Honey bee deformed wing virus structures reveal conformational changes accompany genome release

Running title: DWV capsid structures

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Abstract:

The picornavirus-like deformed wing virus (DWV) has been directly linked to colony collapse; however, little is known about the mechanisms of host attachment or entry for DWV or its molecular and structural details. Here we report the 3-D structures of DWV capsids...
isolated from infected honey bees, including the immature procapsid, genome-filled virion, putative entry intermediate (A-particle), and the empty capsid that remains after genome release. The capsids are decorated by large spikes around the five-fold vertices. For the procapsid and genome filled capsids the five-fold spikes had an open flower-like conformation whereas the putative A-particle and empty capsids that have released the genome had a closed tube-like spike conformation. Between the two conformations the spikes undergo a significant hinge-like movement that we predicted using a Robetta model of the structure comprising the spike. We conclude that the spike structures likely serve a function during host entry, changing conformation to release the genome, and that genome may escape from a five-fold vertex to initiate infection. Finally the structures illustrate that similarly to picornaviruses, DWV forms alternate particle conformations implicated in assembly, host attachment, and RNA release.

Significance:
Honey bees are critical for global agriculture, but dramatic losses of entire hives have been reported in numerous countries since 2006. Deformed wing virus (DWV) and infestation with the ectoparasitic mite Varroa destructor have been linked to colony collapse disorder. DWV was purified from infected adult worker bees to pursue biochemical and structural studies that allow the first glimpse into conformational changes that may be required during transmission and genome release for DWV.

Abstract: 175 words

Significance: 66 words
INTRODUCTION

The European honey bee (*Apis mellifera* L.) is a major agricultural resource critical for the pollination of many important food crops (1, 2). There are concerns about the decline in populations of managed honey bees observed since 2006 (3). Often this decrease in honey bee numbers is attributed to infection with several species of viruses (4) and the widespread ectoparasitic mite *Varroa destructor* (5), which can double as a viral vector. Multiple studies have shown that deformed wing virus (DWV), particularly when associated with high levels of *V. destructor* infestation, is linked to overwintering losses of colonies and “Colony Collapse Disorder” where the number of adults bees in a particular hive plummets dramatically from unexplained factors (3) (6–9). Transmission of DWV to honeybees can occur horizontally, via consumption of contaminated pollen and through direct contact between adult bees (8,10–13). The virus can also be transmitted vertically if an infected drone inseminates a queen and by a DWV positive queen laying infected eggs (13, 14). Infection with DWV during pupation leads to the hallmark appearance of incompletely developed and crumpled wings, which is lethal, as bees do not live long after emergence from the pupal stage, or are directly removed from the hive by other bees (15, 16) (personal observation M.C.P.).

Within the *Picornavirales* order, DWV belongs to the Family *Iflaviridae* (ICVT, 9th Report). The DWV genome consists of positive-strand RNA organized similarly to the mammalian picornaviruses (17) with a 5’ untranslated region that likely functions as an Internal Ribosome Entry Site (IRES) (18), a single open reading frame (orf) encoding a 2893 amino acid polyprotein, with the structural proteins at the N-terminus and the non-structural proteins at the C-terminus (17). Structural proteins are released by cleavage and form a protomer unit
comprised of VP1-VP0-VP3 (17). During picornavirus assembly, five copies of the virus protomer form pentameric structures and twelve of these building blocks incorporate a progeny genome to assemble the infectious virion. Many picornavirus also form an empty capsid of twelve self-assembled pentamers, sometimes called a procapsid, which contains uncleaved VP0. When genomic RNA associates with pentamers, often VP0 is cleaved to produce VP4 and VP2; however, this cleavage has not yet been established for DWV.

The capsid of DWV is a 30nm non-enveloped icosahedron composed of structural proteins VP0 (VP4+VP2), VP1, and VP3 (17). At 44kDa, the VP1 protein of DWV is the largest capsid protein of any virus in the Picornavirales order and sequence alignment indicates a 171-residue C-terminal extension relative to other picornaviruses(17). Structural proteins VP2 and VP3 are more similar in size to other picornavirus capsid proteins (32kDa and 28kDa, respectively). A significant difference between the capsid polyproteins of insect picornaviruses is the location of the VP4 protein, which is located internally and immediately downstream of VP2 rather than at the N-terminus as in picornaviruses (17, 19). Due to this location, the VP4 protein is not myristylated in the insect viruses. During picornavirus entry, capsids undergo a conformational change after binding to the entry receptor that includes the release of some VP4 molecules that form a pore in the host membrane for translocation of the RNA genome. DWV VP4 may function by being inserted directly into cellular membranes during viral entry (20). Many insect viruses share a structural feature called “strand swapping,” which refers to the N-terminus of the VP2 protein connecting two protomers by bridging the icosahedral two-fold. The related Triatoma virus (TrV) also displays a structural feature called a “crown” that is comprised of the VP1 N-termini forming spike-like extensions surrounding the five-fold vertices.
These crowns have also been found in Ljungan virus, a related picornavirus (21) and the insect virus structure that was recently solved for another member of the Iflaviridae family, slow bee paralysis virus (SBPV) (22).

