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Drosophila immunity and homeostasis during viral infection

A dissertation presented

by

Jonathan Chow

to

The Division of Medical Sciences

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Immunology

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Drosophila immunity and homeostasis during viral infection

Abstract

Insects are vectors for human disease and also play a critical role in agriculture. Like humans, insects encounter pathogens such as viruses. Despite having a profound impact on our lives, much has yet to be discovered about how insects respond to viral infection. All organisms have two options when under threat: disease resistance mechanisms and disease tolerance mechanisms. Disease resistance mechanisms are strategies to limit pathogen replication. For insects responding to a viral infection, RNA interference, Toll signaling, IMD signaling, autophagy, and Jak/STAT signaling have been identified as relevant immune mechanisms for restricting pathogen growth. However, each of these mechanisms has been studied to a varying extent, and the relative contribution of each to antiviral immunity has yet to be fully evaluated. The other option that infected insects have is to employ disease tolerance mechanisms. These strategies seek to maintain homeostasis and limit pathology when the animal undergoes stresses such as viral infection without necessarily limiting pathogen replication. Disease tolerance in animals is a nascent field of research, and the few existing experimental systems modeling disease tolerance prove challenging to study. Here, we employ *Drosophila melanogaster* as a genetically tractable model organism to study disease resistance and disease tolerance mechanisms in the context of viral infection. *In vitro* and *in vivo* infections were utilized to evaluate the relative contribution of antiviral resistance mechanisms. RNA interference was found to be the main contributor to antiviral immunity. Additionally, a gene orthologous to STING, a mediator of mammalian antiviral immunity, was found to have antiviral properties in fruit flies. A model for studying disease tolerance was also established. Vesicular stomatitis virus infection renders flies susceptible to coordinative defects only after carbon dioxide exposure, despite being otherwise asymptomatic. We found that the viral glycoprotein alone sufficiently abrogates the ability of flies to tolerate the stress of carbon dioxide anesthesia. The glycoprotein mediates syncytia formation in the nervous system, resulting in instantaneous pathology with ensuing morbidity. The data reported

here demonstrate that disease resistance and tolerance mechanisms support the ability of insects to thrive in the face of pathogenic onslaughts.

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Chapter 1

Disease resistance and tolerance: survival strategies for viral infection

Like humans, insects encounter viral pathogens. Despite having repercussions on human health and disease, many gaps in our knowledge exist on how insects and their viral pathogens interact. *Drosophila melanogaster* serves as an ideal model due to its genetically tractable nature to study host-pathogen interactions. When encountering a pathogen, two major approaches organisms take to fight disease are resistance strategies and tolerance strategies. Disease resistance strategies aim to promote the health of the infected organism by reducing pathogen load. Multiple disease resistance mechanisms have been identified in *Drosophila.* RNA interference (RNAi), autophagy, transcriptional induction via Jak/STAT signaling, Toll signaling, and IMD signaling have been implicated in the reduction of pathogen load in infected flies. Disease tolerance mechanisms are strategies that host organisms use to mitigate the stress and damage incurred by infection and other threats. These disease tolerance mechanisms promote health of the host without necessarily suppressing the proliferation of the pathogen. In this first chapter, disease resistance mechanisms used by fruit flies will be described and will be compared to mechanisms used by mammals. Disease tolerance will then be further explained in the broader context of all animals, as this is a burgeoning field of study. Finally, vesicular stomatitis virus (VSV) and its replication cycle will be described as the scope of this dissertation will mainly focus upon this pathogen in *Drosophila melanogaster*. This chapter will conclude by establishing the scope and objectives of my dissertation research.

RNAi

RNAi is used as an antiviral defense strategy in arthropods, plants, and possibly mammals (Abubaker et al., 2014). Three RNAi pathways exist in arthropods, and each has a distinct function. The microRNA (miRNA) pathway regulates endogenous gene expression. The piwi-interacting RNA (piRNA) pathway inhibits the activity of transposons embedded in the host genome. The small interfering RNA (siRNA) pathway detects viral nucleic acids and restricts viral replication. The siRNA and miRNA pathways have similar mechanisms of action whereas the piRNA pathway has a different method of gene silencing. Each of these pathways and their functions is further described below.

The overall strategy to restrict viral replication by the siRNA pathway requires multiple steps. First, double-stranded RNA (dsRNA) derived from viral sequences must be recognized by Dicer 2 (Dcr2).

Second, Dcr2 loads one strand into Argonaute 2 (Ago2). Finally, Ago2 finds complementary RNA and sequesters or cleaves it to inhibit translation. Because RNAi is an important tool used in genetic screens, the molecular mechanisms of how translation inhibition is mediated have been a focus of research. However, questions still remain to be answered, especially with regards to how viral infections are detected and restricted.

