Musca domestica Salivary Gland Hypertrophy Virus, a Globally Distributed Insect Virus That Infects and Sterilizes Female Houseflies[▽]

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The housefly, *Musca domestica*, is a cosmopolitan pest of livestock and poultry and is of economic, veterinary, and public health importance. Populations of *M. domestica* are naturally infected with *M. domestica* salivary gland hypertrophy virus (MdSGHV), a nonoccluded double-stranded DNA virus that inhibits egg production in infected females and is characterized by salivary gland hypertrophy (SGH) symptoms. MdSGHV has been detected in housefly samples from North America, Europe, Asia, the Caribbean, and the southwestern Pacific. In this study, houseflies were collected from various locations and dissected to observe SGH symptoms, and infected gland pairs were collected for MdSGHV isolation and amplification in laboratory-reared houseflies. Differences among the MdSGHV isolates were examined by using molecular and bioassay approaches. Approximately 600-bp nucleotide sequences from each of five open reading frames having homology to genes encoding DNA polymerase and partial homology to the genes encoding four *per os* infectivity factor proteins (p74, pif-1, pif-2, and pif-3) were selected for phylogenetic analyses. Nucleotide sequences from 16 different geographic isolates were highly homologous, and the polymorphism detected was correlated with geographic source. The virulence of the geographic MdSGHV isolates was evaluated by *per os* treatment of newly emerged and 24-h-old houseflies with homogenates of infected salivary glands. In all cases, 24-h-old flies displayed a resistance to oral infection that was significantly greater than that displayed by newly eclosed adults. Regardless of the MdSGHV isolate tested, all susceptible insects displayed similar degrees of SGH and complete suppression of oogenesis.

Salivary gland hypertrophy virus (SGHV) of *Musca domestica* L. (Diptera: Muscidae) (MdSGHV) was originally detected in adult houseflies collected from dairies located in north Florida (2). Since the original description of this virus, a series of studies have detailed its molecular and biological properties. The circular double-stranded DNA (dsDNA) genome of MdSGHV consists of 124,279 bp and contains over 100 open reading frames (ORFs) (6, 16). The virus, which replicates in the salivary glands of adult flies, is readily transmitted *per os* to healthy conspecific animals (15). During feeding, high numbers of infectious virus particles are deposited on the solid food substrate that is fed upon by healthy houseflies. The development of viremia in female houseflies leads to a shutdown of egg production (7, 14). Associated with female sterility is the downregulation of egg protein gene transcription in the fat body (14). In feral housefly populations, the incidence of infection may peak at 34% at selected sites (7), although at any given sampling time the incidence typically ranges from 1 to 10%. As expected with an orally transmitted

virus, infection frequency is positively correlated with housefly density.

Recently, we initiated a program directed at expanding the current collection of SGHVs associated with *M. domestica*. One goal of this research was to examine the distribution of MdSGHV. The virus host, *M. domestica*, is a cosmopolitan and synanthropic insect that is thought to have originated in Africa and subsequently dispersed worldwide following the movements of early humans (17). Today, *M. domestica* is one of the most abundant and widely distributed animal species on earth, and it can be readily collected on all continents in areas where there is human activity. Coler et al. (2) reported that houseflies displaying salivary gland hypertrophy (SGH) symptoms were collected both in the United States (sites in Massachusetts and Florida) and in Brazil (Rio de Janeiro, Pernambuco, and Rio Grande do Norte) (R. R. Coler, personal communication). Until recently, however, the only research on MdSGHV has been conducted in our laboratory using a strain isolated in 2005 from a dairy in north Florida.

Over the past several years, we have collected houseflies at sites in Europe, Southeast Asia, New Zealand, North and South America, the Caribbean basin, and Africa and examined the flies for the presence of hypertrophied salivary glands. In this report we provide information on the collection and processing of virus strains or viral DNA. Gene-specific primer pairs were designed using MdSGHV ORFs to examine the genetic relatedness of the isolates collected. An additional

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TABLE 1. Partial list of collections of adult houseflies that have been examined for both the incidence of SGH and infectivity for laboratory-reared houseflies

Region	Strain ^a	Collection location	Habitat	Year	Proportion of collected flies with SGH	% of flies with induced infection ^b
USA	MdSGHV1	Alachua, FL	Dairy	1990	Unknown	100
	MdSGHV2	Okeechobee, FL	Dairy	1991	Unknown	100
	MdSGHV3	Alachua, FL	Dairy	2005	34/100	100
	MdSGHV4	Alachua, FL	Dairy	2007	2/100	100
	MdSGHV5	Kansas	Dairy	2008	1/155	100
	—	Virgin Islands	Dairy	2008	5/276	NA
	MdSGHV6	San Bernadino, CA	Dairy	2009	2/100	100
Oceania	MdSGHV7	Green Park, New Zealand	Dairy	2008	0/60	4
	MdSGHV8	Lincoln, New Zealand	Compost	2008	0/100	4
Asia	MdSGHV9	Samut Sakorn, Thailand	Fish market, dumpster	2008	5/45	100
	NA	Prajinburi, Thailand	Military camp, food processing area	2009	0/35	0
	NA	Prajinburi, Thailand	Fresh market, train station	2009	0/32	0
	MdSGHV10	Prajinburi, Thailand	Fresh market, Pak-Plei market	2009	0/40	4
	MdSGHV11	Samut Sakorn, Thailand	Fish market, dumpster	2009	2/110	75
Africa	NA	Bobo Dioulasso, Burkina Faso	Hamster and rabbit cage waste	2009	0/200	0
Europe	MdSGHV12	Slangerup, Zealand, Denmark	Dairy	2007	2/200	100
	MdSGHV13	Havbro, North Jutland, Denmark	Dairy	2008	1/100	75
	MdSGHV14	Tønder, South Jutland, Denmark	Dairy	2008	8/169	100
	MdSGHV15	Morum, North Jutland, Denmark	Dairy	2008	2/200	100

^a Strains for which designations are given represent collections that produced an inoculum that could be amplified by subsequent challenge in healthy laboratory-reared houseflies. Strains in bold type represent preparations that produced very low numbers of infected flies. —, MdSGHV from the Virgin Islands that was detected in ethanol-preserved salivary glands that were used as a source of viral DNA for subsequent genotyping; NA, not applicable.

^b Percentage of flies displaying SGH symptoms after injection of filter-sterilized salivary gland homogenate from field-collected flies. NA, not applicable.

objective was to determine if the new MdSGHV isolates differed in their ability to infect laboratory-reared houseflies *per os* and if one or more of these isolates induced gross symptoms that markedly differed from the symptoms induced by the Florida-2005 strain.

MATERIALS AND METHODS

Housefly rearing for bioassays. Housefly pupae, obtained from colonies maintained at the USDA Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, FL, were placed in rearing cages, provided with deionized water, and maintained under constant conditions (26°C, a photoperiod consisting of 12 h of light and 12 h of darkness, and 40% relative humidity) until adults emerged.

Collection of houseflies from field sites and preparation of MdSGHV isolates. Adult houseflies were collected with a sweep net from different sites in Europe, Southeast Asia, Africa, New Zealand, the Caribbean basin, South America, and North America (Table 1). Samples of hypertrophied salivary glands and asymptomatic glands were dissected from cold-immobilized flies, homogenized in 500 µl of saline, and filtered through a 0.45-µm syringe filter into sterile microcentrifuge tubes. Under these conditions, the MdSGHV remained infectious to houseflies for at least 1 week after storage at room temperature. The different MdSGHV isolates were initially amplified by injecting the filtered homogenates (2.5 µl per fly) into the thoracic cavity of disease-free adult flies (14). One week after injection, flies were dissected, and the condition of the salivary glands and ovaries was recorded. Hypertrophied salivary gland pairs were collected and prepared for microscopic examination, DNA extraction, and *per os* infectivity studies.

PCR amplification of viral genes and sequence analysis. DNA was extracted from the different infected salivary gland samples using a Masterpure DNA purification kit (Epicentre). Extracted DNA samples were suspended in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), treated with an RNase A solution, and extracted with phenol-chloroform. Each ethanol-precipitated DNA sample was suspended in 50 µl of TE buffer and electrophoresed on 0.7% agarose gels to estimate the quality and quantity of the genomic DNA. MdSGHV-specific primer pairs were designed using Primer3 software, and a series of

PCR assays was conducted to amplify five genes, including ORFs MdSGHV001 (forward primer 5'-ATTTCCGCCACACCATACAT-3' and reverse primer 3'-GTCACGCATAACATCCGTTG-5'; 630 bp), MdSGHV039 (forward primer 5'-CCGGATTCCATTATGTGGAC-3' and reverse primer 3'-TTTGACGCGC TTGCATATAG-5'; 669 bp), MdSGHV029 (forward primer 5'-TCTTTCCGG TGTTCGTATCC-3' and reverse primer 3'-GCAAATTGGAACGGTTGACT-5'; 666 bp), MdSGHV089 (forward primer 5'-CCTTTTCTGTTTCGTTTCAG C-3' and reverse primer 3'-CCGAATGAGGCAAATTGTTT-5'; 627 bp), and MdSGHV106 (forward primer 5'-CGGCAGCAACCTTATTCATT-3' and reverse primer 3'-CGTCGTCTCTCTCTTCAG-5'; 608 bp). These five genes were selected because of sequence homology to the DNA polymerase, p74, pif-1, pif-2, and pif-3 genes, respectively, found in other dsDNA insect viruses (5). The 25-µl reaction mixtures used for PCR amplification consisted of 2.5 µl of 10× *Taq* buffer with 25 mM MgCl₂, 0.5 µl of 10 mM solutions of deoxynucleoside triphosphates, 1 µl of a 10 µM forward primer solution, 1 µl of a 10 µM reverse primer solution, 1 µl of salivary gland DNA, and 0.25 µl of *Taq* DNA polymerase. The PCR cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min and then a final extension at 68°C for 7 min. PCR products were purified with a QIAquick PCR kit (Qiagen, Valencia, CA) and subjected to Sanger sequencing.

Reading frame and isolate sequence alignment. The nucleotide reading frame was determined for each of the five protein-encoding partial sequences from 16 geographical MdSGHV isolates. Correct protein translation for each partial sequence was determined by comparison of the BLASTx translation with the corresponding hypothetical protein translation from the complete ORF in the MdSGHV genome (GenBank accession number NC_010671). Outgroups for each MdSGHV partial sequence were identified in each BLASTx search and were obtained from the corresponding ORF in the *Glossina pallidipes* SGHV (GpSGHV) genome (GenBank accession number NC_010356). MdSGHV amino acid sequences, plus the corresponding GpSGHV outgroup for each gene, were aligned using MUSCLE 3.7 (3), and each protein alignment was used to align the nucleotide sequences by triplet, with gaps in the protein alignment equal to three gaps in the corresponding nucleotide alignment. Errors were checked using the DNA to Protein translation tool in Mesquite v2.6 (<http://mesquiteproject.org>) following alignment of nucleotide sequences into a coding frame. The five separate nucleotide alignments were concatenated in Mesquite into a single matrix consisting of 17 taxa and 2,730 characters and were parti-

TABLE 2. Summary of information for the nucleotide polymorphisms detected in the partial sequences from the 16 different MdSGHV isolates

Partial ORF sequence	Length (bp)	No. of single-nucleotide polymorphisms/total no. of nucleotides (%)	No. of silent nucleotide substitutions/total no. of nucleotide substitutions (%)	No. (%) of amino acid mutations
MdSGHV001	555	58/555 (10)	53/58 (91)	4/185 (2)
MdSGHV029	399	64/399 (16)	49/64 (77)	12/133 (9)
MdSGHV039	570	69/570 (12)	67/69 (97)	2/190 (1)
MdSGHV089	534	60/534 (11)	58/60 (97)	2/178 (1)
MdSGHV106	516	36/516 (7)	28/36 (77)	8/172 (5)
Total		286/2,574 (11)	254/286 (89)	28/858 (3)

tioned by gene and by codon position, resulting in 15 (5 genes $\times$ 3 codons) *a priori* partitions.

Optimal partitioning and phylogenetic analysis. The guided clustering method of Li et al. (13) was employed to optimize gene and codon partitions. For each of the 15 data sets generated, ranging from 15 partitions to 1 partition, a partition-wise model was inferred in TreeFinder (10) using the corrected Akaike information criterion as the model selection tool. Phylogenetic analysis of each partitioned data set was carried out with TreeFinder using the corresponding model and maximum likelihood (ML) optimized parameters. As described by Li et al. (13), the optimally performing partitioning scheme for the 15 data sets was identified by the lowest Bayesian information criterion (BIC) score. The topology corresponding to the optimal partition model was subjected to an additional tree search using 10 topologies generated at nearest-neighbor interchange distances of 5 and 10 steps. Because branch lengths between the isolates were short, the optimal ML-generated topology was compared with the topologies generated by a parsimony analysis in TnT v1.1 (8) using sectorial search, tree drift, tree fusing, and parsimony ratchet, with the best score found 1,000 times and with gaps read as an additional state.

Preparation and bioassay of viral inoculum. Five pairs of hypertrophied salivary glands per inoculum per isolate were homogenized in sterile water and centrifuged at $400 \times g$ for 3 min to pellet debris. Supernatants were transferred to an Ultrafree MC Millipore microcentrifuge tube with a 0.45- μ m filter membrane, centrifuged at $8,000 \times g$ for 5 min, and stored at -70°C . The final concentrations of the viral stock preparations were 10 and 50 infected gland pair equivalents (IGE) per ml. These stock preparations were used for bioassays as described below.

Viral stock preparations were diluted 1/10 in a 4% milk powder solution prior to each assay. Newly emerged flies (0 to 2 h postemergence) and 24-h-old adult flies were cold immobilized, and their mouthparts were coated with 0.1- μ l droplets of viral test inoculum. The final dose administered was 10^{-4} or 5×10^{-4} IGE per fly. Control flies were fed the milk solution without a virus. Each viral preparation was tested in three replicate bioassays with 10 treated flies per replicate. Treated flies were maintained in groups in 16-oz plastic cups with food (a 6:6:1 mixture of sugar, powdered milk, and powdered egg) and water and incubated under constant conditions. All flies were dissected 6 days posttreatment to record SGH symptoms. Statistical analyses were conducted using SPSS software (SPSS for Windows, release 15.0.0; SPSS Inc., Chicago, IL). Infection rate data were transformed by the arcsine of the square root prior to correlation analysis and two-way analysis of variance.

Nucleotide sequence accession numbers. All nucleotide sequences have been deposited in the GenBank database under accession numbers GU055219 to GU055298.

RESULTS AND DISCUSSION

SGHV incidence and pathology. Dissection of collected adult houseflies demonstrated the presence of MdSGHV in fly populations in Asia, North America, the Caribbean basin, and Europe. In addition, Coler et al. (2) detected hypertrophied salivary glands in housefly populations sampled at different locations in Brazil, suggesting that the distribution of MdSGHV is worldwide. In our sampling (Table 1), the incidence of SGH ranged from 0% to 34% in houseflies. In general, MdSGHV was detected in flies

collected from dairies, with the exception of the two collections made in Thailand. The data in Table 1 are only a partial list; other samples collected at many sites produced no flies displaying detectable SGH. For example, only one of four dairies sampled in Southern California was found to contain MdSGHV-infected flies. Previous annual surveys have shown that the incidence of MdSGHV infection in different feral housefly populations is highly variable (2, 7) and that the prevalence of the virus fluctuates spatially and temporally at a particular site. The isolates shown in Table 1 were derived from random collections, and it is likely that the incidence of the virus at any of the sites has seasonal fluctuations. Our previous sampling experience suggests that MdSGHV becomes stably associated with housefly populations at specific sites. For example, the dairy reported by Coler et al. (2) to harbor viremic houseflies in 1991 was resampled in 2005, 2006, and 2007 and was found to contain MdSGHV (7) (Table 1). Similarly, the samples taken from the Samut Sakorn (Thailand) site in both 2008 and 2009 contained viremic flies (Table 1).

Sequence comparison and analysis. A total of 2,574 bp ($\sim 2\%$ of the genome), containing trimmed reads for five partial ORFs, was generated for 16 different MdSGHV isolates. Alignment of the different isolate sequences with MUSCLE v3.7 (3) showed that there was 94% to 99% nucleotide identity to the sequenced reference MdSGHV3 isolate (Table 2). Comparisons of the sequence data generated from the 16 DNA preparations demonstrated that the percentage of detectable nucleotide substitutions varied from 7% to 16% for the different ORFs (Table 2). For the aligned 16 MdSGHV isolates, the majority of nucleotide polymorphisms detected at specific loci were due to a single base substitution, whereas only 12 of 286 loci had multiple nucleotide substitutions. On average, 89% of the substitutions detected were synonymous or neutral; the 11% that were nonsynonymous caused 28 amino acid substitutions in the compiled contigs generated for the 16 isolates. It should be noted that the lack of a suitable host cell culture precluded cloning of MdSGHV, and therefore all isolates should be considered populations. Close examination of the sequence chromatograms for the loci responsible for nonsynonymous substitutions revealed that many isolates produced multiple peaks for one or more loci, suggesting the presence of a mixture of viral genotypes. Only the predominant genotypes, represented by loci producing the major peaks, were aligned,

FIG. 1. Phylogenetic relationships among the 16 geographic isolates of MdSGHV. Isolates grouped predominantly by geographic region, with the exception of the Virgin Islands isolate, whose position in the best tree was not supported by resampling analyses. The values to the left of the slashes are node support values based on 100 ML bootstrap replicates. The values to the right of the slash are branch length support values based on 1,000 replicates of the expected likelihood weights determined by the local rearrangement (LR-ELW) method derived from the method of Strimmer and Rambaut (18). Dashes indicate values that are less than 50.

and only clean sequence reads were subjected to phylogenetic analysis.

Phylogenetic analysis. Phylogenetic analysis using ML was carried out with TreeFinder for each data set inferred by the guided clustering method (13), and a concurrent analysis using parsimony was carried out with TnT v1.1 (8) using a new technology search, which produced five equally parsimonious trees (length, 1,985; consistency index, 0.88; retention index, 0.67). The guided clustering method identified a six-partition model as the optimal model (log likelihood, $-9,112.045$; BIC, 18,825.41) and produced a phylogeny with clades generally separated by geographic region; the Virgin Islands isolate was the basal isolate in a United States isolate clade (Fig. 1). The exception was the clustering of isolates MdSGHV7, MdSGHV8, and MdSGHV10 with the MdSGHV3. The ML-generated topology was also recovered as one of the five most parsimonious trees.

We examined the utility of a novel partition selection heuristic for a data set for viral geographic isolates with a high degree of sequence similarity and site heterogeneity and compared the results of the partitioning protocol to the results obtained with a total-evidence approach (12) using parsimony. We considered a parsimony-based phylogeny alongside a model-based approach because of the degree of sequence similarity among the MdSGHV isolates. A model-based approach emphasizing congruence among loci was critical due to the possible presence of a conflicting phylogenetic signal, given the

highly heterogeneous clustering patterns for codon positions. Guided clustering showed that our MdSGHV data set was more “complex” than that of Li et al. (13) in that the evolutionary rates of codons were not conserved across the partial sequences of the five genes examined. Although clustering, a distance-like method, does not consider global optimality, the differences in site-specific rates that we observed persisted despite exploratory changes in the clustering method.

The source of possibly conflicting signals in our data set is unclear. The GpSGHV outgroup, despite the fact that it was the taxon most closely related to MdSGHV (1, 5), was present on a branch that was at least 3 orders of magnitude longer than the branches between the MdSGHV isolates, which were extremely short. Whereas the sequence-level and syntenic differences between these two members of the *Hytrosaviridae* have been described well elsewhere (5), we observed that the level of sequence identity for MdSGHV geographic isolates was high. However, this observation may be misleading since our analysis utilized partial ORFs, for each of which the average coverage of the complete protein-encoding sequence was as low as 22%. Similarly, low phylogenetic resolution may be due to analysis of isolates from geographically disparate locations. Our study examined isolates from the southern, central, and Pacific United States, the Caribbean, Scandinavia, the Indo-Pacific region, and the southwestern Pacific Ocean, but MdSGHV has also been observed in *M. domestica* populations in four cities in Brazil and in Massachusetts (R. R. Coler, personal communication). Future research should include these and other isolates, as it is postulated that MdSGHV, unlike GpSGHV, has a worldwide distribution.

Per os infectivity of the MdSGHV isolates. The symptoms exhibited by flies challenged with all of the infectious isolates mimicked the symptoms observed with the well-studied MdSGHV3 Florida-2005 isolate in that enlarged salivary glands were formed within 2 days after infection (data not shown). In addition, all virus preparations induced a complete shutdown of ovarian development in adult females displaying SGH symptoms (14). The results of *per os* infection bioassays demonstrated that the 15 MdSGHV isolates tested were infectious for laboratory-reared houseflies (Table 3). One of the 16 isolates collected was detected in ethanol-preserved salivary glands and therefore could not be used to test infectivity. When newly emerged flies were orally challenged with 10^{-4} IGE/fly, the average infection rates at 6 days posttreatment ranged from $30\% \pm 6\%$ to $83\% \pm 3\%$ for the different isolates. Statistically, MdSGHV6 was the most infectious of the 15 isolates ($F = 2.929$; $df = 14, 54$; $P < 0.05$). Several isolates that were tested using two different doses (Table 3) produced similar infection rates that were not significantly different ($t = -0.107$; $df = 136$; $P > 0.05$). In no case could 100% SGH be produced by *per os* challenge. These findings agree with the results of previous assays conducted with the MdSGHV3 isolate (7, 15). However, all MdSGHV preparations originating from primary or secondary hypertrophied salivary glands induced 100% infection when they were injected into adult flies. These results suggest that adult houseflies possess a mechanism of resistance to oral infection with MdSGHV and that this mechanism is circumvented when the virus is injected directly into the hemocoel. It should be noted that the newly emerged houseflies were challenged between 0 and 2 h after eclosion, an age at which they

TABLE 3. *Per os* infectivity of various MdSGHV strains for newly emerged and 24-h-old flies expressed as the mean percentage of SGH detected 6 days after oral treatment

Isolate	Dose administered (IGE/fly) ^a	% SGH (mean $\pm$ SE) ^b	
		Newly emerged flies	24-h-old flies
MdSGHV1	1×10^{-4}	30 ± 6	0
MdSGHV2	1×10^{-4}	43 ± 18	2 ± 2
MdSGHV3	1×10^{-4}	64 ± 6	3 ± 3
	5×10^{-4}	57 ± 9	10 ± 10
MdSGHV4	1×10^{-4}	65 ± 13	14 ± 9
	5×10^{-4}	45 ± 10	3 ± 3
MdSGHV5	1×10^{-4}	38 ± 8	7 ± 3
MdSGHV6	1×10^{-4}	67 ± 9	7 ± 3
	5×10^{-4}	83 ± 3	7 ± 7
MdSGHV7	1×10^{-4}	79 ± 6	16 ± 3
	5×10^{-4}	65 ± 5	3 ± 3
MdSGHV8	1×10^{-4}	52 ± 14	8 ± 4
	5×10^{-4}	63 ± 9	7 ± 3
MdSGHV9	1×10^{-4}	35 ± 8	0
MdSGHV10	1×10^{-4}	60 ± 13	17 ± 6
	5×10^{-4}	37 ± 8	10 ± 0
MdSGHV11	1×10^{-4}	48 ± 2	7 ± 3
	5×10^{-4}	58 ± 4	13 ± 9
MdSGHV12	1×10^{-4}	42 ± 7	16 ± 10
MdSGHV13	1×10^{-4}	57 ± 9	3 ± 3
MdSGHV14	1×10^{-4}	61 ± 6	8 ± 4
	5×10^{-4}	57 ± 3	0
MdSGHV15	1×10^{-4}	42 ± 11	26 ± 14

^a Viral stock preparations containing 10 and 50 infected gland pair equivalents (IGE)/ml were used for oral treatments.

^b The means are based on the results of three replicate bioassays performed with 10 treated flies per replicate. For statistical analysis, the mean SGH data for newly emerged and 24-h-old flies were transformed by the arcsine of the square root and compared by Student's *t* test analysis. The data were significantly different at a *P* value of < 0.05 for all isolates.

have not developed a peritrophic matrix in the midgut yet (P. Prompiboon, unpublished data).

When adult houseflies were treated *per os* with an MdSGHV inoculum 24 h after eclosion, the resistance to viral infection increased significantly ($F = 3.862$; $df = 136$; $P < 0.05$) (Table 3). The average rate of infection for these older flies ($8.3\% \pm 1.3\%$) was 6-fold less than that observed for newly emerged flies ($53.3\% \pm 2.3\%$), although all flies were fed the same preparations in the replicated assays conducted with the 15 MdSGHV isolates. These bioassay results point to what appears to be a paradox: a fly is most susceptible to *per os* infection during a developmental window in which it is not yet ready to feed. Although these results may explain why infection levels in the field are generally low, the factors that promote occasional spikes of MdSGHV infection in field populations remain unknown. Age- or development-related changes in susceptibility or resistance to viral infection are a well-established phenomenon and have been documented in various insects (4, 9, 11, 20). Differences in susceptibility may occur among life stages (i.e., egg, larva, pupa, and adult), among larval instars, or within a given instar (19).

In summary, our results demonstrate that MdSGHV has a cosmopolitan distribution. MdSGHV isolates originating from field sites in Europe, Southeast Asia, North America, and the Caribbean were shown to be closely related and to produce the

same pathogenesis in laboratory-reared houseflies, and all isolates induced development of hypertrophied salivary glands and inhibited female ovarian development.

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REFERENCES

1. Abd-Alla, A., J. M. Vlask, M. Bergoin, J. E. Maruniak, A. Parker, J. Burand, J. A. Jehle, and D. Boucias. 2009. Hytrosaviridae: a proposal for classification and nomenclature of a new insect virus family. *Arch. Virol.* **154**:909–918.
2. Coler, R. R., D. G. Boucias, J. H. Frank, J. E. Maruniak, A. Garcia-Canedo, and J. C. Pendlant. 1993. Characterization and description of a virus causing salivary gland hyperplasia in the housefly, *Musca domestica*. *Med. Vet. Entomol.* **7**:275–282.
3. Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**:1792–1797.
4. Engelhard, E. K., and L. E. Volkman. 1995. Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. *Virology* **209**:384–389.
5. Garcia-Maruniak, A., A. Abd-Alla, T. Z. Salem, A. Parker, V.-U. Lietze, M. M. van Oers, J. E. Maruniak, W. Kim, J. P. Burand, F. Cousserans, A. Robinson, J. M. Vlask, M. Bergoin, and D. G. Boucias. 2009. Two viruses that cause salivary gland hypertrophy in *Glossina pallidipes* and *Musca domestica* are related and form a distinct phylogenetic clade. *J. Gen. Virol.* **90**:334–346.
6. Garcia-Maruniak, A., J. E. Maruniak, W. Farmerie, and D. G. Boucias. 2008. Sequence analysis of a non-classified, non-occluded DNA virus that causes salivary gland hypertrophy of *Musca domestica*, MdSGHV. *Virology* **377**:184–196.
7. Geden, C. J., V.-U. Lietze, and D. Boucias. 2008. Seasonal prevalence and transmission of salivary gland hypertrophy virus of house flies (Diptera: Muscidae). *J. Med. Entomol.* **45**:42–51.
8. Goloboff, P. A., J. S. Farris, and K. C. Nixon. 2008. TNT, a free program for phylogenetic analysis. *Cladistics* **24**:774–786.
9. Grove, M. J., and K. Hoover. 2007. Intrastadial developmental resistance of third instar gypsy moths (*Lymantria dispar* L.) to *L. dispar* nucleopolyhedrovirus. *Biol. Control* **40**:355–361.
10. Jobb, G., A. von Haeseler, and K. Strimmer. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol. Biol.* **4**:18.
11. Kirkpatrick, B. A., J. O. Washburn, and L. E. Volkman. 1998. AcMNPV pathogenesis and developmental resistance in fifth instar *Heliothis virescens*. *J. Invertebr. Pathol.* **72**:63–72.
12. Kluge, A. G. 1989. A concern for evidence and a phylogenetic hypothesis of relationships among Epicrates (Boidae, Serpentes). *Syst. Zool.* **38**:7–25.
13. Li, C., G. Lu, and G. Orti. 2008. Optimal data partitioning and a test case for ray-finned fishes (Actinopterygii) based on ten nuclear loci. *Syst. Biol.* **57**:519–539.
14. Lietze, V.-U., C. J. Geden, P. Blackburn, and D. G. Boucias. 2007. Effects of salivary gland hypertrophy virus on the reproductive behavior of the housefly, *Musca domestica*. *Appl. Environ. Microbiol.* **73**:6811–6818.
15. Lietze, V.-U., K. R. Sims, T. Z. Salem, C. J. Geden, and D. G. Boucias. 2009. Transmission of MdSGHV among adult house flies, *Musca domestica* (Diptera: Muscidae), occurs via salivary secretions and excreta. *J. Invertebr. Pathol.* **101**:49–55.
16. Salem, T. Z., A. Garcia-Maruniak, V.-U. Lietze, J. E. Maruniak, and D. G. Boucias. 2009. Analysis of transcripts from predicted open reading frames of the *Musca domestica* salivary gland hypertrophy virus. *J. Gen. Virol.* **90**:1270–1280.
17. Skidmore, P. 1985. The biology of the Muscidae of the world. Series Entomologica, vol. 29. W. Junk, Dordrecht, the Netherlands.
18. Strimmer, K., and A. Rambaut. 2002. Inferring confidence sets of possibly misspecified gene trees. *Proc. R. Soc. Lond. B* **269**:137–142.
19. Tanada, Y., and H. K. Kaya. 1993. Insect pathology. Academic Press, San Diego, CA.
20. Washburn, J. O., B. A. Kirkpatrick, and L. E. Volkman. 1995. Comparative pathogenesis of *Autographa californica* M nuclear polyhedrosis virus in larvae of *Trichoplusia ni* and *Heliothis virescens*. *Virology* **209**:561–568.