Very little is known about the DWV host attachment, entry, and genome release. Based on sequence alignment, the most closely related viruses are Ljungan, TrV, and hepatitis A virus (HAV). TrV has several picornavirus-like features including the production of a procapsid, a naturally occurring empty capsid made during assembly (23). During picornavirus entry the infectious virus undergoes a conformational change during host entry forming an altered entry intermediate or A-particle. After releasing the genome an empty capsid remains that sediments at a different buoyant density (80S) than the other forms of the capsid. These picornavirus entry steps can be mimicked in the laboratory setting by heating infectious particles to produce A-particle and 80S. Heating TrV virus particles has also been shown to release the genome, resulting in a stable empty capsid (24).

Here we report the first high-resolution cryo-EM structure of the honey bee virus, DWV, isolated from infected honey bees. The honey bee DWV lysate was infectious and by TEM both RNA-filled and empty capsids were visualized. Upon purification, two structurally distinct empty capsid forms were isolated. Although comprised of the same capsid proteins, the capsids have unique buoyant densities and capsid topologies. The cryo-EM density maps show that DWV capsids have “strand swapping” of the VP2 protein that crosses from one asymmetric unit to the next. Spikes composed of the extended VP1 C-terminus decorate the five fold vertices; however these spikes exist in two distinct conformations that we describe as open and closed. Further analysis identified the conformation of the genome filled capsid and a putative A-particle, suggesting a model for host attachment and entry of DWV. Based on this first structural
characterization of an important agricultural pathogen, we propose that DWV shares features with picornaviruses, including the assembly of a procapsid and the formation of an 80S-like empty capsid after genome release that is accompanied by large conformational movements of the VP1 C-terminal spikes.

Methods

Processing of infected honey bees

In August of 2014, adult worker honey bees with obvious wing deformities were collected from brood combs originating from colonies containing an egg-laying queen (queen-right) in the Bucknell University Apiary and immediately stored at -80°C. Approximately ten bees with deformed-wings were homogenized in 30% (w/v) mixture containing phosphate buffered saline (PBS). The mixture was clarified by centrifugation at 10,000xg in a JS-13 rotor for 15 minutes at 4°C. A portion of the clarified bee homogenate was passed through a 0.22 µm filter for further purification. Both filtered and unfiltered bee homogenate were stored at -80°C until used for RNA and virion purification.

Quantitative RT-PCR detection of virus

Total RNA was purified from a 400 µL aliquot of the filtered and unfiltered bee homogenate using Trizol (Invitrogen) followed by the Direct-zol RNA MiniPrep kit and DNAseI treatment (Zymo Research) according to the manufacturer’s instructions. RNA concentrations were measured using a Nanodrop spectrophotometer and confirmed using a Quibit RNA BR assay kit (Invitrogen). Two µg of total RNA were converted to cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems). Real-time PCR was carried out on the cDNA samples using the Roche Light Cycler 96 instrument. Each 20 µL reaction contained 1x Fast Start
Essential DNA Green Supermix (Roche), 0.25μM primers, 0.2 μL of cDNA sample and was heated to 95°C for 10 mins followed by 45 cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 15 sec. Primers used to detect levels of DWV and other honey bee viruses were as follows:

- DWV-F, 5’-AGCATGGGTGAAGGAATGTC-3’; DWV-R, 5’-ATATGAATGTGCCGCAAACA-3’;
- ABPV-F, 5’-TGAAACGGAACAAATCACCA-3’; ABPV-R, 5’-GGGGCGTTGTAAAAACTGAA-3’;
- BQCV-F, 5’-CTCTAAGACAGCGAGCTT-3’; BQCV-R, 5’-CGCTCCAGATTTGAGGAAAG; KBV-F, 5’-ATGCAGAGACCGGAGAAAAA’3’; KBV-R, 5’-TGGCAGACTCATCTCGACAC-3’;
- SBV-F, 5’-GATTGGTTGGTGCGAAGTT-3’; SBV-R, 5’-CGCAAAGATCCTACCTCAGC-3’; Am-rp49-F, 5’-CGTCATATGTTGCCAACTGGT-3’, Am-rp49-R, 5’-

Negative controls consisted of RNA minus reverse transcriptase.