In *Drosophila*, the siRNA pathway resists infection by multiple classes of viruses. Of the viruses composed of an RNA genome, the siRNA pathway can inhibit infection by non-enveloped virus and enveloped, single-stranded RNA viruses of both polarities (negative sense and positive sense) (Galiana-Arnoux et al., 2006). Double-stranded RNA virus replication is also inhibited by the siRNA pathway (Zambon et al., 2006). In *Drosophila*, the DNA virus, invertebrate iridescent virus 6 (IIV-6), is restricted by the siRNA pathway (Bronkhorst et al., 2012). Depending on the composition of the genome, viruses have different methods of replication. Despite these viral pathogens having varied strategies for replication, the siRNA pathway is capable of restricting them.

The ability of the siRNA pathway to recognize broad classes of viruses could be partly due to its cellular localization. In mammalian cells, Ago2 localizes to processing bodies (P-bodies) along with siRNA and cognate mRNA transcripts suggesting that P-bodies facilitate RNA silencing (Jagannath and Wood, 2009). Additionally, Dcr2 and Ago2 have been found to be localized to the cytoplasm and the nucleus (Cernilogar et al., 2011). However, the relationship between subcellular localization and function has not been fully explored. Nuclear localization of Dcr2/Ago2 was studied in the context of host gene transcription and epigenetic regulation (Cernilogar et al., 2011). mRNA processing machinery such as the cap binding complex has also been associated with siRNA components (Sabin et al., 2009). How these processes such as chromatin regulation and mRNA capping promote gene silencing is unclear.

Another factor that may contribute to the broad applicability of the siRNA pathway to a variety of viruses is the selectivity of substrates by Dcr2. Dcr2 recognizes long double-stranded RNA (dsRNA) (Lee et al., 2004b). This RNA binding is mediated cooperatively with the accessory protein R2D2 (Liu et al., 2006). Upon RNA binding to the Dcr2/R2D2 complex, Dcr2 dices RNA to a 21-nucleotide length with a 2-nucleotide overhang (Elbashir et al., 2001a). However, the origin of long dsRNA substrates recognized by the siRNA pathway is unclear. During viral replication, dsRNA must be produced in order for Dcr2 to

recognize it. One proposed model of nucleic acid recognition from high throughput RNA sequencing suggests that transcription in opposing directions of a viral genome produces complementary strands (Aliyari et al., 2008; Sabin et al., 2013). These complementary strands could potentially bind together to create dsRNA which is then recognized by Dcr2. However, this model of pathogen recognition is potentially problematic and requires more research. These transcripts would arise from positive strand and negative strand templates, which might be produced at different times in the viral replication cycle. Also, these complementary strands might be produced in different subcellular locations. It is unclear how the complementary strands would traffic and physically bind each other. Another proposed model is that the template RNA strand and the newly transcribed RNA strand bind each other, allowing for Dcr2 recognition (Sabin et al., 2013). This too is a challenging model because viral genomes are often associated with viral proteins, which could inhibit complementary binding. Finally, a third possibility is that viral RNA could be self-complementary, creating a hairpin structure. If the complementary sequence is long enough, Dcr2 could potentially recognize it as a substrate (Sabin et al., 2013). However, such hairpin structures ideal for Dcr2 recognition might not exist in every virus. The selection of viral RNA as Dcr2 substrates likely depends on multiple factors. Although ambiguity remains in the model for how dsRNA is detected by Dcr2, this RNAse enzyme is well established to be integral to the siRNA pathway.

In addition to Dcr2, additional proteins are necessary for dsRNA recognition, processing, and loading into Ago2 (Iwasaki et al., 2015). Loquacious was identified *in vivo* to serve a non-redundant role in facilitating the dicing of dsRNA to create a duplex of the correct length for Ago2 loading (Marques et al., 2009). R2D2 is essential in RNA binding and processing activities (Liu et al., 2006). The binding preference of R2D2 determines which strand of the dsRNA is the passenger strand and which is the guide strand that is loaded onto Ago2 (Tomari et al., 2004). R2D2 orients the dsRNA for loading into Ago2 depending upon the relative binding strength of each 5' end (Tomari et al., 2004). The dsRNA is then unwound, and the guide strand is loaded into Ago2 to create the RNAi silencing complex (RISC) (Tomari et al., 2004). A complex called C3PO, composed of Translin and Trax, facilitate the unwinding and subsequent removal of cleaved passenger strand RNA (Liu et al., 2009). RISC is then ready to silence mRNA transcripts.

