# Discovery and characterization of a novel picorna-like RNA virus in the cotton bollworm *Helicoverpa armigera*

**Xianming Yang[1¶], Pengjun Xu[2¶], He Yuan[1], Robert I. Graham[3], Kenneth Wilson[4] and Kongming Wu[1*]**

[1] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Key Laboratory of Integrated Pest Management in Crops, Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, P.R. China;

[2] Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, P.R. China;

[3] Crop and Environment Sciences, Harper Adams University, Edgmond, Shropshire, UK;

[4] Lancaster Environment Centre, Lancaster University, Lancaster, UK

[¶] These authors equally contributed to this paper.

*Corresponding author:
Mailing address: State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R. China; Phone: +86-10-82105551; Fax: +86-10-62896114; E-mail: kmwu@ippcaas.cn.

## Abstract

We characterize a novel picorna-like virus, named *Helicoverpa armigera Nora virus* (HaNV), with a genome length of 11,200 nts, the sequence of which was isolated from the lepidopteran host cotton bollworm *Helicoverpa armigera*, using RNA-Seq. Phylogenetic analysis, using the putative amino acid sequence of the conserved RNA-dependent RNA polymerase (RdRp) domain, indicated that HaNV clustered with *Spodoptera exigua Nora virus*, *Drosophila Nora virus* and *Nasonia vitripennis* virus-3 with a high bootstrap value (100%), which might indicate a new viral family within the order *Picornavirales*. HaNV was efficiently horizontally transmitted between hosts via contaminated food, and transmission was found to be dose-dependent (up to 100% efficiency with $10^9$ viral copy number/µl). HaNV was also found to be transmitted vertically from parent to offspring, mainly through *transovum* transmission (virus contamination on the surface of the eggs), but having a lower transmission efficiency (around 43%). Infection distribution within the host was also investigated, with HaNV mainly found in only the gut of both adult moths and larvae (>90%). Moreover, our results showed that HaNV appears not to be an overtly pathogenic virus to its host.



Keywords: *Helicoverpa armigera*; cotton bollworm; picorna-like viruses; transmission strategy; covert virus

## 1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner), is a key pest of multiple crops throughout the world. In China, resistance to synthetic pyrethroids and organophosphate insecticides in the field has caused considerable outbreaks of cotton bollworm since the early 1990s (Wu and Guo, 2005). In the late 1990s, the introduction of Bt-cotton successfully suppressed the cotton bollworm populations in the field (Wu et al., 2008). However, extensive cultivation of Bt-cotton might impose the risk of Bt-resistance evolving in field populations of *H. armigera*, and alternative control strategies are needed (Jin et al., 2015). In 1993, the baculovirus *H. armigera* nucleopolyhedrovirus (HaNPV) was authorized as a commercial biopesticide and has since become the most abundantly produced viral insecticide in China (Sun 2015). But, like other baculovirus products in the world, the application of HaNPV as a biopesticide has not yet reached its full potential. It is becoming more apparent that the susceptibility of host insects to baculoviruses might be impacted by the interactions between virus-host or microbe-virus interactions: for example, the endocytoplasmic bacterium *Wolbachia* increases the susceptibility of the African armyworm, *Spodoptera exempta*, to its endemic baculovirus, *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) (Graham et al., 2012); whereas the densovirus HaDV-2 in *H. armigera* appears to allow its host to grow faster and potentially escape infection by the baculovirus HaNPV (Xu et al., 2014). Hence, virus-host and virus-microbe interactions might be more complex than originally thought.

The recent advent of next generation sequencing technology has facilitated the

discovery of many novel viruses, especially covert viruses displaying no obvious pathological or beneficial effects to their hosts. These recent discoveries include various viruses belonging to the order Picornavirales (picorna-like viruses) (Ho and Tzanetakis, 2014; Webster et al., 2015). Insect picorna-like viruses possess a positive-sense, single-stranded RNA genome which replicates in the host-cell cytoplasm, and which is translated into one or two polyproteins cleaved into individual structural and non-structural proteins (Habayeb et al., 2009; Le Gall et al., 2008). Most picorna-like viruses known to colonize insect hosts belong to the families Dicistroviridae or Iflaviridae (Le Gall et al., 2008; Moore and Tinsley 1982; Carrillo-Tripp et al. 2015), and their pathogenicity can vary broadly from lethal to persistent commensal infections (Oliveira et al., 2010). For example, *Drosophila* C virus and slow bee paralysis virus are pathogenic to *D. melanogaster* and honey bees *Apis mellifera*, respectively (de Miranda et al., 2010; Ferreira et al., 2014); whereas examples of persistent commensal infections include *Nasonia vitripennis* virus-1 and *Spodoptera exigua* iflavirus-1 in the hosts *N. vitripennis* and *S. exigua*, respectively (Jakubowska et al., 2014; Oliveira et al., 2010). In addition, *Drosophila Nora virus* can establish infections in laboratory strains that persist for several years without seemingly causing any obvious pathological effects (Habayeb et al., 2006; Habayeb et al., 2009). Knowledge of the interactions between recently discovered viruses and their hosts should generate fresh perspectives on insect-host biology and pest management.

In this study, we characterize a new picorna-like virus in *H. armigera*, named *Helicoverpa armigera Nora virus* (HaNV), using RNA-Seq technology and laboratory bioassays. HaNV was found to show highest identities with *Spodoptera exigua Nora virus* (SeNV) (79% nucleotide identity and 90% amino acid identity). Together with *Drosophila Nora virus* and *N. vitripennis* virus-3, we propose that these viruses might form a new viral family in the order *Picornavirales*.

## 2. Materials and Methods

### 2.1 Colony maintenance

Cotton bollworm (*H. armigera*) originally collected in Langfang (Hebei province, China) in 2005 (LF2005) were reared using artificial diet (Liang et al. 1999) at 25 ± 1 °C with a 14:10, light:dark photoperiod. Adult moths were provided with 10% sugar and 2% vitamin complex.

### 2.2 RNA-Seq and virus detection

Briefly, RNA was isolated from first instar larvae, fifth instar larvae and adults using TRIzol (Invitrogen). Poly(A) mRNA was isolated from total RNA using Oligo (dT) magnetic beads and was broken into short fragments (about 200bp). The mRNA samples were used to construct the cDNA library, and the mRNA-Seq assay was performed by Novogene (Beijing, China). The libraries were sequenced using HiSeq2000 (Illumina) in paired-end mode, creating reads with a length of 101 bp (HiSeq2000, accession number: GSE86914). Adaptor sequences and low-quality reads were trimmed and clean reads were used for de novo assembly using Trinity

(Grabherr et al., 2011). Assembled contigs were annotated using BLASTx to align with the NR database. As a result of the RNA-Seq process, we found an assembled contig showing high identity with SeNV. This contig sequence was confirmed by sequencing eight overlapping amplicons covering the full genome. All primers used in this study are listed in Table 1. The ORFs were identified using ORF Finder (https://www.ncbi.nlm.nih.gov/gorf/gorf.html). Neighbor-joining trees with Poisson-corrected distances for the amino acid sequences of RdRp were constructed using MEGA 6.0 software (Tamura et al., 2013). The conserved RdRp domains were predicted using NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

**2.3. Electron microscopy**

Adult moths from the P-strain (HaNV-infected strain, see 2.5) were collected. Then the virus particles were isolated and purified using the Sucrose Density Gradient Centrifugation method 1 (La Fauce et al (2007). Each 1 ml centrifuged materials was collected from top to bottom of the 50 ml centrifuge tube. Then, collected materials were checked by RT-PCR using the HaNV-specific primers NoraPF/NoraPR (Table 1). Purified materials that were HaNV-positive were concentrated and used for the electron microscope analysis. Purified particles were negatively stained with 2% sodium phosphotungstate at pH 6.8 and observed with a transmission electron microscope.

**2.4 Uninfected colony construction and PCR protocols**

An N-strain (Non-infected) laboratory culture of *H. armigera*, uninfected with known

viruses *Helicoverpa armigera* densovirus-2 (HaDV2), *Helicoverpa armigera iflavirus* (HaIV), HaNPV, HaNV and known bacterium *Wolbachia*, was established as described previously (Xu et al., 2014; Yuan et al., 2017; Zhou et al. 1998). NoraPF/NoraPR primers were used to check the HaNV-infection status of the N-strain laboratory culture. The PCR program for the HaNV detection was: 30 s at 94 $^{o}$C, 30 s at 53 $^{o}$C, and 30 s at 72 $^{o}$C for 35 cycles.

**2.5 HaNV transmission and host tissue distribution**

A HaNV-infected line (called P-strain) of *H. armigera* was also established by orally inoculating newly hatched N-strain larvae with HaNV-infected filtered liquid from LF2005, at $10^9$ copy number/μl, which was found to yield a 100% infection rate. Briefly, individual adults were homogenized in liquid nitrogen. Part of the homogenate was subsequently used to extract DNA and RNA, which was used for cDNA synthesis. PCR was then undertaken to detect the presence of HaDV-2, HaNPV and *Wolbachia* using DNA template and HaNV and HaIV using cDNA template, respectively. Subsequently, the remaining homogenate of HaNV-positive and HaDV-2, HaNPV, *Wolbachia* and HaIV-negative individuals was used to prepare a filtered liquid, containing an unpurified form of virus. Briefly, this method involved transferring the homogenate to 1 ml PBS buffer (0.01M, pH 7.4). The homogenate was centrifuged at 6500×g for 15 min at 4°C, and the liquid supernatant subsequently filtered with Sartorius Minisart ® 0.2μm PES (Invitrogen, Grand Island, USA). The homogenates of individuals uninfected with HaDV2, HaIV, HaNPV, HaNV and known bacteria *Wolbachia* were filtered using the same method. Quantification of the

viruses was performed using the absolute qPCR method described below. Primers PF/PR was used to generate a standard curve and NoraF/NoraR/Nora-probe was used to quantify HaNV virus copy. All the HaNV-infected filtered liquids were stored at -80°C.

To examine virus replication within the host at different developmental stages, larvae 24 h to 240 h post-infection (1st-5th instar), pupae and newly eclosed adults were sampled. HaNV copy numbers was quantified by an absolute quantification qPCR methodology using a standard curve (Wong and Medrano, 2005). To ensure reproducibility, each sample was carried out in three biological replicates and in three technical replicates.

Individuals from both N-strain and P-strain were used to determine the transmission modes of HaNV. To examine the possibility of horizontal transmission through the ingestion of contaminated foodplants (a possibility in the field populations), a diet contamination assay was performed. Infected individuals from the P-strain were reared from 48-hour old larvae in diet cells until the start of the $4^{th}$ instar and then immediately removed from the rearing cell. Non-infected $1^{st}$ – $5^{th}$ instar N-strain larvae were then placed in the vacated cells and reared to the pupal stage. Newly-eclosed moths were subsequently collected and HaNV-infection status was probed using PCR (as described above). For the quantification of the virus titer in the feces, the infected $4^{th}$ instar larvae (about 10 days old) were subsequently placed into a single diet cell for 6 hours and feces were collected. For vertical transmission, ♀-/♂-, ♀+/♂-, ♀-/♂+ and ♀+/♂+ pairs were crossed and RNA from $5^{th}$ instar offspring

184 larvae used to probe for HaNV.

185 Tissue dissection for HaNV-infected individuals of cotton bollworm (both larval
186 and adult stages) was performed as in Yang et al. (2017). RNA was extracted from
187 body tissues (gut, malpighian tubules, fat body, haemolymph, muscle, brain or
188 reproductive organs) and the copy numbers of HaNV were quantified by qPCR. To
189 account for individual variation, we first calculated the copy numbers per milligram
190 of tissue and then summed all the copy numbers from different tissues from the same
191 individual and the percentage of each tissue was statistically analyzed (larvae: n = 15;
192 adult males: n = 9; adult females: n = 9).

193 HaNV infection was quantified in *H. armigera* eggs, primarily to distinguish
194 between transovarial and transovum infection routes. Eggs from HaNV-infected
195 P-strain breeding pairs were submerged in 1% sodium hypochlorite for 10 minutes.
196 They were then filtered through a damp cloth, thoroughly rinsed, and allowed to dry.
197 Three groups of hypochlorite-treated eggs (n = 100 eggs per group) were tested
198 against non-treated eggs (control) and HaNV infections tested by qPCR.

199 **2.6 Effects of HaNV infection on the survival of *H. armigera***

200 To test the impact of HaNV infection on the survival of its host, neonate N-strain
201 larvae were first orally-inoculated with either filtered-liquid containing HaNV, or
202 filtered-liquid from non-infected individuals (control). One hundred N-strain neonates
203 were placed in each treatment Petri-dish for 2 days to ensure that larvae ingested the
204 treated diet. They were then transferred to a 24-well plate (one individual per well:
205 diameter = 1.5 cm; height = 2 cm) until the 5th larval instar; larvae were then

individually reared in glass tubes until eclosion (diameter = 2 cm; height = 7.5 cm). The larval mortality, pupation and eclosion rate was recorded. Fifth-instar larvae were randomly selected to estimate the infection rate of HaNV during the experiment. Our results showed that the larvae were successfully infected by HaNV using the above protocol. This bioassay was replicated twice. Individuals dying within 24 hours of the experimental set up were considered handling deaths, and excluded from the analysis.

## 3. Results and Discussion

### 3.1 Molecular characterization of HaNV

Using RNA-Seq, a novel virus sequence, named *Helicoverpa armigera Nora virus* (HaNV), was isolated from the cotton bollworm, comprising a whole genome sequence of 11,200 nts in length, excluding the poly (A) tail (Genbank No. MK033133). Analysis of the genomic structure indicated that HaNV contained five deduced open reading frames (ORFs) (Fig 1A). ORF1 and ORF3 showed highest identities with ORF1 and ORF3 of SeNV (75% and 68% amino acid (aa) identity, respectively). ORF2 was the largest open reading frame, encoding a putative protein of 1857 aa, containing a conserved picornavirus-like helicase-protease-replicase (H-P-Rep) cassette together with conserved domains of RNA-dependent RNA polymerase (RdRp), RNA helicase and protease. ORF4 and ORF5 encoded putative structural proteins with homology to *Drosophila Nora virus* capsid protein 4 (VP4) and showed highest identities to ORF4 and ORF5 of SeNV (95% and 91% aa identity, respectively). Like the SeNV genome, ORFs 2-5 of HaNV appear to overlap with each other (Fig 1A). Phylogenetic analysis based on the putative amino acid

sequences of the conserved RdRp domain indicated that HaNV clustered with SeNV, *Drosophila Nora virus* and *N. vitripennis* virus-3 (Fig 1B) with a high bootstrap value (100%) for that grouping, suggesting a possible unclassified virus family within the order *Picornavirales* (Jakubowska et al. 2014).

Electron microscopy showed that HaNV particles had an isometric appearance, with a diameter of approximately 30 nm (Fig 2).

**3.2 HaNV transmission strategies**

Viruses show great diversity in their transmission strategies and efficiencies (Chen et al., 2006; Zhou et al., 2005). For example, HaDV2 could be efficiently vertically transmitted (around 100%) and horizontally transmitted via contaminated food (Xu et al., 2014); whereas the picorna virus *Drosophila Nora virus*, HaIV and baculovirus pathogen HaNPV can be efficiently horizontally transmitted by ingestion of virus-contaminated food (Fuxa, 2004; Habayeb et al., 2009; Yuan et al., 2017). For HaNV, the N-strain larvae were allowed to develop through to pupation and newly-eclosed adults were tested for the presence of HaNV. All adults were found to be positive for HaNV (100% infection; Table 2), suggesting a possible food-borne or oral-fecal transmission pathway.

The quantity of viral RNA in the feces produced by a single $4^{th}$-instar larva during six hours was approximately $1.5\times10^8$ viral genome copies. The HaNV infection rate of $5^{th}$-instar larvae was positively correlated with the magnitude of the challenge dose, with 100% infection rate at a dose of $10^9$ copy number/μl (Table 3), suggesting an efficient horizontal transmission strategy. Hence, the HaNV virus can

252 be horizontally-transmitted by ingestion of virus-contaminated food. HaNV copy
253 number increased over time in the larval stage of *H. armigera*, peaking in the last
254 instar before pupation. There were lower viral loads in pupal and adult stages of the
255 host (Fig 3).
256 The infection rate of the offspring from infected parents was around 43%, but
257 almost no HaNV was detected when only one parent was infected (Table 2). This
258 suggests that vertical transmission is most efficient only when both parents are
259 infected. However, the virus titer in each sex and replicate were not determined and
260 might contribute to the difference between crosses. The low transmission efficiency of
261 the HaNV virus observed in this study is similar to other picorna-like viruses, e.g.
262 *Iflavirus* in cotton bollworm (HaIV, around 28%) (Yuan et al., 2017) and *Drosophila*
263 *Nora virus* (around ##%) (Habayeb et al., 2009). In addition, the HaNV titre of
264 sodium hypochlorite-treated eggs was significantly lower than for non-treated eggs ($t = 2.840$, $df = 4$, $P = 0.047$; Fig 4), suggesting that the vertical transmission is mainly
266 due to *transovum* transmission (i.e. virus contamination on the surface of eggs) and
267 not *transovarial* (within-egg) transmission, which is similar to HaIV (Yuan et al.,
268 2017). This is, however, different from the *transovarial*-transmitted densovirus
269 HaDV2 in cotton bollworm, which can be efficiently vertical transmitted (Xu et al.,
270 2014).
271 **3.3 Within-host distribution of HaNV infection**
272 In both life-stages, HaNV viral load was significantly higher in the intestinal tract
273 than in other tissues (malpighian tubules, fat body, haemolymph, muscle, brain or

reproductive organs) (larvae: $F = 7.75$, $df = 5,29$, $P < 0.001$; female adults: $F = 1095.70$, $df = 5,17$, $P < 0.001$; male adults: $F = 5057.20$, $df = 5,17$, $P < 0.001$; Fig 5). The high HaNV abundance in the intestinal tract of both adult males and females (>90%), which could explain the observed low vertical-transmission efficiency of HaNV. Aggregation of infection within the intestinal tract is similar to the distributions observed for other picorna-like viruses, such as the *Drosophila* Nora virus (Habayeb et al., 2009) and a picorna-like virus in *Pectinophora gossypiella* (Monsarrat et al., 1995). However, it is a different distribution pattern to that of SeNV, which showed homogenous distribution in midgut, fat body and hemolymph (Jakubowska et al., 2014), and that of *Iflavirus* HaIV and *densovirus* HaDV2 in cotton bollworm, both mainly distributed in the fat body (Yuan et al., 2017).

With the advance in technology, more insect picorna-like viruses are likely to be detected in other species, which will be useful in unveiling the complicated interaction between viruses and their hosts, as well as among viruses within the same hosts. Firstly, many novel pathogenic viruses could be used directly to control pests, as has been seen with baculoviruses. Secondly, although nonpathogenic viruses may not directly impact host mortality, the interactions between them and other virus species residing in their host should be studied further as multiple-species interactions might occur, e.g. *Iflavirus* was shown to reduce baculovirus *Spodoptera exigua* multiple nucleopolyhedrovirus pathogenicity and affect occlusion bodies production (Carballo et al., 2017; Jakubowska et al., 2016); densovirus HaDV2 in *H. armigera* appears to offer its host some protection, increasing host resistance to the baculovirus

296 HaNPV (Xu et al., 2014). As HaNV was found in seemingly healthy individuals of
297 cotton bollworm (showing no pathology), HaNV appears not to be an overtly
298 pathogenic virus and might have a mild or non-pathogenic effect on its host. This
299 hypothesis was confirmed by our results that the larval mortality, pupation and
300 eclosion rates of HaNV-positive insects did not differ significantly from
301 HaNV-negative individuals (Table 4). As vertical transmission of HaNV (from parents
302 to offspring) was inefficient, and larvae probably have little chance to ingest high
303 titers of virus from contaminated food plants, a low prevalence of HaNV in the field
304 could be expected and should be studied in the field populations. Conversely, the
305 virus HaDV2 (which was found to protect its host from the baculovirus; Xu et al.,
306 2014) was found to be highly efficient at vertical transmission and to have high
307 frequency in the field (around 80%; Xu et al., 2014). However, the frequency of
308 HaNV in field populations and its effect on other co-infected bacteria or viruses
309 remained unexplored and need to be studied further in the future.
310

311 **4. Conclusions**

312 A new picorna-like virus in *H. armigera* named HaNV has been characterised, with a
313 genome of 11,200 nts in length containing the complete coding regions. Phylogenetic
314 analysis clustered HaNV with SeNV, *Drosophila* Nora virus and *N. vitripennis*
315 virus-3, which might indicate a new viral family in the order Picornavirales. HaNV
316 can be efficiently horizontally transmitted following a typical dose-dependent
317 response, and vertically transmitted but with low efficiency.

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421 **Table 1** Primers used in this study

| Code | Primer Name | Primer sequence (5'-3') | Instruction |
|---|---|---|---|
| 1 | NRV-14 | CTGAAGCACTGCACCTAAG | Amplification of the HaNV genome |
| | NRV-1279 | GGATACTTGGCAATAGGAC | |
| 2 | NRV-731 | CGTTTGTGAGTCATCTGCC | |
| | NRV-1568 | TTTGGTTCAGTTGCCCAC | |
| 3 | NRV-1386 | TCTTCATTGATACACTGGACCC | |
| | NRV-4219 | ACCTTACCCTCATAGGAGCG | |
| 4 | NRV-3950 | CACCATTTACCGCACTTTAC | |
| | NRV-6522 | GTCAACAACCCAAGACCTAATC | |
| 5 | NRV-5309 | ACAGTGAAGGATGGGTCTC | |
| | NRV-7299 | TGGTATCAAGCAACCGAG | |
| 6 | NRV-7021 | GGGAGGGAATACTATGAATCG | |
| | NRV-8582 | TGGCATCTAACTCGGGTG | |
| 7 | NRV1-7615 | AACAACAGCAGATACGGC | |
| | NRV1-10207 | CCAACACTTTAGCGAGTC | |
| 8 | NRV-9026 | AAGAAACCTGATTCATATGC | |
| | NRV-11200 | TTCACAAAACGTTTTCAAG | |
| 9 | NoraPF | TGGACCTGAAATTGGCACAT | Detection of HaNV |
| | NoraPR | CATGTCGAAGAGATAGCTCA | |
| 10 | PF | ATATGCAGGTAAAACAAAG | HaNV standard curve assay |
| | PR | CAAAACGTTTTCAAGAATAT | |
| 11 | NoraF | GCTTGATGCGAATTCTGATGAC | Quantification of HaNV |
| | NoraR | GGTTATCTCCCCAACATGTTTCA | |
| | Nora-probe | AAGAAGCTCCTGCTTTAGGCCCCGA | |

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426 **Table 2** Transmission efficiency of HaNV virus. "+" = infected individuals, "-" =
427 non-infected individuals.

| Transmission mode | Individuals | Transmission Efficiency |
|---|---|---|
| Horizontal[a] | 1st | 100% (n = 24) |
| | 2nd | 100% (n = 24) |
| | 3rd | 100% (n = 24) |
| | 4th | 100% (n = 24) |
| | 5th | 100% (n = 24) |
| | Negative control | 0% (n = 24) |
| Vertical | ♀+ /♂+ | 43.1% (n = 44) |
| | ♀+ /♂- | 3.5% (n = 64) |
| | ♀- /♂+ | 0% (n = 48) |
| | ♀- /♂- | 0% (n = 24) |

428 [a] Individuals at 1st-5th instars transferred to the contaminated artificial diet previously hosted
429 infected individuals.
430
431
432

433 **Table 3** Detection of HaNV infecting newly-hatched larvae dosed at a range of
434 concentrations.

| Virus concentration (copy number/µL) | Number testing positive | Number testing negative | Infection rate (%) |
|---|---|---|---|
| $10^9$ | 24 | 0 | 100.0% |
| $10^8$ | 8 | 7 | 53.3% |
| $10^7$ | 2 | 10 | 16.7% |
| $10^6$ | 1 | 11 | 8.3% |
| $10^5$ | 1 | 11 | 8.3% |

435 Infected individuals = "+ve", uninfected individuals = ''-ve''.
436
437
438
439 **Table 4** Influence of HaNV on selected life-history traits of cotton bollworm.

| Index | HaNV+ (%) | HaNV- (%) | n† | $\chi^2$ | P value |
|---|---|---|---|---|---|
| Larval mortality | 2.8 ( ± 0.8) | 1.9 ( ± 0.6) | 28 | 0.320 | 0.572 |
| Pupation rate | 94.7 ( ± 1.2) | 95.6 (± 1.3) | 28 | 0.416 | 0.519 |
| Eclosion rate | 91.9 ( ± 1.5) | 89.2 (± 2.0) | 28 | 1.645 | 0.200 |

440 HaNV+ = HaNV-infected; HaNV- = non-infected individuals; Larval mortality = proportion of
441 larvae dying before pupation; pupation rate = proportion of surviving larvae that successfully
442 pupated; pupae deformity rate = proportion of pupae that were deformed; eclosion rate =
443 proportion of pupae that successfully eclosed; adult deformity rate = proportion of adults that were
444 deformed. †n = number of batches of 24 larvae
445
446

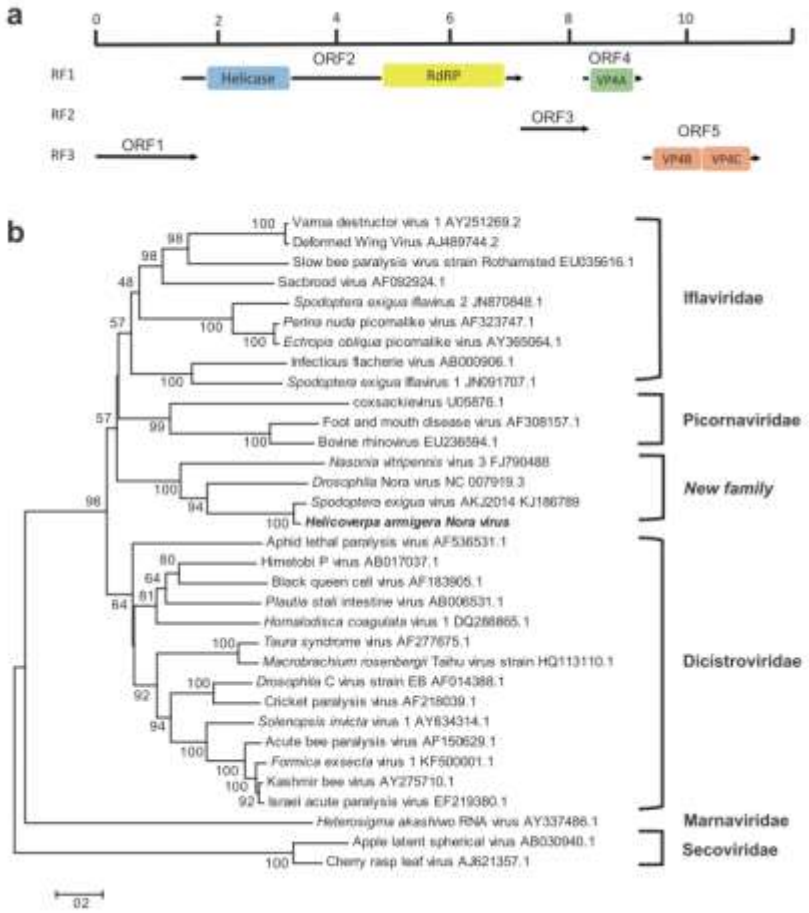


**Fig 1.** Characterization of the HaNV virus. (**a**) The genomic structure of HaNV. ORF=open reading frame, RdRp = RNA-dependent RNA polymerase, Structural proteins: VP4A, VP4B and VP4C. (**b**) Tree based analysis based on the putative conservered RdRp amino acids of HaNV and 32 other picorna-like viruses. Neighbor-joining tree with 1000 replications was constructed using MEGA 6. Bootstrap values (1000 pseudoreplicates) are indicated on the nodes.

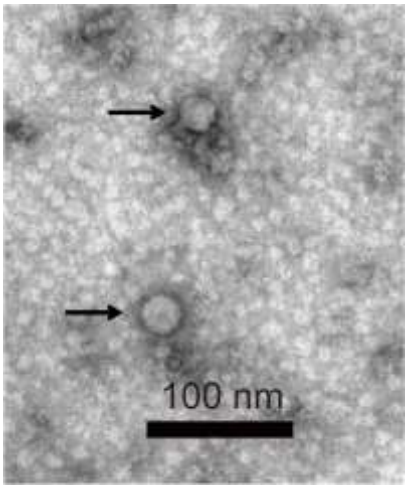


**Fig 2.** Electron microscopy of purified virus particles. Arrows point to virus particle.

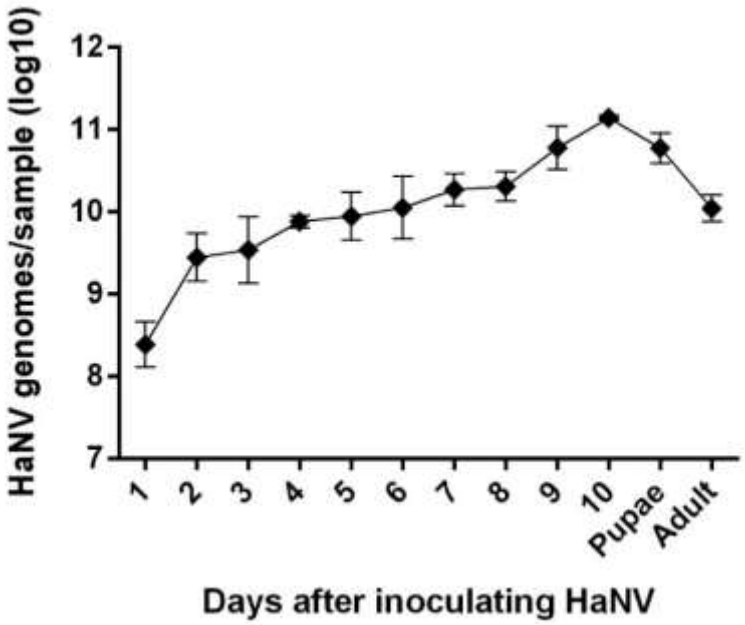


**Fig 3.** HaNV copy numbers at different stages. The line connects the average values for each time point and standard deviation values were shown. Three batches of 3 individuals were shown for each time point.

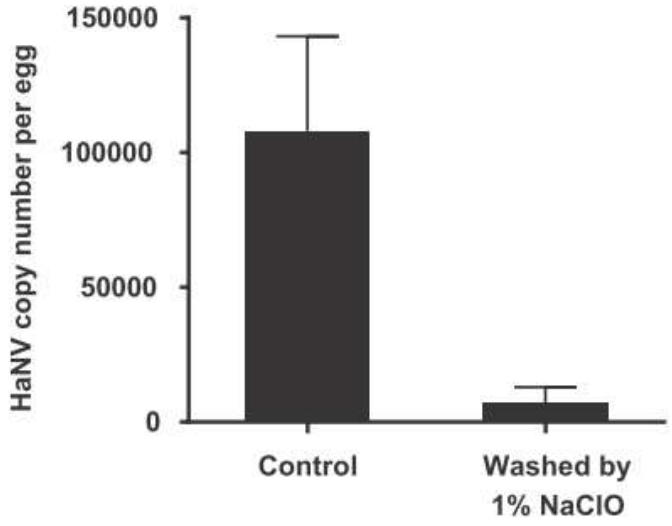


**Fig 4.** Viral load of HaNV in cotton bollworm eggs. Absolute quantification of HaNV copy number per eggs washed or non-washed in 1% sodium hypochlorite (3 groups of 100 eggs). Means ± SE. * = $P < 0.05$.

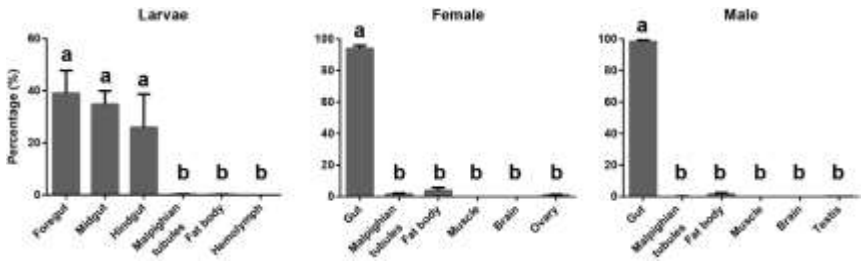


**Fig 5.** Tissue distribution of HaNV in cotton bollworms of larvae, adult females and adult males. Within each figure, significant differences ascribed using Tukey post-hoc tests are shown using different letters. Percentage (%) = the ratio of HaNV in different tissues (per mg), as described in Methods (Means ± SE. Larvae: n = 15; adult males: n = 9; adult females: n = 9).