**DWV purification and characterization**

For DWV1 and DWV2, two mL of unfiltered DWV infected bee homogenate was applied to 2 mL of 30% sucrose (w/v) in PBS in a red-capped Beckman tube. Viral capsids were pelleted through the sucrose cushion in a 50.2Ti rotor at 48k rpm for two hours at 4°C. The pellet was resuspended in 2 mL PBS and transferred to a 10%-35% sucrose (w/v) in PBS with 0.1% Tween gradient for ultracentrifugation in a SW41 rotor for two hours at 4°C and a speed of 36k rpm. Two protein bands were purified and each band was extracted via side puncture. The material from each band was stored overnight and the following day, was washed five times with excess PBS in 10 kDa cutoff centrifugation filters to remove the sucrose and concentrate each sample for transmission electron microscopy (TEM). Material from each of band was stained...
samples were imaged at 60 kV accelerating voltage in a JEOL 1400 TEM (Peabody, MA) at the Penn State College of Medicine Imaging Core Facility.

From each of the two gradient bands a 16 μL aliquot was mixed with 4 μL of 5X Laemmli running buffer and incubated at 95°C for 5 minutes before transfer to a Mini-PROTEAN TGX precast gel (Bio-Rad). The Precision Plus protein standard (Bio-Rad) was added as a size ladder and electrophoresis was conducted at 200 V and 30 mA for 40 minutes. The gel was stained with Coomassie blue.

For the second purification protocol that isolated the RNA-filled virus, one mL of DWV infected bee homogenate was purified and imaged as described above with the exception of transfer to a 10%-35% sucrose (w/v) in PBS gradient for ultracentrifugation, buffer exchange was to PBS, pH 7.4, and the sample was not stored, but imaged immediately by TEM. After imaging, an aliquot was incubated at 37°C for 24 hours prior to TEM imaging, vitrification, and cryo-EM data collection.

**Microscopy for negative stain TEM**

For all negative stain TEM, three microliters of sample was applied to a freshly glow-discharged continuous carbon-coated copper EM grid negatively stained with 3 μL of uranyl formate. The grids were imaged with a JEOL 2100 (Peabody MA) transmission electron microscope housed in the imaging facility at The Pennsylvania State University College of Medicine.

**Microscopy for first sample preparation conditions**
For cryo-EM, aliquots of DWV capsids from the top band (DWV1) and lower band (DWV2) were applied to freshly glow-discharged holey carbon Quantifoil EM grids (Quantifoil Micro Tools GmbH, Jena, Germany) to which a thin layer of continuous carbon support film was applied. Grids were blotted and plunge frozen in liquid ethane using a Gatan CP3 robot (Pleasanton, CA). Vitrified grids were screened for ice quality and sample concentration at the Penn State College of Medicine Imaging Core Facility using a JEOL (Peabody MA) 2100 LaB6 cryo-electron microscope (cryo-EM).

Grids of virus from DWV1 were cryo-shipped to the University of Virginia School of Medicine Molecular Electron Microscopy Core. Low dose micrographs were recorded using an FEI (Hillsboro OR) Titan Krios cryo-EM operating at an accelerating voltage of 300 kV and a nominal magnification of 59,000x with defocus values ranging from -0.74 to -5.58 μm. Data were collected under EPU software control using an FEI Falcon 2 direct electron detector operating in movie mode. The post column magnification of 1.6x yielded a calibrated pixel size at the sample of 1.40 Å.

Grids from DWV2 were transported to University of Pittsburgh for data collection using an FEI Polara G2 microscope operating at 300 kV and a nominal magnification of 78,000x with defocus values ranging from -0.19 to -5.76 μm using an FEI Falcon 2 direct electron detector with post-column magnification of 1.4x yielding a calibrated pixel size at the sample of 1.37 Å.

**Microscopy for second sample preparation conditions**

Aliquots from the top band and lower band were imaged by negative stain (see above). From the lower band, 3.5 μL of sample was applied to freshly glow-discharged holey carbon Quantifoil EM grids (Quantifoil Micro Tools GmbH, Jena, Germany) to which a thin layer of
continuous carbon support film was applied. Grids were blotted and plunge frozen in liquid ethane using a Gatan CP3 robot (Pleasanton, CA). Vitrified grids from the RNA-filled capsids were recorded at the Penn State College of Medicine Imaging Core Facility using low dose conditions on an Ultrascan 4000 charged coupled-device (CCD) camera (Gatan, Pleasanton, CA). The JEOL 2100 microscope was operating at 200 kV and equipped with a Gatan 626 cryo-holder.

**Image processing and three-dimensional reconstruction**

Reconstructions for the DWV1 and DWV2 from the first purification and the partially-RNA-filled viruses that had been incubated overnight at 37°C were initiated separately with drift correction using the DriftCorr program(25). Defocus estimation was performed with CTFFIND4(26). Particles were automatically selected from each micrograph using the EMAN2.1 program(27). The selected particles were normalized, linearized, and apodized prior to image reconstruction. AUTO3DEM operating in “gold standard” mode divided each data set into two halves to generate random models, determine particle orientations, calculate the final reconstructions, and assess the resolution at a Fourier shell correlation (FSC) cutoff of 0.143 (28)( Table 1). The negative stain particles were also processed similarly except the contrast was inverted before initiating auto3DEM.

Fitting analysis and density difference map calculations were completed using Situs and Chimera (29, 30). Handedness of the maps was assigned based on the correlation coefficient of the fitted structures into flipped and unflipped maps. Local resolution estimations were calculated with ResMap (31). Sequence alignments for Fig. 5 and Fig. 8 were performed using Clustal-Omega (32). Maps of slow bee paralysis virus were generated from the crystal structures.
(PDB ID: 5J96 and 5J98) using pdb2vol from the Situs package with a Gaussian smoothing kernel, resolution = 7Å, and pixel size = 1.37 Å/pixel (29). The crystal structures (PDB ID: 5J96 and 5J98) were fit into DWV1 and DWV2 using Chimera fit-in-map with icosahedral symmetry operators applied to generate all 60 asymmetric units.

Results and Discussion

Detection of DWV

Homogenates made from adult worker honey bees with obvious deformed wings were assessed by qRT-PCR using primers for DWV and other honey bee viruses. The only virus detected in samples was DWV (Fig. 1A). When these infectious bee homogenates were examined by negative stain transmission electron microscopy (TEM) both RNA-filled and empty virus capsids were seen (Fig. 1B). Since RNA-filled capsids and procapsids are often found in lysates from picornaviruses (21, 33)(34), this finding suggests that DWV makes a procapsid along with the RNA-packaged infectious virus (Fig. 1C). After differential centrifugation of the homogenate, two protein bands were observed as has been seen previously for large volume picornavirus preparations where the top band corresponds to the procapsid and the lower band to the genome packaged infectious viron (35). However after the bands were collected, incubated overnight at 4°C, and the buffer exchanged for examination by negative stain TEM (Fig. 2B,C) both bands were found to contain ~30 nm diameter empty capsids. Thus upon further handling of the two virus capsid types, the RNA packaged virus lost the genome resulting in an empty capsid. At this point we could not definitively distinguish between preexisting procapsids and newly formed 80S-like empty capsids (36). SDS-PAGE analysis of the bands confirmed the
presence of virus proteins, but also did not allow differentiation between procapsid and the 80S particle (Fig. 2A).

Top and lower bands contain empty virus capsids with different structures

Samples from top and bottom bands were vitrified for cryo-EM data collection and icosahedral image reconstruction (Fig. 3 and Table 1). The 3-D reconstruction of the top band, DWV1, showed capsids with pentameric units joined by minimal contacts at the icosahedral three-fold axes and more substantial connections at the two-fold axes. A striking feature is the spikes of density on the external capsid surface around, but at some distance from each icosahedral five-fold vertex and arranged in an open conformation (Fig. 3C, D; blue spikes). The five-fold vertex is closed by a strong density plug at the inner capsid surface that has multiple thick connections to the capsid shell (Fig. 3G, H).

The density map from the lower band, DWV2, was also decorated with spikes at each five-fold vertex; however, the protrusions were much closer to the axis and formed a closed ring structure (Fig. 3E, F; blue spikes). The inter-pentamer bridge at the two-fold axis was present whereas the connections between pentamers at the three-fold axis seemed more substantial. There was an open pore through the capsid shell at each five-fold vertex that together with the ring structure forms a tube-like extension (Fig. 3I, J). A plug of density is also present beneath the five-fold pore, but held in place with only tenuous density connections to the capsid shell (Fig 3J). The local resolution of the maps shows that the spike density has lower resolution suggesting flexibility. The density displayed in the central sections attests to the resolutions of 6.1 and 7.6 Å for DWV1 and DWV2, respectively (Fig. 4).
Capsid composition

Compared to related viruses, DWV has a significantly longer VP1 C-terminus, 171 residues longer than that of the related Ljungan virus, which has 49% sequence similarity based on alignment of the region encoding the structural proteins (Methods and Fig. 5). The Ljungan virus capsid structure (PDB ID 3JB4) was fitted into the DWV1 (Fig. 6) and DWV2 cryo-EM maps (21). The last C-terminal residue of Ljungan VP1 mapped to the base of the DWV spike, leaving most of the spike density unfilled – 1.22x10^5 Å^3 for both DWV1 and DWV2. Apart from the spikes, there was no other DWV density left unfilled from the fitted Ljungan virus structure that might accommodate the DWV VP1 C-terminal extension. The entire DWV VP1 sequence was submitted to the structure prediction server Robetta (http://robetta.bakerlab.org) (37, 38) and the resulting robetta model was aligned with the Ljungan VP1 structure. The first 221 amino acids of the predicted DWV protein had a similar structure to that of Ljungan VP1. The unique 171 C-terminal residues of DWV model contained two helices (residue 338-355 and 358-361). The Robetta model was fitted into both DWV1 (Fig. 6B) and DWV2 to predict the movement of the spikes during genome release (Movie 1). The 171 C-terminal spike domain residues of our model appear to rock ~40° about the hinge at the base with residues Ser236 - Thr243 appearing to be the pivot point. The large movement of the spikes produces the effect of opening the vertex channel through the capsid shell.

A defining characteristic of related insect virus capsids is the linking of pentamers by a strand swapping mechanism that occurs when the N-terminus of a VP2 protein extends across the icosahedral two-fold axis to interact with residues in the neighboring pentamer. This extension creates a density bridge. The pentamer that receives the VP2 N-terminus donates a VP2 N-terminus back across that same bridge creating an effective “protein staple” across each
two-fold. Ljungan virus also uses strand swapping to connect pentameric units as indicated by the fitted Ljungan VP2 that passed through a specific bridge of DWV density located at the two-fold axis in both DWV maps (Fig. 6A).

Fitting of the Ljungan virus structure aided in interpretation of the DWV capsid composition; however, the structure of a more closely related virus, slow bee paralysis virus (SBPV), was recently solved (22). Although sequence similarity is lower compared to Ljungan virus (33%), the capsid structure of SBPV shares similar characteristics with DWV, including the five fold spikes and VP2 strand swapping (Fig. 7). Fitting the SBPV structures (PDB ID 5J98 and 5J96) into DWV1 and DWV2 resulted in relatively poor correlation coefficients (0.35 and 0.59, respectively), likely due to spike and five-fold related structures that are out of density (Fig 7 CD). The fittings did reveal that the N-termini of VP1 maps to the strong density plugs beneath the five-fold channel. The VP1 N-termini are mostly out of density wrapping around the five fold vertex in an intertwined configuration, much like the placement of VP4 in picornavirus structures. The five-fold density plug remains unfilled, although the strength of the cryo-EM density (equal to that of the strongest capsid features) suggests it might correspond to RNA or VP4, it cannot yet be interpreted. The exact location of DWV VP4, or even if it has been cleaved from VP0, remains unknown.

During the DWV and SBPV comparisons several inconsistencies in nomenclature were found due to the use of different conventions by different researchers. Previously during annotations of the DWV and the SBPV genomes the virus proteins were named according to a molecular weight convention resulting in a DWV gene order of VP 2, 4, 1, 3 and a SBPV gene order of VP 3, 4, 1, 2 (Fig. 8) (17, 39). However, in the recent SBPV crystal structure the virus proteins were labeled according to structural homology with picornaviruses, which resulted in
assigning VP1, 2, and 3 as VP3, 1, 2, respectively. Since SBPV VP1 (46kDa), 2 (29kDa), and 3 (27kDa) had been characterized previously (39), we used the previous assignment here when comparing to DWV. To provide consistency and clarity, the alignment of DWV with SBPV was color coded (Fig. 8) and the DWV annotation published in 2007 (17) was used throughout.

**DWV1 is the procapsid and DWV2 is an 80S-like empty capsid**

The two different empty capsid forms identified from the top band and lower band are likely procapsid and an 80S-like particle that resulted from the virion releasing genome upon further handling during the purification. The major structural difference between the two is in the position of the five-fold spikes, suggesting that the emptied virion has undergone significant conformational changes in order to release the genome. To learn more about the capsid conformational changes of DWV, the preparations of infectious honey bee homogenate were reexamined by negative stain TEM (Fig. 9A). Approximately 200 RNA-filled virion were selected for reconstruction, and despite the limitation on resolution, the density map clearly revealed five-fold spikes in the open conformation (Fig. 9B, C) similarly to DWV1. This result suggests that the procapsid (DWV1) and the RNA containing virus capsid share the same conformation whereas DWV2 is likely an empty 80S-like particle that has undergone a conformational transformation in order to release its RNA.

**RNA filled virus releases genome upon incubation**

With the aim to stabilize the RNA filled virus capsids, additional DWV infectious bee homogenate was purified using new purification buffer with a pH 7.4. Again ultracentrifugation resulted in distinct top and lower bands, which were collected, buffer exchanged and viewed by
TEM (Fig 10A, B). Whereas only empty capsids were seen again in the top band, the lower band was found to contain a mixed population of filled and empty capsids in a ratio of approximately 50:50. The presence of filled and empty capsids suggested that infectious virus might be releasing RNA. To test this possibility, an aliquot from the lower band was incubated at 37°C overnight and viewed the next day by TEM (Fig. 10C). After the incubation the population was characterized by slightly filled particles, reminiscent of A-particles (40–42) and empty capsids. The sample was vitrified and a data set collected using the home source cryo-EM (Methods), resulting in a cryo-EM map of modest resolution. The density map showed a virus particle with density corresponding to RNA in the interior and the five-fold spike conformation in the closed ring-like form (Fig 10D, E).

Model for necessary conformational changes of DWV

Bee homogenate is infectious (43–45) and we could detect DWV virions with packaged RNA prior to purification by ultracentrifugation. Currently it is unknown which step in the purification process triggers infectious DWV to change from the open to the closed conformation that accompanies genome release. Similar conversion of full picornavirus to other intermediate forms of capsid during purification has been seen before (46). Heat, receptor interactions, and pH changes can trigger picornavirus genome release, providing several parameters for modification of the DWV purification protocol.

Ultracentrifugation reproducibly resulted in two distinct bands. Gel electrophoresis analysis demonstrated that VP1 (or VP0), VP2, and VP3 were present in all four bands. As yet, it is not known whether the VP0 protein of DWV cleaves into VP4 and 1 upon RNA incorporation. Further confounding the issue, the DWV VP4 sequence corresponds to a peptide of 2.3kDa that
is undetectable by PAGE and makes VP1 indistinguishable from VP0. In the absence of any antibodies directed to VP4 to probe the identity of protein bands we could not yet conclusively identify the proteins by electrophoresis.

The two predominate bands that formed during repeated DWV purifications, were similar to purification for many picornaviruses that assemble both procapsid and infectious virus. Furthermore the appearance of an empty capsid in the top band persisted, whereas changes to the purification protocol allowed us to capture intact virion in the lower band. However, our conditions need further optimization as RNA packaged virus converted to capsid forms downstream in the virus lifecycle, i.e. a putative A-particle and 80S capsid. Nevertheless, we propose a model for DWV that consists of assembly of both a naturally occurring empty capsid (procapsid) and an RNA filled infectious virion. Furthermore, DWV likely proceeds with infection through an A-particle intermediate before releasing the genome to leave behind an empty 80S-like capsid. The large conformational changes to the five fold spikes that accompany the release of genome suggest that the change has function during host interaction or genome release (Fig. 11).

Although it seems that DWV capsids undergo changes similar to that of picornaviruses, the structures reveal some notable differences. DWV employs strand swapping to maintain capsid structure, a strong structural feature that occurs across the icosahedral two-fold axis, which is the proposed site of genome release for picornaviruses (40, 47–49). The site for DWV genome release may be the open pore at the five-fold vertex. In both capsids there is a plug of density below the five-fold vertex at the capsid interior; however, for the 80S-like capsid the density is only loosely tethered. Perhaps the plug of density hanging below the opening is missing altogether at the vertex through which the RNA egressed. The use of a five-fold pore to
package and release the virus genome is a known feature of a parvovirus, minute virus of mice (MVM), another small non-enveloped icosahedral virus (50).

The movement of the spikes from open to closed illustrates a major conformational change that may accompany genome release or host attachment. The spikes in the closed conformation may function as a tube at the five-fold vertex through which to translocate the RNA. Another insect virus, Helicoverpa armigera stunt virus (HaSV), binds asymmetrically and nonspecifically to host cells to create a pore in the cell membrane and allow genome egress likely through a modified five-fold vertex (51, 52). Similarly the DWV spikes may function during attachment and contribute to the remarkably broad tropism observed for this virus, since DWV can infect myriad bee tissues from the gut to the brain, as well as other species, including the Varroa mite and bumble bees (11, 53–55).

Given the importance of honey bees to global agriculture, continuing structural and molecular genetics research will be imperative for characterizing the DWV pathogen. Solving the atomic resolution structure of the RNA filled virus is a clear objective, as is understanding the proteolytic status of the VP0 protein and the process of RNA packaging. Beyond that, compelling future directions are to understand the function of elements such as the VP1 C-terminal spikes, to explore the effect on tropism and the process of genome release into the host.

Acknowledgments: This work was supported in part by Pennsylvania Department of Health CURE funds. Research reported in this publication was also supported by the Office Of The Director, National Institutes of Health, under Award Numbers S10OD019995 and S10OD011986, as well as NIH grants R01AI107121 (SH) and T32CA060395 (LJO). The content is solely the responsibility of the authors and does not necessarily represent the official
views of the National Institutes of Health. The UVA MEMC equipment was partially funded by grants from NIH for the Titan Krios (S10-RR025067) and the Falcon II direct detector (S10-OD018149).

Accession numbers: The cryo-EM maps for the DWV1 and DWV2 were deposited in the EM data bank (www.emdatabank.org/) under accession numbers EMD-8463 and EMD-8464, respectively.

The authors declare that there are no potential conflicts of interest.


REFERENCES:


Table 1: Summary of data for 3-D reconstructions.

<table>
<thead>
<tr>
<th>Capsid Type</th>
<th>Microscope*</th>
<th>Method</th>
<th>Number of Micrographs</th>
<th>Number of Particles</th>
<th>Number of Particles Used</th>
<th>Defocus Range (μ)</th>
<th>Resolution (Å)</th>
</tr>
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<tbody>
<tr>
<td>DWV1 DWV2</td>
<td>Polara 300</td>
<td>Cryo-EM</td>
<td>3,357</td>
<td>99938</td>
<td>79958</td>
<td>0.74 to 5.58</td>
<td>6.09</td>
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<tr>
<td>RNA Filled</td>
<td>JEM1400</td>
<td>Negative Stain</td>
<td>101</td>
<td>204</td>
<td>102</td>
<td>0.47 to 4.73</td>
<td>23</td>
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<td>A-particle</td>
<td>JEM2100</td>
<td>Cryo-EM</td>
<td>380</td>
<td>1893</td>
<td>1860</td>
<td>0.05 to 5.47</td>
<td>18</td>
</tr>
</tbody>
</table>

*Polara, 300kV cryo-EM with field emission gun and Falcon2 direct electron detector
JEM1400, 160kV TEM with tungsten filament for negative stain data collection with CCD camera
JEM2100, 200kV cryo-EM with LaB6 and CCD camera
**Figure Legends:**

**Figure 1.** Detection of DWV in honey bee lysate. A) Quantitative Real-Time PCR of DWV infected honey bee homogenates. Unfiltered and filtered honey bee extracts were subjected to qRT-PCR using primers corresponding to four different common honey bee viruses including DWV, black queen cell virus (BQCV), Kashmir bee virus (KBV), and sacbrood virus (SBV). The relative ratio of each virus RNA compared to an internal control gene is shown. DWV was the only virus detected and the amount of virus detected was unaffected by the filtration process. B) Visualization of DWV capsids. Negative stained TEM of DWV-infected bee homogenate shows a mixture of full capsids (black arrow) and empty capsids (white arrow). C) A schematic showing that for picornaviruses two types of capsids are assembled, procapsid and genome filled virus. During host attachment and entry an entry intermediate, the A-particle is formed and then triggered to release genome, leaving an empty capsid referred to as 80S.

**Figure 2:** (A) SDS-PAGE stained with Coomassie blue shows that proteins corresponding to VP1/0, VP2, and VP3 can be detected in the top and lower bands. A protein standard is used as a sizing ladder (lane one). (B-C) Material from each of the two bands that formed during sucrose gradient purification were negatively stained and imaged by TEM. The top (B) and lower (C) bands both contain empty capsids.

**Figure 3:** Cryo-EM reconstructions of two different types of DWV capsids. Micrographs of (A) DWV1 and (B) DWV2 show empty capsids. The DWV1 (C,D,G,H) (6.1Å) and DWV2 (E,F,I,J) (7.6Å) cryo-EM reconstructions are visualized at a contour level of 1σ, surface rendered, and
colored radially according to the scale bar. (G-J) Cut away and close-up views of the virus five-fold vertices show the different conformations of the density spikes (blue) and the five-fold density plug (red arrow) that may block access to the capsid interior. (J) Tenuous connections (black arrows) seem to hold a five-fold density plug (red arrow) at the inner capsid surface although there is a clear opening through the capsid shell at each five fold vertex, which is better visualized with a continuous color scheme for DWV1 (yellow) and DVV2 (green) in the zoomed views.

Figure 4: Quality and resolution of the DWV cryo-EM reconstructions. (A, B) DWV1 and (E, F) DWV2 are colored according to local resolution estimations (see scale bars). The capsid shells reach higher resolutions than the flexible five-fold decorations with DWV1 spikes at lower resolution than the DWV2 spikes. The map central sections (protein is black) of DWV1 (C) and DWV2 (G) show the quality of the maps. Because the data sets were split initially and the halves reconstructed separately the resolution for each reconstruction was assessed using the gold standard FSC cut-off of 0.143 yielding 6.1Å and 7.6 Å resolutions for DWV1 (D) and DWV2 (H), respectively.

Figure 5: Alignment of DWV and Ljungan virus VP1 residues shows C-terminal extension of DWV VP1. The two viruses share 49% sequence identity.

Figure 6: DWV capsid composition and spike movement. A) The Ljungan virus structure (PDB ID 3JB4) (blue and green ribbon) fitted into the DWV1 map illustrates that the last C-terminal residue of VP1 maps to the base of the five fold spike density (blue sphere). Symmetry axes are...
indicated and the strand swapping mechanism can be seen where VP2 ribbons cross the two-fold density bridge. B) The last 171 C-terminal VP1 residues of DWV were predicted to form a helical-loop structure (yellow) that was fitted into the spike density. (C) The predicted model of DWV VP1 C-terminal extension has a similar structure compared to the VP1 crystal structure of slow bee paralysis virus (PDB ID 5J98) (22).

Figure 7: A and B) The structures of slow bee paralysis virus (PDB ID 5J98 and 5J96) (22) were used to calculate ~7Å surface rendered radially colored (see key) maps to compare gross surface topologies with DWV1 and DWV2, respectively. C and D) The SBPV structures (color coded: VP1, 2, 3 as blue, green, and red according to gene order reported in Fig. 7) were fitted into the DWV cryo-EM maps (transparent grey) and correlation coefficients were obtained that agreed with a moderately poor fit despite the obvious overall similarities. E and F) The zoomed view shows DWV (grey) internal surface at the five-fold vertex with SBPV structures fitted to show VP1 N-termini (blue) surround the five-fold. If VP0 is not cleaved, the VP4 portion would map to this region, which is similar to the location of VP4 in picornaviruses. The large grey plug of unfilled density is equal in magnitude to the capsid density.

Figure 8: The gene order for SBPV, DWV, and picornaviruses are shown with boxes color coded to represent VP1, 2, 3, and 4 (blue, green, red, and yellow respectively). The alignment of the P1 region encoding the structural proteins for DWV and SBPV (33% sequence similarity) has been similarly color coded with lines to indicate viral proteins. For consistency, the DWV published gene order and the picornavirus color code were used throughout this work.
Figure 9: The structure of the RNA-filled DWV capsid from infectious bee lysates. (A) Negatively stained micrographs of infected bee homogenate were used to select 102 genome-filled infectious virus capsids for a low resolution (∼25Å resolution) negative stain reconstruction to reveal the spike conformation. (B, C) The RNA-filled capsids have spikes in the open conformation, similar to the DWV1 reconstruction.

Figure 10: Results from the second purification of infectious honey bee lysate. Negative stain TEM images of the (A) top and (B) lower bands. The top band remained empty as before, whereas the lower band contained approximately 50% genome-filled virus. (C) Negatively stained micrograph of an aliquot from the lower band after incubation at 37°C for 24 hours shows different distribution, as most of the filled capsids have lost the genome (blue arrows). The filled virus that retain the genome appear to have less dense centers than previous observed (red arrows), consistent with A-particles. (D) The low resolution (18Å) cryo-EM reconstruction and (E) central section of putative A-particle (C) reveal the spikes have undergone a conformational changed into the closed tube-like form after incubation.

Figure 11: Similarly to picornavirus, DWV assembles an empty capsid in addition to RNA-filled capsids. This procapsid structure resembles the infectious virus, whereas the putative A-particle resembles the 80S empty capsid. The putative A-particle and 80S-like empty capsid have a different conformation for the five-fold spikes.
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