After an RNA strand is loaded into Ago2 forming RISC, Ago2 searches for complementary RNA sequences to silence. Gene silencing can occur by one of two ways: slicing or obstruction of translation elongation of mRNA transcripts. Ago2 slicing activity requires that sequences are complementary to the siRNA, particularly the middle sequence region where slicing occurs (Elbashir et al., 2001a, 2001b). The cleaved mRNA can no longer be used for translation and is quickly degraded due to a lack of a 5' cap or a 3' poly-A tail (Elbashir et al., 2001a). If the mRNA cannot be sliced, RISC can still interfere with translation by obstructing translation elongation (Olsen and Ambros, 1999). These RISC-bound transcripts which can no longer be translated are then transported to P-bodies where they are degraded (Eulalio et al., 2007).

RNAi is a cell intrinsic mechanism of gene regulation, and the siRNA pathway specifically restricts the expression of viral genes inside the cell. However, the siRNA pathway uniquely functions in a cell extrinsic manner as well. Using a recombinant sindbis virus expressing a GFP reporter gene, gene silencing was discovered to outpace the spread of virus, suggesting that RNAi targeting specific genes can be transferred from one cell to another (Saleh et al., 2009). Additional studies suggest that RISC can be transported between cells via nanotube-like structures and that RNA binding proteins could mediate the endocytosis of the RNA (Karlikow et al., 2016; Saleh et al., 2006). Thus, the intercellular spread of siRNA pathway components could prepare uninfected cells against potential viral infection.

The importance of the RNAi pathway in antiviral defense is highlighted by viral evasion mechanisms used to thwart the host. The B2 protein is encoded by flock house virus and efficiently inhibits RNAi activity by two mechanisms (Li et al., 2002). First, B2 protein can bind dsRNA, thereby inhibiting Dcr2 recognition (Chao et al., 2005; Lingel et al., 2005). Second, B2 can directly interact with Dcr2 via its helicase domain in order to prevent recognition of viral RNA (Singh et al., 2009). In addition to B2, other insect viruses encode proteins that inhibit RNAi. Drosophila C virus also encodes an RNAi suppressor gene that binds to dsRNA (van Rij et al., 2006). Cricket paralysis virus encodes the 1a protein which antagonizes Ago2 function (Nayak et al., 2010). Mechanisms to inhibit siRNA function demonstrate the importance of RNAi in resisting viral infection.

The miRNA pathway is different from the siRNA pathway but shares some commonalities. One major difference is that the miRNA pathway regulates endogenous gene expression and the source of the

miRNA is from the genome. miRNA arise from intergenic sequences transcribed by RNA polymerase II, although they can come from intronic sequences as well (Rodriguez et al., 2004). Sometimes, these miRNA genes are clustered together, and the transcripts can be polycistronic, producing multiple miRNA (Lee et al., 2002). These transcripts have a 5' cap and a 3' tail, like mRNA transcripts (Lee et al., 2004a). The resulting primary miRNA transcripts have secondary stem-loop structures, base-pairing *in cis* and resulting in dsRNA.

These stem-loop structures are recognized by Pasha which recruits Drosha, an RNAse III enzyme (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003). Drosha cleaves the primary miRNA, releasing the stem loop structure from the rest of the transcript. The stem-loop structure is approximately 30 base pairs in length. Two nucleotides overhang the 3' end of the stem-loop structure, and they are necessary for export into the cytoplasm (Bohnsack et al., 2004; Lund et al., 2004a; Yi et al., 2003). Additionally, proteins recognizing the 3' overhang which mediate the transport protect the RNA from degradation by exonucleases (Lund et al., 2004a; Yi et al., 2003).

In the cytoplasm, Dicer-1 (Dcr1) recognizes the dsRNA and cleaves the terminal loop structure (Bernstein et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Dcr2 and Dcr1 have distinct substrates. Dcr2 recognizes long dsRNA with the help of R2D2 (Cenik et al., 2011) whereas Dcr1 recognizes the 3' overhang and measures the distance to the loop structure (Tsutsumi et al., 2011). After the loop structure has been cleaved off, a duplex of miRNA-miRNA* (guide and passenger RNA, respectively) remains. Depending on the characteristics of the two strands, one will be loaded into the silencing complex while the other one is degraded. Dcr1 can load miRNA into either Ago1 or Ago2. The preferred strand for Ago1 loading contains a 5' uracil and mismatches at nucleotides 7-11, whereas Ago2 substrates tend to have base pairing at nucleotides 9-10 (Förstemann et al., 2007; Okamura et al., 2009; Tomari et al., 2007). Thus, while the siRNA pathway uses Ago2 exclusively, the miRNA pathway can use either Ago1 or Ago2. After loading into the RISC complex, the miRNA can be further processed by Nibbler, an exonuclease that degrades RNA in the 3' to 5' direction (Han et al., 2011).

