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Drosophila melanogaster Mounts a Unique Immune Response to the Rhabdovirus *Sigma virus*[▽]

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Rhabdoviruses are important pathogens of humans, livestock, and plants that are often vectored by insects. Rhabdovirus particles have a characteristic bullet shape with a lipid envelope and surface-exposed transmembrane glycoproteins. *Sigma virus* (SIGMAV) is a member of the *Rhabdoviridae* and is a naturally occurring disease agent of *Drosophila melanogaster*. The infection is maintained in *Drosophila* populations through vertical transmission via germ cells. We report here the nature of the *Drosophila* innate immune response to SIGMAV infection as revealed by quantitative reverse transcription-PCR analysis of differentially expressed genes identified by microarray analysis. We have also compared and contrasted the immune response of the host with respect to two nonenveloped viruses, *Drosophila* C virus (DCV) and *Drosophila* X virus (DXV). We determined that SIGMAV infection upregulates expression of the peptidoglycan receptor protein genes *PGRP-SB1* and *PGRP-SD* and the antimicrobial peptide (AMP) genes *Diptericin-A*, *Attacin-A*, *Attacin-B*, *Cecropin-A1*, and *Drosocin*. SIGMAV infection did not induce *PGRP-SA* and the AMP genes *Drosomycin-B*, *Metchnikowin*, and *Defensin* that are upregulated in DCV and/or DXV infections. Expression levels of the Toll and Imd signaling cascade genes are not significantly altered by SIGMAV infection. These results highlight shared and unique aspects of the *Drosophila* immune response to the three viruses and may shed light on the nature of the interaction with the host and the evolution of these associations.

Sigma virus (SIGMAV; family *Rhabdoviridae*) occurs naturally in *Drosophila melanogaster* and is maintained in fly populations through vertical transmission via germ cells (31). Other viruses in this family are known pathogens of humans, livestock, fish, and plants (33). Insects commonly serve as vectors and replication hosts for many livestock and all well-characterized plant rhabdoviruses. Black flies, sand flies, and mosquitoes, for example, transmit vertebrate-infecting rhabdoviruses, e.g., *Vesicular stomatitis virus* and *Bovine ephemeral fever virus* (12, 27), whereas aphids, leafhoppers, and planthoppers vector plant rhabdoviruses (17, 19).

While rhabdoviruses can infect a variety of tissues in their invertebrate hosts, they appear to predominantly invade the central nervous system. In humans and other vertebrates, *Rabies virus* spreads throughout the body, including the central nervous system, and most importantly for transmission, the salivary glands (12). SIGMAV and some plant rhabdoviruses have been shown to replicate in neural and other tissues of *Drosophila* and their insect vectors (1, 2, 17, 31). SIGMAV does not appear to adversely affect *Drosophila* in their natural environment; however, SIGMAV-infected flies remain irreversibly paralyzed and die after CO_2 anesthetization (7, 31). Vesiculoviruses also confer similar CO_2 sensitivity to their black fly hosts (7).

Drosophila immune responses to various bacterial and fungal pathogens are well characterized at the molecular level. The elucidation of *Drosophila* antiviral immune responses began only recently and has focused on two other naturally occurring viruses, *Drosophila* C virus (DCV; family *Dicistroviridae*) (13, 28) and *Drosophila* X virus (DXV; family *Birnaviridae*) (35). SIGMAV differs from these two viruses in its mode of transmission, morphology, tissue tropism, and virulence (8, 16, 21, 31, 32, 35) (Table 1). Given SIGMAV's unique biology, we predicted that the *Drosophila* immune response might also differ with respect to this virus. Using quantitative reverse transcription-PCR (qRT-PCR) approaches, we have examined the expression of a number of innate immune genes in SIGMAV-infected *Drosophila* insects relative to uninfected flies. We have compared these patterns of transcription to those in response to DCV and DXV with the aim of shedding some light on how *Drosophila* responds to diverse viral infections.

MATERIALS AND METHODS

***Drosophila* stocks.** The *D. melanogaster* Fe strain (SIGMAV infected) and Canton-S strain (SIGMAV free) were used as starting stocks. All *Drosophila* stocks were maintained at 25°C in 70% humidity with a 12-h light-dark cycle on standard cornmeal-yeast medium. To minimize genetic background effects, Canton-S females were crossed with Fe males, and then the progeny females of each generation were backcrossed against Fe males for four generations to create a BC4 strain with 97% Fe background. A small portion of BC4 flies remained infected with SIGMAV because paternal transmission is possible although it is less efficient than maternal transmission (31). SIGMAV-infected BC4 flies

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TABLE 1. Characteristics of DCV (*Dicistroviridae*), DXV (*Birnaviridae*), and SIGMAV (*Rhabdoviridae*)

Virus	Characteristic (reference)			
	Structure	Mode of transmission	Tissue tropism	Effect(s)/virulence
DCV	Nonenveloped, isometric, positive-sense single-stranded RNA genome (16)	Horizontal, ingestion (9, 15)	Reproductive tissue, fat body, thoracic muscle, tracheal cells, digestive tract (21)	Mortality, faster developmental time, increased daily fecundity (20)
DXV	Nonenveloped, icosahedral nucleocapsid, double-stranded RNA genome (35)	Horizontal, contact (32)	Brain, thorax, reproductive tissue, malpighian tubules, trachea, muscle sheath (32)	Anoxia sensitivity, mortality (32)
SIGMAV	Enveloped bullet-shaped, transmembrane glycoprotein protruding from lipid envelope, negative sense single stranded RNA genome (27)	Vertical, via germ cells (31)	All tissues except muscle, especially thoracic and cephalic ganglia (31)	CO_2 sensitivity, reduced egg viability (31)

were removed from the population using a CO_2 sensitivity assay (see below). SIGMAV-negative samples were also screened via qRT-PCR using SIGMAV-specific primers (see below).

Confirming SIGMAV infection in flies by immunofluorescence microscopy. Three-day-old adult *Drosophila* SIGMAV-infected (Fe) or virus-free (BC4) flies were treated with CO_2 gas and kept on ice for 10 min. The SIGMAV-infected flies remained irreversibly paralyzed whereas the virus-free flies recovered from anesthetization after returning to fresh air and room temperature. Subsequently, the heads of tested flies were separated from the bodies (thorax and abdomen) under a stereomicroscope. The bodies of tested flies were stored in RNA*later* (Ambion, Austin, TX) at 4°C for subsequent RNA isolation and qRT-PCR whereas the heads were used to confirm the presence of SIGMAV in the brain and other tissues by immunofluorescence confocal laser scanning microscopy (iCLSM) (Fig. 1). To achieve this goal, each *Drosophila* head was split transversely with a sharp razor blade (to expose internal structures), kept in fixative (4% paraformaldehyde in 0.1 M phosphate buffer [pH 7.4], 0.1% Triton X-100) at 4°C overnight, and subsequently processed and examined by iCLSM as described previously (1). Briefly, the split heads were incubated with anti-SIGMAV antiserum (diluted 1/200) for 3 h and then incubated in a 1/600 dilution of the secondary antibody (goat anti-rabbit Alexa Fluor 488; Invitrogen Corp., Carlsbad, CA) for 1 h; samples were immersed first in the nuclear stain propidium iodide (Invitrogen Corp.) for 5 min and then in the actin stain phalloidin for 1 h before being examined by CLSM (Leica TCS SP). A subset of samples exhibiting substantial levels of infection (Fig. 1A) was then selected for downstream analysis. The status of SIGMAV-negative samples (Fig. 1B) was further confirmed with qRT-PCR (see below) using primers designed to amplify a fragment spanning the SIGMAV N and P genes (Table 2). Relative SIGMAV abundance per sample was compared following normalization against the host gene, *Actin 88F* (Table 2).

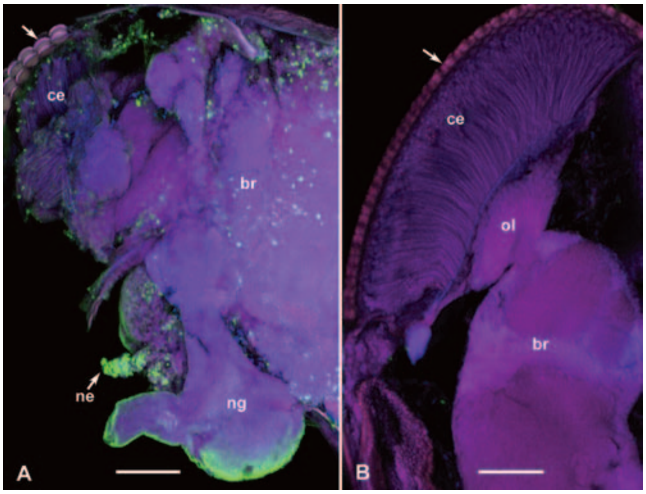

FIG. 1. SIGMAV infection (green fluorescence) in the compound eye (ce), brain (br), other nerve ganglia (ng), and nerves (ne) in the head of an infected *Drosophila* (A) compared to that of a noninfected fly (B). In each case, the head was split transversely to expose internal structures and immediately fixed and processed for iCLSM using SIGMAV antiserum as a primary antibody, Alexa Fluor 488 as a secondary antibody, the nuclear stain propidium iodide (red), and the actin stain phalloidin (blue or purple). Arrows indicate compound eye lenses. ol, optic lobe. Scale bar, 50 μm.

Sample preparation and qRT-PCR. A total of six samples were prepared for analysis for each SIGMAV-infected and uninfected *Drosophila* line. Each sample was comprised of paired male and female flies. Total RNA was extracted using Trizol (Invitrogen Corp.) according to the manufacturer's instructions. Initial homogenization was carried out using a Mini BeadBeater (BioSpec Products, Inc. Bartlesville, OK). The integrity and concentration of the RNA were determined spectrophotometrically using a NanoDrop and associated software, version 1000, (NanoDrop Technologies, Wilmington, DE). Extractions were treated with Turbo DNA-free (Ambion), and concentration was determined using a Quant-iT RiboGreen RNA reagent kit (Molecular Probes, Eugene, OR).

A SuperScript III Platinum Two-Step qRT-PCR kit with Sybr Green (Invitrogen Corp.) was used according to the manufacturer's protocol. cDNA was generated for each sample using random primers. Gene-specific primers were subsequently utilized for qRT-PCRs in a Rotor-Gene 3000 thermal cycler (Corbett Research, Brisbane, Australia). Real-time PCR primers (Table 2) were designed using Primer Express, version 1.5, software (Applied Biosystems, Foster City, CA) to yield 100- to 200-bp amplicons with a thermal denaturation midpoint temperature of $\geq$80°C. Threshold cycle values were normalized against *Actin 88F* as an internal control, and the $\Delta\Delta C_T$ (where C_T threshold cycle) method was used to calculate relative concentrations of target mRNA using Rest 2005, version 1.9.12, software (Corbett Research) (26). Two assay replicates and five to six biological replicates were compiled and averaged for each treatment.

RESULTS

Relative SIGMAV abundance. The mean relative values of SIGMAV infection, as revealed by qRT-PCR across the six samples that were found positive in the iCLSM study (Fig. 1A), was 2.3 ± 0.76 (mean ± standard error of the mean) with a range of 0.8 to 6.0 (Fig. 2). Of the six putative SIGMAV-negative samples based on the iCLSM study (Fig. 1B), one sample was apparently infected with SIGMAV, as revealed by qRT-PCR using SIGMAV primers (data not shown), and therefore was excluded for further analyses. Thus, iCLSM detected only relatively high levels of SIGMAV infection, which is reflected in the narrow range of qRT-PCR numbers for the six samples found positive in iCLSM (Fig. 2).

In pilot experiments prior to employing selection of SIGMAV-positive samples by iCLSM, extremely variable results were obtained with respect to the transcriptional profiles of various immune genes. This variation can be explained by a polymor-

TABLE 2. Oligonucleotide primers for qRT-PCR

Gene(s)	GeneID[a]	Primer name	Nucleotide sequence (5′ to 3′)	Amplicon size (bp)
Actin88F	CG5178	Actin88 305F	ATCGAGCACGGCATCATCAC	78
		Actin88 349R	CACGCGCAGCTCGTTGTA	
Attacin-A, *Attacin-B*	CG10146, CG18372	AttB 362F	GGCCCATGCCAATTTATTCA	101
		AttB 435R	CATTGCGCTGGAACTCGAA	
Cecropin-A	CG1365	CecA 91F	TCTTCGTTTTCGTCGCTCTC	144
		CecA 234R	CTTGTTGAGCGATTCCCAGT	
Defensin	CG1385	Def 146F	GCCAGAAGCGAGCCACAT	54
		Def 181R	CGGTGTGGTTCCAGTTCCA	
Diptericin-A	CG12763	Dipt 226F	AGGTGTGGACCAGCGACAA	61
		Dipt 265R	TGCTGTCCATATCCTCCATTCA	
Drosocin	CG10816	Dro 30F	GCACAATGAAGTTCACCATCGT	60
		Dro 71R	CCACACCCATGGCAAAAAC	
Drosomycin-B	CG10810	Dros 29F	CTCCGTGAGAACCTTTTCCA	120
		Dros 149R	GTATCTTCCGGACAGGCAGT	
Metchnikowin	CG8175	Mtk 85F	GCTACATCAGTGCTGGCAGA	102
		Mtk 187R	AATAAATTGGACCCGGTCT	
Toll	CG5490	Toll 2201F	AACTTGGGCAACCTTGTGAC	180
		Toll 2380R	GTAACCAAACGGGGAGTTGA	
PGRP-SA	CG11709	PGRPSA 30F	CTGCGGCTGTTATCAGTGAA	144
		PGRPSA 155R	TGATGGAATTTCCGCTTTTC	
PGRP-SB1	CG9681	PGRPSB 27F	TGTGGCCGCTTTAGTGCTT	57
		PGRPSB 65R	TCAATCTGCAGGGCATTGG	
PGRP-SC1a, *PGRP-SC1b*	CG14746, CG8577	SC1 330F	CGAGTGGAACCCCTACAGCAT	65
		SC1 408R	GCTCCAGGGTGTCCCAGTT	
PGRP-SD	CG7496	PGRPSD 128F	CCTTGCCACGTGCTGTGA	59
		PGRPSD 165R	TGTAACATCATCCGCACAAGCT	
PGRP-LC	CG4432	PGRPLC 211F	ACGGAATCCAAGCGTATCAG	165
		PGRPLC 356R	GGCCTCCGAATCACTATCAA	
Relish	CG11992	Rel 2916F	TCCTTAATGGAGTGCCAACC	181
		Rel 3097R	TGCCATGTGGAGTG ATTAT	
SIGMAV N and P genes	NA	SIGMAV 1343F	ATGTAACTCGGGTGTGACAG	154
		SIGMAV 1496R	CCTTCGTTCATCCTCCTGAG	
vir-1	CG31764	vir-1 1361F	TGTGCCCATTGACCTATCCA	109
		vir-1 1450R	GATTACAGCTGGGTGCACAA	

[a] NA, not applicable.

phism for both infection status and viral titer in laboratory stocks. The CO_2 sensitivity assays are also not 100% accurate in identifying SIGMAV-free flies. Hence, we decided to focus on comparing the transcription profiles of highly infected flies and SIGMAV-negative flies as determined by iCLSM and qRT-PCR.

Expression of innate immunity-associated genes. We tested the transcription levels of 15 immunity-related genes relative to the internal control gene *Actin 88F* by qRT-PCR. This indicated that six of the immune genes showed a consistent and statistically significant upregulation in the six SIGMAV-infected samples versus the five samples of uninfected flies. For the upstream genes involved in receptor activity and signaling, the peptidoglycan recognition protein (PGRP) genes *PGRP-SB1* and *PGRP-SD* showed clear upregulation in infected flies (Fig. 3A and Table 3), whereas *PGRP-LC*, *PGRP-SC1*, and *PGRP-SA* were not upregulated (Fig. 3A and Table 3). Expression levels of *PGRP-SB1* were particularly high (23.3-fold uninfected) whereas the expression level of *PGRP-SD* was only slightly higher (3.5-fold uninfected) (Table 3). Expression of *Toll*, *Relish*, and *vir-1* showed increases in expression in SIGMAV-infected flies, but these increases were not statistically significant (Fig. 3B). For the genes encoding antimicrobial peptides (AMPs), significant upregulation was found for *Attacin-A*, *Attacin-B*, *Cecropin-A1*, *Diptericin-A*, and *Drosocin* in SIGMAV-infected flies but not for *Defensin*, *Drosomycin-B*, and *Metchnikowin* (Fig. 3C). *PGRP-SB1*, *PGRP-SD*, and *Diptericin-A* are primarily regulated by *Relish* of the Imd pathway, whereas *Attacin-A*, *Attacin-B*, *Cecropin-A1*, and *Drosocin* are regulated by Relish of the Imd pathway and Spaetzle of the Toll pathway (11). However, we find no evidence that SIGMAV infection induces expression of *Toll* and *Relish* (Table 3). SIGMAV infection also does not activate *vir-1* of the Jak-STAT pathway (Table 3).

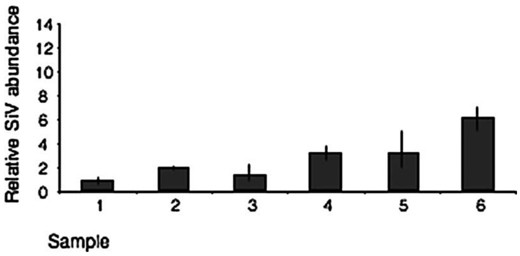

FIG. 2. Relative abundance of SIGMAV (SiV) per sample based on the expression of the SIGMAV N and P genes normalized against host *Actin 88F* expression. Error bars represent the range from assay replicates.

Comparison of *Drosophila* immune responses toward SIGMAV, DCV, and DXV. Signaling pathways controlling the

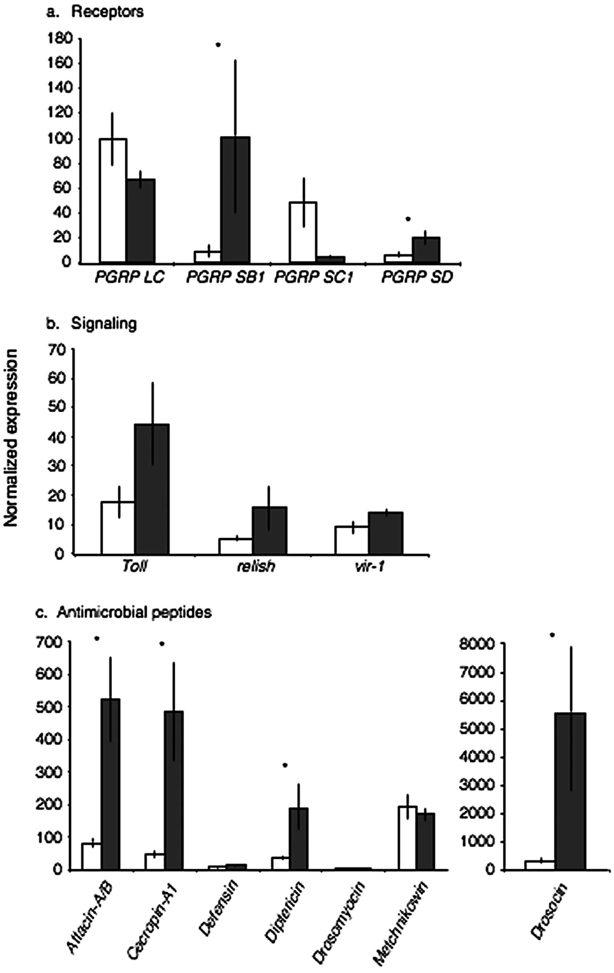

FIG. 3. Innate immune gene expression normalized against host *Actin 88F* by functional group. Values are mean ± standard error of the mean. SIGMAV-negative (white) and SIGMAV-positive (gray) bars represent results of five and six samples, respectively. *, $P < 0.05$ for the difference between SIGMAV-negative and SIGMAV-positive flies.

Drosophila humoral defense have been well described (6, 11, 22), but an understanding of the antiviral response is less complete (9). There have been several transcriptional profiles generated of *Drosophila* in response to the viruses DCV (13, 28) and DXV (35) that we have attempted to summarize (Table 3). The platforms/methods utilized vary across these studies as do the genes compared. Additionally, the fly response to DCV differs whether the virus is administered orally (28) or by intrathoracic injection (13). In general, the transcriptional responses of flies infected with DCV and DXV appear to be more similar to each other than to flies infected with SIGMAV. SIGMAV heavily induces *PGRP-SB1* and *PGRP-SD* while DCV increases transcription of only *PGRP-SA* (13). DCV also induces expression of *Relish* and other signaling cascade genes (13), which is not the case for SIGMAV (Table 3). Lastly, SIGMAV infection did not upregulate the AMP-encoding genes *Drosomycin-B*, *Defensin*, and *Metchnikowin* (Table 3) whereas DCV and DXV infections did (Table 3) (13, 28, 35). SIGMAV- and DCV-infected flies (13, 28) share upregulated expression of the *Attacin-A*, *Attacin-B*, and *Cecropin A* genes. SIGMAV- and DXV-infected flies (35) share upregulated expression of *Drosocin*, *Diptericin-A*, *Attacin-A*, and *Attacin-B*.

DISCUSSION

The pattern of induced PGRP gene expression by SIGMAV is distinct from that of other viruses, where only *PGRP-SA* shows induced transcription by DCV. SIGMAV induces both *PGRP-SD* and, more notably in terms of the magnitude of expression, *PGRP-SB1*. These two members of the short class of PGRP genes share a number of characteristics. Both genes exhibit low-level constitutive expression in adult *Drosophila* insects, are highly inducible in response to bacterial infection, are expressed mainly in the fat body, and encode proteins that are likely exported from the cell (34). *PGRP-SB1* has also been shown to have amidase activity and bactericidal properties (25). Unlike DCV and DXV that have proteinaceous capsids, SIGMAV particles are surrounded by a lipid bilayer with glycoprotein spikes. PGRPs are the first receptors that recognize, bind, or catalytically cleave specific surface components of bacterial cell membranes (22, 29). Thus, the differential induction of the PGRPs among the viruses may be an indication of the different virus surface properties.

TABLE 3. Mean ratios of expression for virus-infected relative to virus-free *Drosophila*

Gene function and name	GeneID	Relative expression level (virus-infected flies/virus-free flies)[a]			
		SIGMAV[b]	DCV		DXV[e]
			Oral infection route[c]	Intrathoracic infection route[d]	
Upstream genes and receptors					
PGRP-SA	CG11709	x	–	3.2	ND
PGRP-SB1	CG9681	23.5	–	–	ND
PGRP-SC1a, *PGRP-SC1b*	CG14746, CG8577	–	–	–	ND
PGRP-SD	CG7496	3.5	–	–	ND
PGRP-LC	CG4432	–	–	–	ND
Toll	CG5490	–	–	–	ND
Spaetzle	CG6134	ND	–	3.0	ND
Signaling cascade					
Relish	CG11992	–	–	3.5	ND
vir-1	CG31764	–	–	6.4	ND
Antimicrobial peptides					
Drosomycin-B	CG10810	–	3.0	2.1	70
Defensin	CG1385	–	–	+	4.8
Metchnikowin	CG8175	–	–	3.0	60
Drosocin	CG10816	10.3	–	–	3.2
Diptericin-A	CG12763	5.6	–	–	3.2
Diptericin-B	CG10794	ND	–	5.0	ND
Attacin-A	CG10146	7.2	6.3	8.7	2.5
Attacin-B	CG18372	ND	–	4.7	ND
Attacin-C	CG4740	ND	–	2.7	ND
Attacin-D	CG7629	ND	–	+	ND
Cecropin-A1	CG1365	8.8	2.6	–	1.8
Cecropin-A2	CG1367	ND	3.3	–	ND
Cecropin-B	CG1878	ND	–	+	ND
Cecropin-C	CG1373	ND	–	+	ND

[a] –, no difference in expression levels; x, no detectable expression; ND, not determined; +, induced expression relatively to virus-free flies.

[b] qRT-PCR data from this study; data reported for P values <0.05.

[c] Microarray data from reference 28.

[d] Microarray data from reference 13.

[e] qRT-PCR data from reference 35.

Unlike the case with DCV (13), we found little evidence of increased transcription in the signaling cascade genes of the Imd, Toll, and Jak-STAT pathways. However, one would expect a chance in expression of these signaling genes, because PGRP-SB1 and PGRP-SD expressions are primarily regulated by Relish of the Imd pathway (11), and PGRP-SD function is required for activation of the Toll pathway (4, 30). Also, *Diptericin-A* is primarily regulated by Relish of the Imd pathway, whereas *Attacin-A*, *Attacin-B*, *Cecropin-A1*, and *Drosocin* are regulated by Relish of the Imd pathway and Spaetzle of the Toll pathway (11). On the other hand, we did not find upregulation of *Metchnikowin*, which is also induced by both the Toll and Imd pathways (23). The type of infection dictates how the Toll and Imd pathways contribute to the expression of each AMP gene (11). For the AMP gene expression levels, the SIGMAV infection appears to be most similar to that of the gram-negative bacteria that also induce *Diptericin*, *Attacin*, *Cecropin*, and *Drosocin* but not *Drosomycin* and *Metchnikowin* (18). The outcomes of the *Drosophila* immune response to SIGMAV and gram-negative bacteria may be similar because both microbes have outer lipid bilayers and glucose.

Since SIGMAV is a vertically transmitted parasite, there would be substantial selection pressure for reduced virulence and for evasion of the host immune response in the virus (14). Like SIGMAV, DCV has an old and established relationship with *Drosophila*. Laboratory experiments comparing responses of flies following infection by intrathoracic injection with the more natural route of feeding indicate a weaker Toll response in the latter case (9, 13, 28). This may be due entirely to differences in the mode of immune system activation via the gut but could also reveal a history of adaptation. The constitutive upregulation of immunity genes in SIGMAV-infected flies nonetheless indicates evidence of host recognition and energetic investment in fighting the SIGMAV infection. Extreme overactivation of the Imd pathway has been associated with developmental defects and larval death (5), and a number of published works reveal tradeoffs between immune function and fitness in insects (3, 10, 24). Not surprisingly, SIGMAV has been shown to cause mild reductions in host egg viability; however, the 10 to 20% SIGMAV infection frequency in natural *Drosophila* populations (31) suggests that infected flies can compete in terms of fitness to some degree with virus-free flies.

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