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Potential for the *Anopheles gambiae* Densonucleosis Virus To Act as an "Evolution-Proof" Biopesticide▽

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"Evolution-proof" or "late-life-acting" insecticides (LLAIs) preferentially kill older adult mosquitoes and are of extreme interest to control vector-borne diseases such as malaria. We used quantitative PCR to assess whether the *Anopheles gambiae* densonucleosis virus (AgDNV) had potential as an LLAI. After infection, AgDNV titers increased modestly during larval development but replicated slower than the host cells, resulting in a significant decrease in the normalized virus titer during larval and pupal development. Normalized virus titers dramatically increased after adult emergence, peaking in 7- to 10-day-old adults. Unlike other DNVs, AgDNV does not significantly replicate in preadult mosquitoes but rather preferentially replicates in older adults. The natural dynamics of AgDNV make it ideal for expression of insect-specific toxin genes as a biological LLAI.

Malaria infects several hundred million people and results in over one million deaths annually. Vector control is a major component of current malaria control strategies. However, the evolution of insecticide resistance by *Anopheles* mosquito vectors has severely hampered control efforts (5, 13, 14). Although novel chemistries are being explored, new insecticides will face similar problems with resistance evolution. Recently, late-life-acting insecticides (LLAIs) have been proposed as novel agents to control vector-borne diseases such as malaria (1, 10). LLAIs selectively kill the older mosquitoes responsible for the bulk of parasite transmission while allowing for reproduction of the younger age classes that contribute to the bulk of evolutionary fitness, i.e., there is an optimal window of time wherein mosquitoes live long enough to reproduce but not long enough to transmit pathogens (8, 9). Reproduction allows for relaxation of evolutionary pressures that select for resistance to the agent. If resistance alleles exert fitness costs, there are theoretical scenarios under which resistance is not expected to evolve, leading some to provocatively term LLAIs as "evolution-proof" (1, 10).

LLAI do not have to be conventional chemical pesticides. Like all organisms, mosquitoes can be infected with pathogens of their own (including fungi, bacteria, or viruses) that can be exploited to shorten mosquito life span to control disease (6, 9–12). Some pathogens can be vertically transmitted (9, 11), which not only results in relaxed selection pressure but also allows for their transmission and spread into the vector population.

Densonucleosis viruses (or densoviruses [DNVs]) are icosahedral, nonenveloped parvoviruses that have been identified from many invertebrate taxa, including multiple mosquito species (2, 11). Naturally occurring DNVs typically infect mosquitoes during the aquatic larval phase. Infection of young larvae (first or second instar) is generally lethal, resulting in virus amplification and release into the larval environment. Larvae infected at later time points (third or fourth instar) develop into infected adults that inoculate virus vertically and horizontally into the larval environment during oviposition, completing the virus life cycle (2, 11).

The *Aedes aegypti* densovirus (AeDNV) is generally lethal to *Ae. aegypti* larvae in a dose-dependent manner, and high virus titers in larvae are observed both by quantitative PCR (qPCR) and by expression of foreign transgenes such as green fluorescent protein (GFP) (2, 8, 15). In contrast, the *Anopheles gambiae* densovirus (AgDNV) is not lethal to *An. gambiae* larvae (11). Using GFP-transducing virus and epifluorescence microscopy, we have also never seen GFP expression in larvae, pupae, or young adults; GFP is not observable until the adults are ~1 week postemergence (J. L. Rasgon and X. Ren, unpublished observations) (Fig. 1). Based on these observations, we hypothesized that AgDNV has different replication kinetics in *An. gambiae* than AeDNV has in *Ae. aegypti*, remaining at relatively low titers in the immature life stages but replicating to high titers after adult emergence.

MATERIALS AND METHODS

Determination of AgDNV replication in MOS.55 cells. *An. gambiae* MOS.55 cells were cultured and transfected with 2 μg of the AgDNV infectious clone (pBAgα) using Effectene Transfection Reagent (Qiagen) as described previously (11) and then passaged into 150-cm^2 culture flasks (three replicate flasks). We used 10-fold dilutions of the pBagα plasmid as a copy number standard to create a standard curve for viral quantitation as described previously (11) (slope, −3.37; $R^2 = 0.997$). At 5 days and 14 days posttransfection, the cells in the culture flasks were suspended, and 1-ml samples were collected. Viral DNA was extracted from the samples, and the virus titers were determined by quantitative real-time PCR as described previously (11, 16). Since DNVs do not form plaques, the virus titers are reported as viral genome equivalents (vge), which represent the num-

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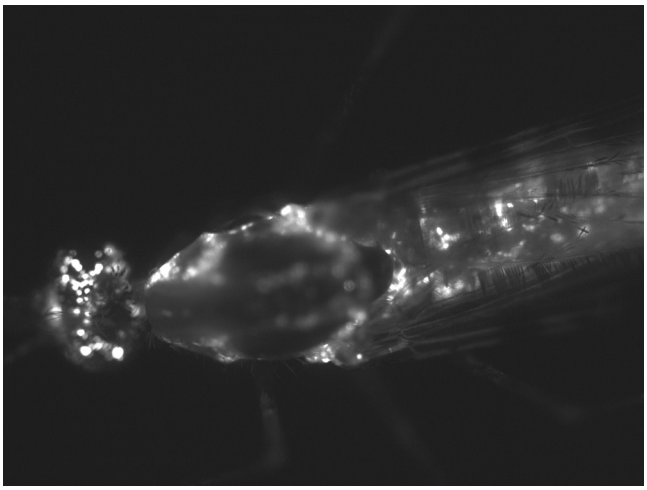

FIG. 1. Epifluorescent image (dorsal view) of a 10-day-old adult *An. gambiae* female infected with GFP-expressing AgDNV (as described in reference 11). GFP is not visible in infected mosquitoes until 7 to 10 days postemergence.

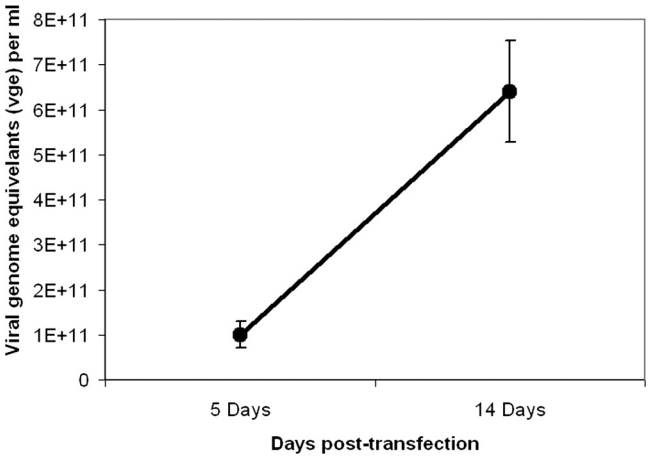

FIG. 2. AgDNV replication in MOS.55 cells ($n = 3$ data points per time point). Significant viral replication occurred between time points (Mann-Whitney U, $P = 0.05$). Error bars represent the standard deviation.

ber of viral genomes rather than the number of viable virions. The data were analyzed by using the Mann-Whitney U test.

***An. gambiae* mosquito infection.** MOS.55 cells were transfected with 2 μg of pBAgα as described above. Transfected cells were passaged once and grown for 2 weeks to produce virus for mosquito infection. Infected cells were harvested from the culture flasks by scraping and pelleted by centrifugation, and the pellets were resuspended in 20 ml of sterile water. The cells were lysed by vortexing with sterile 3-mm borosilicate glass beads for 5 min. The cell lysate was added to sterile water for a final volume of 90 ml. Two hundred newly hatched first-instar *An. gambiae* larvae (Keele strain) were added to the lysate mix. The larvae were exposed to virus for 48 h and then removed from the cell lysate, rinsed with water three times, transferred to pans with clean water, and fed as described previously (11). Mosquitoes were collected for qPCR at the following time points: 5-day-old larvae (5 days postinfection [dpi]), 7-day-old larvae, 7-day-old pupae, and 1-day-old adults (all 7 dpi due to natural variation in rearing); 7-day-old adults (13 dpi); 10-day-old adults (16 dpi) (which were bloodfed using a membrane feeder); and 14-day-old adults (20 dpi). For qPCR, each biological replicate was a pool of five mosquitoes, with three biological replicates per time point. The entire experiment was replicated three times.

Determination of relative AgDNV titers in infected mosquitoes. We used qPCR to investigate AgDNV infection dynamics during development in *An. gambiae* mosquitoes. Mosquito genomic DNA (gDNA) was extracted by using DNeasy blood and tissue kits (Qiagen). gDNAs were adjusted to 100 ng/μl by using a Nanodrop spectrophotometer. The relative abundance of AgDNV in each mosquito life stage was assessed by comparing the abundance of the AgDNV genome (forward, 5′-CAT-ACT-ACA-CAT-TCG-TCC-TCC-ACA-A-3′; reverse, 5′-CTTG-GTG-ATT-CTG-GTT-CTG-ACT-CTT-3′) to that of the nuclear single-copy *An. gambiae* ribosomal S7 gene (forward, 5′-TCC-TGG-AGC-TGG-AGA-TGA-AC-3′; reverse, 5′-GAC-GGG-TCT-GTA-CCT-TCT-GG-3′). Duplicate reactions were performed for every PCR. Uninfected mosquitoes were included as negative controls. qPCR was performed by using an ABI Prism 7300 detection system (Applied Biosystems) with the QuantiTect SYBR green PCR kit (Qiagen). Determinations of relative abundance of AgDNV in each collection group were calculated as previously described (6, 11, 16).

For the three experimental repeats, AgDNV titers used for mosquito infections were as follows: 7.8×10^{11} vge/ml, 8.1×10^{11} vge/ml, and 3.1×10^{11} vge/ml. Since the variances of the data did not differ significantly between experimental repeats (squared-ranks test [4], $P > 0.05$), the data were pooled for analysis (nine data points per life stage). The data were analyzed by Kruskal-Wallace test using the Conover-Inman method for pairwise contrasts (4) using StatsDirect statistical software (StatsDirect, Ltd.).

RESULTS

AgDNV replication in MOS.55 cells. After transfection with pBagα, viable AgDNV was rescued from the plasmid and significantly increased in titer from 5 to 14 days posttransfection (Fig. 2) (Mann-Whitney U, $P < 0.05$). This indicated that recombinant AgDNV is able to replicate in MOS.55 cells.

AgDNV replication in mosquitoes. Figure 3A shows the relative fold change in both the *Anopheles* nuclear genome (as indicated by the single-copy S7 gene) and AgDNV. The collections start at 5 dpi when mosquitoes are second to third-instar larvae. At 7 dpi, mosquitoes were asynchronous in development with a mix of fourth-instar larvae, pupae, and adults. S7 increased in titer during larval development, peaked in the pupal stage, and remained relatively constant through the remainder of adult life. In contrast, AgDNV increased in titer modestly during larval development but did not increase further in the pupal or early adult stages. AgDNV titers then dramatically increased ~100-fold 7 days postemergence and remained constant thereafter (Fig. 3A).

By comparing the relative abundance of both the S7 gene and the AgDNV genome, we can assess normalized AgDNV dynamics during mosquito development. The normalized abundance of AgDNV in each mosquito life stage is shown in Fig. 3B. Kruskal-Wallace test indicated that the normalized AgDNV titer of at least one time point differed statistically ($P < 0.0001$). Although the virus titers increased modestly during immature development, the even greater replication of the *Anopheles* nuclear genome resulted in a net drop in normalized virus titer during the larval stage. All mosquitoes at 7 dpi had similar normalized AgDNV titers regardless of life stage (larvae, pupae, or adult) that were significantly lower than those of mosquitoes at 5 dpi. By 1 week postemergence (13 dpi), normalized AgDNV titers drastically increased to approximately 10,000 vge per host genome and remained at that level thereafter (Fig. 3B).