After miRNA loading RISC can silence gene expression multiple ways like in the siRNA pathway. Cap-dependent translation inhibition occurs by Ago binding to the 5' cap of complementary mRNA, thus inhibiting engagement by translation initiation factor eIF4E (Kiriakidou et al., 2007). The mRNA can be brought to P-bodies, where decapping enzymes and nucleases degrade the transcripts (Rehwinkel et al., 2005). Additional factors, such as Armitage, also support the silencing of miRNA target transcripts (Cook et al., 2004). However, the mechanism by which these factors mediate silencing is unclear.

The piRNA pathway is quite different from the siRNA and miRNA pathways. Neither of the two Dicer proteins encoded in the genome is required (Vagin et al., 2006). Instead, three argonaute family proteins, Piwi, Aubergine (Aub), and Ago3, suppress expression of transposons in the germline. Indeed, Aub and Ago3 expression has exclusively been identified in nurse cells (Brennecke et al., 2007). The small RNAs synthesized in the piRNA pathway are characteristically longer than those created by the siRNA and miRNA pathways. piRNAs are 23-30 nucleotides in length, whereas miRNA and siRNA are 21-23 nucleotides in length (Aravin et al., 2003, 2004). While many aspects of the piRNA pathway have yet to be fully explored, the pathway is clearly important for passing stable genetic information trans-generationally.

Substrates for the piRNA pathway are encoded in the genome and are transcribed by RNA polymerase II. Multiple piRNA products can be encoded in a single transcript. The selection of transcripts for processing by piRNA components is unclear since they look like typical transcripts with a 5' cap and 3' tail. However, DNA sequences encoding piRNA substrates are mostly clustered together within the genome (Brennecke et al., 2007). Additionally, epigenetic modifications are required for piRNA biogenesis (Rangan et al., 2011). Thus, a prevailing theory is that specific transcription factors associated with these regions are involved in the selection of transcripts for piRNA production, but these transcription factors have yet to be identified.

The primary transcripts traffic to the cytoplasm in order to be processed. Zucchini cuts the transcript at the 5' end (Pane et al., 2007). Uridine preferentially occupies the first position at the 5' end of piRNA (Brennecke et al., 2007; Gunawardane et al., 2007). After initial processing, the immature piRNA is bound to Piwi and is trafficked to Yb bodies. The Yb bodies contain Yb protein in addition to other proteins and are localized to the perinuclear region (Murota et al., 2014). In the Yb bodies, Nibbler is an

exonuclease that cuts nucleic acids in the 3' to 5' direction, thus trimming the piRNA to the proper length (Feltzin et al., 2015). Hen-1 then methylates the 2' oxygen to fully mature the piRNA (Horwich et al., 2007).

The Piwi RISC complex then translocates into the nucleus where it mediates gene silencing. This nuclear localization is important to its function, as localization mutants are incapable of mediating gene silencing (Klenov et al., 2011; Saito et al., 2009; Sienski et al., 2012). Additionally, the slicer activity of Piwi was determined to be unnecessary for gene silencing (Saito et al., 2009; Sienski et al., 2012). Instead, Piwi seems to mediate gene silencing by recognizing nascent, complementary transcripts and recruiting additional factors to epigenetically silence the locus. H4K9me3 modifications are made to inhibit further transcription.

The additional argonaute proteins, Aub and Ago3, are involved in the secondary biogenesis pathway also known as ping-pong amplification (Brennecke et al., 2007; Gunawardane et al., 2007). Unlike Piwi which is localizes to the cytoplasm and nucleus, Aub and Ago3 are only found in the cytoplasm (Brennecke et al., 2007). This secondary pathway creates a feed-forward loop that generates increasing amounts of piRNA. The process begins when Aub binds an anti-sense piRNA. Upon recognition of a complementary sequence derived from a transposon transcript, the slicing activity of Aub cleaves the transcript. The cleaved transcript then becomes a substrate for Ago3. After being loaded onto Ago3, the RISC recognizes anti-sense sequences that are then sliced. The cleaved products can then serve as substrates for Aub, thus continuing a feed-forward amplification loop. Collectively, the three RNAi pathways regulate gene expression, but only the siRNA pathway functions as an antiviral mechanism.

Autophagy

Autophagy is a cellular process whereby cytoplasmic contents can be captured and degraded in lysosomes (Zirin and Perrimon, 2010). A phagophore, also known as an isolation membrane, forms, elongates and engulfs the cargo to be degraded. In the process, the characteristic double membrane of autophagosomes forms. Autophagy has been studied in number of model organisms including yeast, flies, and mice because the process and genes involved are highly conserved (Reggiori and Klionsky,

2002). The use of multiple organisms has led to standardization of the nomenclature (Klionsky et al., 2003, 2011).

Many proteins are involved in autophagy. Initiation of autophagy involves a complex of proteins including Atg1 and Atg13 which become activated by upstream signals such as metabolic shifts (Kamada et al., 2000; Meléndez et al., 2003; Scott et al., 2004). The activation of the Atg1 complex leads to the activation of another complex of proteins including Vps34, which produces phosphatidylinositol-3-phosphate (Simonsen and Tooze, 2009). This step results in the nucleation of the phagophore which in turn recruits more proteins such as Atg18 (Berry and Baehrecke, 2007; Pace et al., 2002). Once the phagophore has been created, it must elongate in order to engulf its intended cargo. This elongation step is mediated by two conjugation systems. In the first conjugation system Atg7 and Atg10 mediate covalent binding of Atg5 to Atg12, and Atg5 and Atg12 form a complex with Atg16 (Fujita et al., 2008; Kuma et al., 2002; Mizushima et al., 1998; Shintani et al., 1999). The second conjugation system links Atg8 to phosphatidylethanolamine (PE) via an amide bond (Ichimura et al., 2000). The conjugation of Atg8 to PE involves Atg4, Atg7, and Atg3 which bind and transfer Atg8 akin to the ubiquitin ligation system (Kirisako et al., 2000). Once the phagophore completely envelopes its cargo, the autophagosome fuses with endosomes and then lysosomes to degrade the contents.

In *Drosophila*, autophagy was demonstrated to be an antiviral defense. A genomic screen in the *Drosophila* S2 cell line revealed that VSV infection was poorly restricted in cells where autophagy genes were knocked down (Shelly et al., 2009). When flies were infected, knockdown or mutation of autophagy genes resulted in worse survival outcomes compared to control flies (Shelly et al., 2009). In a subsequent study, Toll-7 was implicated in the detection of VSV glycoprotein (G), resulting in the induction of autophagy (Nakamoto et al., 2012). Although Toll-7 is homologous to Toll, downstream mediators of Toll signaling (dMyD88 and DIF) were not involved in restricting VSV infection, suggesting that Toll-7 induced autophagy noncanonically (Nakamoto et al., 2012). In addition to VSV, Rift Valley Fever virus infection is sensitive to autophagy as an antiviral defense (Moy et al., 2013). In the case of Rift Valley Fever virus, Toll-7 was also responsible for virus recognition and induction of autophagy (Moy et al., 2013). However, another group found no evidence for Toll-7 restricting VSV infection (Lamiable et al., 2016a). They did indeed confirm that autophagy is an antiviral defense mechanism (Lamiable et al., 2016a). Interestingly,

flock house virus was less virulent when Atg7 was mutated, suggesting that some viruses can use the autophagy pathway to their advantage (Lamiable et al., 2016a). The importance of autophagy in resisting viral infection is likely pathogen and context dependent but will require more work to ascertain.

Jak/STAT

The Jak/STAT signaling pathway was originally identified in the pursuit to understand Type I interferon signaling in mammals (Stark and Darnell, 2012). Type I interferons are signaling molecules that induce the transcription of genes whose products antagonize viral replication in numerous ways, thus creating an antiviral state in mammals (Raftery and Stevenson, 2017). Signaling is initiated when extracellular interferon binds to the interferon receptor at the plasma membrane. In order for downstream signaling to occur, a class of cytoplasmic proteins called Janus kinases (Jaks) must become activated. Jaks are associated with signaling receptors via protein-protein interactions, and they operate in pairs to phosphorylate each other, thus becoming activated and poised to activate downstream STAT proteins via phosphorylation (Argetsinger et al., 1993; Muller et al., 1993). Activated STAT proteins serve as transcription factors, translocating to the nucleus to initiate gene transcription. In addition to playing a critical role in Type I interferon signaling, Jak and STAT proteins also mediate signal transduction downstream of Type II and Type III interferons and cytokines (Banerjee et al., 2017; Lin and Young, 2014).

In contrast to mammals which have four Jaks and seven STATs, *Drosophila* have only one Jak and one STAT, hopscotch and STAT92E respectively (Binari and Perrimon, 1994; Hou et al., 1996; Yan et al., 1996). Only one receptor, domeless, has been identified to activate hopscotch and STAT92E (Brown et al., 2001; Chen et al., 2002). The Jak/STAT pathway in *Drosophila* is important for development and tissue homeostasis, particularly in regulating cell proliferation (Zeidler et al., 2000). Like mammalian Jak/STAT signaling, the *Drosophila* counterpart is involved in hematopoiesis (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1995). Additionally, it is involved in cellular proliferation in the fly gut, which undergoes a high rate of cellular turnover (Jiang et al., 2009). While interferons do not exist in flies, three cytokine-like molecules have been identified to activate the receptor domeless: unpaired, unpaired2 and unpaired3 (upd, upd2, and upd3 respectively) (Agaisse et al., 2003; Harrison et

al., 1998). The differences between these signaling molecules is still unclear, but it has been proposed that they have differential binding affinities for domeless and differential binding for the extracellular matrix (Wright et al., 2011). A fourth potential ligand, vago, induces Jak/STAT signaling in mosquitoes (Paradkar et al., 2012). Vago mediates antiviral activity in *Drosophila*, but it has yet to be confirmed to induce Jak/STAT signaling in flies as well (Deddouche et al., 2008).

The antiviral function of the Jak/STAT pathway in insects is an area of active investigation. Drosophila C virus infection induces Jak/STAT signaling, and loss of function mutations in Jak result in decreased viability of infected flies (Dostert et al., 2005). Similarly, flies with mutations in Jak are also more susceptible to infection by cricket paralysis virus and Drosophila X virus (Kemp et al., 2013). While Jak/STAT signaling is required to maintain viability during viral infection, Jak/STAT signaling was insufficient in itself (Dostert et al., 2005), thus suggesting that Jak/STAT signaling works cooperatively with additional defense mechanisms. Like autophagy, the relative importance of Jak/STAT signaling during viral infection requires further study.

Toll

The Toll pathway was originally discovered in a forward-genetic screen for genes involved in dorsal-ventral patterning of the *Drosophila* embryo (Anderson et al., 1985a, 1985b; Nusslein-Volhard, 1980). Later, Toll pathway mutants were discovered to be susceptible to fungal and bacterial infections (Lemaitre et al., 1996). In *Drosophila*, the Toll receptor is activated by the extracellular ligand, spatzle (Anderson and Nusslein-Volhard, 1984), which is cleaved into its active form by proteases (Morisato and Anderson, 1994; Schneider et al., 1994; Stein and Nusslein-Volhard, 1992). The proteases are activated by peptidoglycan recognition proteins (PGRP) that recognize microbe-derived molecules (Bischoff et al., 2004; Garver et al., 2006; Michel et al., 2001). Downstream of the Toll receptor, protein-protein interactions mediate signaling. *Drosophila* MyD88 (dMyD88) is localized to the plasma membrane via protein-lipid interactions and binds to the cytoplasmic tail of the Toll receptor which recruits tube and pelle (Horng and Medzhitov, 2001; Marek and Kagan, 2012; Sun et al., 2002, 2004). Pelle is a serine/threonine kinase that mediates the phosphorylation and degradation of cactus (Hecht and Anderson, 1993; Shelton and Wasserman, 1993). Cactus inhibits transcription factors, dorsal and dorsal-related immunity factor

(DIF) (Kidd, 1992). DIF and dorsal are NF-κB-family transcription factors that mediate the anti-microbial immune response and activate genes that define dorsal-ventral patterning of the embryo, respectively (Anderson et al., 1985a; Lemaitre et al., 1996). Thus, the two transcription factors differentiate the functions of Toll signaling.

Toll signaling is involved in some antiviral responses. *Drosophila* X virus infection induces the production of antimicrobial peptides (Zambon et al., 2005). Further analysis demonstrated that a loss of function mutant in DIF and a constitutively active mutant of the Toll receptor resulted in poorer survival outcomes compared to wild type animals (Zambon et al., 2005). Both loss of function and constitutive signaling of the Toll pathway resulted in decreased viability of infected animals. Thus in the context of *Drosophila* X virus infection, the role of Toll signaling is complicated and requires further study. Toll signaling also seems to be involved in viral infection via the oral route. When flies were fed *Drosophila* C virus, Toll pathway mutants succumbed to infection faster than wild type animals (Ferreira et al., 2014). Similar survival defects were also observed with flock house virus, cricket paralysis virus, and nora virus oral infections (Ferreira et al., 2014).

Homologs of the Toll receptor also exist, numbered 2 through 9. These additional Toll receptors have been studied for their potential involvement in immune defense. As described above, Toll-7 had been implicated in inducing autophagy downstream of VSV and Rift Valley fever virus (Moy et al., 2013; Nakamoto et al., 2012). Additionally, Toll-2 gene silencing resulted in increased VSV infectivity of S2 cells compared to control knockdowns (Nakamoto et al., 2012). To elucidate potential immune functions, the Toll receptors have been genetically modified by replacing their ectodomain with that of a constitutively active Toll mutant ectodomain. Only Toll-5 was observed to express the antimicrobial peptide drosomycin (Tauszig et al., 2000). In an independent study, Toll-3, Toll-5 and Toll-9 induced antimicrobial peptides (Ooi et al., 2002). Although a second paper confirmed an immunological role for Toll-9 (Bettencourt et al., 2004), a third study suggested no defects were present in Toll-9 mutant flies (Narbonne-Reveau et al., 2011). Toll-2 mutants are defective in activating the transcription factor DIF for expression of the antimicrobial peptide attacin (Williams et al., 1997). In trachea, Toll-8 had been found to negatively regulate antimicrobial peptide production (Akhouayri et al., 2011).

In addition to their potential roles in immune defense, the Toll receptors regulate development and mediate cell adhesion. Toll-2 has been implicated in the development of legs, salivary glands, fat body, and follicle cells (Eldon et al., 1994; Kleve et al., 2006; Kolesnikov and Beckendorf, 2007; Ligoxygakis et al., 2002). Toll-8 is involved in neuronal glycosylation and leg development, and it antagonizes the function of decapentaplegic during wing formation (Kim et al., 2006; Seppo et al., 2003; Yagi et al., 2010). Toll-6 and Toll-7 have been found to cooperate in the wiring of the olfactory system (Ward et al., 2015). Only Toll-4 has no identified function. Given that Toll signaling has dual roles in development and immunity, the other Toll receptor family members might have undiscovered functions. Even for Toll, Toll-2, and Toll-7, which have already been implicated in antiviral immunity, require further study to understand their mechanisms of action.

IMD

The IMD pathway is another *Drosophila* immune defense mechanism. IMD signaling confers protection against bacterial pathogens by activating the transcription of antimicrobial peptides (Lemaitre et al., 1995). In addition to its role during bacterial infection, the IMD pathway also affects the health of animals at steady state. In the *Drosophila* gut, the IMD pathway maintains homeostasis of the barrier epithelium with its associated commensal bacteria (Bosco-Drayon et al., 2012; Ryu et al., 2008). The IMD pathway also mediates a similar role in maintaining the health of epithelia of the trachea, the respiratory tract of insects (Tzou et al., 2000). In the nervous system, some IMD pathway components such as DREDD and relish have been implicated in mediating neurodegeneration (Cao et al., 2013; Chinchore et al., 2012; Petersen et al., 2013). Hyperactive signaling of the IMD pathway associated with aging can lead to shortened lifespan, similar to chronic inflammation in mammals (Landis et al., 2004; Pletcher et al., 2002; Valtonen et al., 2010; Zerofsky et al., 2005). Thus, the IMD pathway is important during both healthy and diseased states.

Sensing of bacteria triggers a signaling cascade resulting in the activation of Relish, an NF-κB-like transcription factor. To initiate the IMD signaling pathway, the receptors PGRP-LE and PGRP-LC detect bacteria-derived peptidoglycans (Kaneko et al., 2006; Lim et al., 2006; Stenbak et al., 2004; Takehana et al., 2002, 2004; Werner et al., 2000). PGRP-LC has multiple splice variants, resulting in

transmembrane proteins that all have the same cytoplasmic and transmembrane domains but different PGRP ectodomains (Werner et al., 2003). Unlike PGRP-LC, PGRP-LE is not anchored to the plasma membrane (Kaneko et al., 2006; Werner et al., 2000). Intracellular bacteria such as *Listeria monocytogenes* can be detected by PGRP-LE, but the detection of extracellular bacteria by PGRP-LE can also occur via unclear mechanisms (Kaneko et al., 2006; Yano et al., 2008). Recognition of bacterial ligands by PGRP-LC or PGRP-LE leads to the activation of IMD, although how signal transduction from the receptor to IMD occurs is unclear. Homotypic death domain interactions between IMD and dFADD lead to dFADD activation (Naitza et al., 2002). dFADD then recruits and activates the caspase DREDD (Hu and Yang, 2000). Activated DREDD has proteolytic activity that cleaves IMD and the inhibitory domain of Relish (Erturk-Hasdemir et al., 2009; Leulier et al., 2000; Paquette et al., 2010; Stoven et al., 2003; Stöven et al., 2000). The cleaved IMD protein proceeds to activate the mitogen activated protein kinase (MAPK), Jun kinase (JNK), via dTAK1. Cleaved Relish, which is released from its inhibitory domain, translocates to the nucleus to induce gene expression (Silverman et al., 2003).

Although the IMD pathway has been mainly studied for its antibacterial properties, this pathway may also serve as an antiviral defense. Flies with genetic mutations in *relish, PGRP-LC, dTak1, ird5,* and *kenny,* all of which are in the IMD pathway, were more susceptible to cricket paralysis virus infection (Costa et al., 2009). The IMD pathway also restricts sindbis virus (SINV) replication (Avadhanula et al., 2009). In a subsequent study, diptericin B, an antimicrobial peptide induced by the IMD pathway, protected against SINV infection (Huang et al., 2013). Whether additional genes regulated by the IMD pathway also conferred resistance was unclear, and the mechanism of how diptericin B inhibited infection remains to be examined. A conflicting report did not find kenny and IMD mutant flies to have decreased survival compared to wild type flies during SINV infection (Lamiable et al., 2016b). Contrary to previous work, a knockout of diedel, a negative regulator of the IMD pathway, resulted in decreased survival of SINV infected flies (Lamiable et al., 2016b). This finding would suggest that activating the IMD pathway during SINV infection results in poorer outcomes for the flies. However, a group of insect DNA viruses were found to encode genes homologous to diedel, a negative regulator of the IMD pathway (Lamiable et al., 2016b). The existence of viral mimics would suggest that the pathway to which the host gene belongs

is important in regulating viral infection. Although the IMD pathway seems to have an effect on viral infections, the consequences of IMD signaling are not entirely clear and may be virus-specific.

Similarities between the *Drosophila* immune system and the mammalian innate immune system

As described above, *Drosophila* have numerous ways of fighting pathogens. Some of these disease resistance mechanisms are evolutionarily conserved across species. Mammals such as humans and mice share some of these disease resistance mechanisms in common with flies. Comparisons across species offer the opportunity to determine the most important features and strategies of the immune system. Also intriguing are the disease resistance mechanisms that have not been conserved between flies and mammals.

For example, the *Drosophila* Toll pathway shares similarities with its mammalian counterpart, the Toll-like receptors (TLRs). Broadly, both are used for the detection of microbes and result in the activation of NF-κB proteins to initiate a transcriptional immune response. TLRs are expressed on the plasma membrane or endosomal compartments, directly detecting pathogen associated molecular patterns (PAMPs). In contrast, only Toll has been well-characterized for its immune function, and detection of PAMPs by *Drosophila* Toll is not direct. Instead, an upstream receptor detects PAMPs and subsequently promotes the cleavage of the pro-form of spatzle by the spatzle processing enzyme in order to activate the Toll receptor. Thus, the *Drosophila* Toll receptor signaling pathway involves upstream mediators which are not involved in mammalian TLR signaling.

Downstream of TLRs are adaptor proteins that recruit kinases and additional factors, forming a supramolecular organizational center (SMOC) that initiates inflammatory and defense-associated gene expression (Kagan et al., 2014). *Drosophila* Toll also requires adaptors, dMyD88 and tube, and a kinase, pelle. The mammalian and insect systems are similar in that they require protein-protein interactions mediated by protein domains such as the TIR and death domains. Interestingly, the functionality of the adaptor molecules seems to be conserved across species. TIRAP is a mammalian adaptor molecule localized to the plasma membrane and endosomes via lipid interaction while MyD88 is cytoplasmic (Bonham et al., 2014; Kagan and Medzhitov, 2006). In the *Drosophila* system, dMyD88 is localized to the plasma membrane and tube is cytoplasmic (Marek and Kagan, 2012). Although the composition of

structural domains between proteins in the mammalian and insect systems differ, the localization of the adaptor molecules and their recruitment of downstream kinases are conserved.

TLR signaling differs from *Drosophila* Toll signaling in that MAPKs are activated in mammals (Brown et al., 2011; Wang et al., 2001). No MAPK pathway has been identified to be initiated downstream of *Drosophila* Toll signaling. However, MAPKs are indeed part of the response to pathogens in flies. In the IMD pathway, dTAK1 mediates signaling to JNK (Silverman et al., 2003). Another MAPK pathway, ERK, is involved in defense against VSV infection via the oral route (Xu et al., 2013). Thus, MAPK signaling is involved in the immune response in both insects and mammals but the signaling pathways might not function identically.

While TLRs survey the extracellular environment for potential pathogens, PRRs also exist inside the cytoplasm. One such family is the NOD-like receptor (NLR) family. Some NLRs are involved in recognizing PAMPs and other danger signals such as potassium efflux (Muñoz-Planillo et al., 2013). Upon activation, PAMP-detecting NLRs oligomerize, forming specialized SMOCs called inflammasomes that ultimately lead to caspase activation. Caspases cleave proteins such as the cytokine pro-IL-1ß which is then secreted from the cell to initiate an inflammatory response in the tissue. Although caspases exist in *Drosophila* and are involved in apoptotic cell death, NLRs do not exist in *Drosophila.* It is interesting to note that receptors upstream of the Toll receptor are responsible for direct detection of PAMPs. Their activation leads to the cleavage of spatzle which then initiates Toll signaling. The cascade of events leading to spatzle cleavage is reminiscent of the activation of IL-1ß in mammals. Although spatzle activation occurs in the extracellular space and not in the cytoplasm, the two pathways are reminiscent of each other. However, similarities between PGRPs and NLRs remain to be seen. For example, whether PGRPs oligomerize like inflammasomes is unknown.

Another family of mammalian cytoplasmic receptors is the RIG-I-like receptor (RLR) family. The RLR family includes RIG-I, MDA5, and LGP2 receptors. RIG-I and MDA5 recognize viral RNA, and LGP2 is a proposed regulator of signaling (Rothenfusser et al., 2005; Satoh et al., 2010; