChsneo Δ2-3 were mixed and diluted in injection buffer (0.1 mM sodium phosphate buffer, 5 mM KCl) to a final concentration of 250 ng/μL and 50 ng/μL, respectively. Seven hundred and ninety seven eggs of the $W^{1118}$ strain of *D. melanogaster* (pre-treated with tetracycline to remove *Wolbachia*) were microinjected in the posterior end within 1 h of being laid, using a pulled glass needle attached to a Narishige microinjector, and after 24 h they were heatshocked at 37°C for 1 h. Eclosing adults were mated with $W^{1118}$ males and virgin females. One mating resulted in red-eyed progeny. These were mated with 2nd and 3rd chromosome balancer lines (CyO/Gla and TM3/TM6B, respectively) to determine the position of insertion of UAS-Vn50 which allowed production, through mating, of a fly line homozygous for UAS-Vn50 (Vn50-Vn50). This line was crossed with a da-GAL (P{Gal4-da.G32}UH1; Bloomington Stock Centre) line to enable ubiquitous expression of Vn50 in the cells of the progeny.

### *Confirmation of expression*

Confirmation of transposition of UAS-Vn50 into the flies' genome and, consequently, expression of Vn50 was first carried out using RT-PCR. The primers used were the forward primer described above and the internal reverse primer GCGCGGTACCTTATCTAAAACCTACACCAT. RNA samples extracted from flies were treated with DNase I (Promega, Madison, WI, USA) prior to cDNA synthesis to ensure removal of contaminating DNA. Samples were run on a 1% agarose gel and visualized under UV light. In addition, expression of Vn50 gene was confirmed using Northern blotting. Complementary DNA to Vn50 gene labelled with [$^{32}$P]-dCTPs was used as a probe. Hybridizations and washings were carried out under stringent conditions (65°C).

### *Prophenoloxidase enzyme activity*

To determine PO activity in transformed and control fly lines, a haemolymph phenoloxidase assay was carried out similar to that described previously (Asgari *et al*., 2003) but with slight modifications. Flies were collected daily to control ages of flies and were used when aged between 4 and 8 days. Five male or female 4- to 8-day-old flies were added to 100 μL of ice cold PBS and crushed. These were then centrifuged at 13 000 ×*g* for 15 min to separate haemolymph from cell debris. After 10 min L-3,4-dihydroxyphenylalanine (L-DOPA) was added to PBS to 20 mM final concentration, and vortexed to dissolve. The first 10 μL of haemolymph was discarded to avoid floating debris. In each well of a 96-well plate, 10 μL of haemolymph was mixed with 90 μL of 20 mM L-DOPA by pipetting. Absorbance at 485 nm was then measured every 10 min for 300 min. Experiments were repeated at least three times and data were analysed using Microsoft Excel.

### *Treatment with* Serratia

An overnight suspension of *Serratia* in LB was centrifuged and resuspended to a final O.D. of 0.5. Each 4- to 8-day-old fly was first anaesthetized with $CO_2$ and then stabbed in the side of the abdomen with a thin needle which had been dipped in the suspension. Care was taken to be as gentle as possible while ensuring that the needle pierced the exoskeleton. Control flies were stabbed with medium only. Flies that did not survive treatment were removed after the first day and mortality was recorded everyday afterwards. Twenty flies were used for each treatment.

 

Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

*Treatment with* Beauveria

Treatment of flies with *Beauveria* also involved anaesthetizing 4- to 8-day-old flies with $CO_2$ but on a piece of filter paper to soak up excess liquid. 0.5 μL of a $1 \times 10^{10}$ spores/ml of *Beauveria* (Strain GHA, BotaniGard) in sunflower oil was pipetted onto the abdomen of each fly. Control flies were treated with sunflower oil only. Flies that did not survive treatment were removed after the first day and mortality was recorded everyday afterwards. Twenty flies were used for each treatment. Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

*Treatment with Drosophila C virus*

Drosophila 6 to 7 days old were infected with DCV or mock infected by micro-injection of virus or PBS as previously described (Osborne *et al*., 2009). For each fly line assayed, two groups of 15 flies were injected with virus and one group of 15 flies was injected with PBS. Mortality that occurred within one day of injection was deemed to be due to injury. Survival curves were compared using Kaplan-Meier analysis and log-rank statistics reported (GraphPad Prism).

*Larval development*

Three-day-old virgin flies were mated for two days and then placed in plastic bottles with molasses lids. These had some yeast paste (yeast and water) placed on the middle of the plate and were stored upside-down. After 24 h, eggs were collected and placed, 20 each, in fly food vials. The number of Vn50-expressing and control pupae formed and adults eclosing were recorded daily.

*Oviposition*

Ten 4-day-old, Vn50-expressing male flies and 10 females were placed in each fly food vial. These were left for 3 days before they were removed from the vials. The same was carried out for control flies. The number of progeny from these flies was recorded as the total number of pupae that formed in these vials.

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