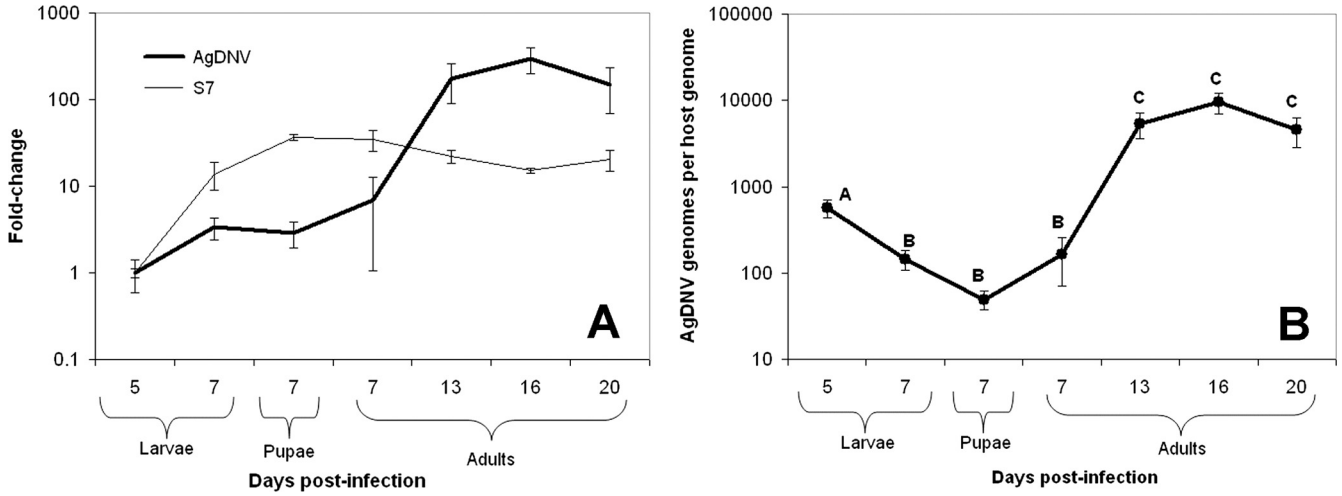

FIG. 3. Dynamics of AgDNV titer in *An. gambiae* (n = 9 data points per time point). (A) Fold change in S7 and AgDNV during mosquito development. (B) Changes in relative AgDNV titer during mosquito development. Time points with the same letter do not differ statistically (see the text). Error bars represent the standard errors of the mean.

DISCUSSION

Our data indicate that, unlike AeDNV in *Ae. aegypti*, AgDNV does not replicate substantially in the immature or early adult life stages of *An. gambiae*. Normalized virus titers decreased significantly during larval development and reached the lowest titers in the pupal stage (~50 vge per host genome). After adult emergence, the virus titers rapidly increased, approaching 10,000 vge per host genome by the time the mosquitoes were 7 to 10 days old. These results mirror our observations using GFP-expressing AgDNV, where we believe a low virus titer results in levels of GFP expression that are too low to be observed using epifluorescence microscopy. Using more sensitive techniques such as PCR, we can detect GFP DNA and transcript during this period (Rasgon and Ren, unpublished).

The data suggest that AgDNV has replication tropism for tissues in the adult rather than the larvae or pupae, which we suspect is partially responsible for the lack of observed larval mortality and visible GFP expression in early life stages. This result is puzzling because, like all parvoviruses, DNVs rely on the host replication machinery and only replicate in cells that are actively undergoing mitosis (7). Thus, we would expect AgDNV to dramatically increase in titer during the mosquito larval stage when cell replication is very high. However, while the mass of the larvae can increase >50-fold during development, not all larval tissues increase in size by cell replication. Organs such as the anterior intestine, salivary glands, rectum, and anal papillae grow by an increase in cell size rather than cell replication (3). Some of these organs, such as the malpighian tubules and anterior intestine, are carried over into the adult stage during metamorphosis (3). We hypothesize that in the larval stage, AgDNV has tropism for non-cell-dividing tissues that persist in the adult stage, while in the adult AgDNV infects metabolically active and rapidly dividing cells in tissues such as the fat body.

The intrinsic dynamics of viral replication make AgDNV ideal as the basis of an LLAI or "evolution-proof" biocontrol agent to selectively kill the older *Anopheles* mosquitoes that are responsible for the majority of malaria transmission. Although wild-type AgDNV is not lethal, recombinant AgDNV that expresses insect-specific toxin or "death" genes should be able to infect larvae but not express the toxic gene at an effective level until the mosquitoes are approximately 7 to 10 days postemergence, similar to observations using GFP (Fig. 1). This late-acting lethality is predicted to be extremely efficient at controlling malaria incidence (1, 9, 10), but since mosquitoes are able to reproduce, the evolution of resistance will be significantly slowed or even halted under some circumstances (1, 10). We envision using AgDNV as a biological microbial pesticide that can be applied to the larval habitat. The virus will infect mosquitoes and establish an infection that will kill the mosquito as an adult. As a bonus, the virus can be transmitted to larvae by vertical and/or horizontal transmission, leading to increased coverage and efficacy (since the mosquitoes themselves can distribute the agent to new oviposition sites). Ultimately, we expect that a properly engineered LLAI AgDNV can be deployed in the field to significantly modulate malaria transmission.

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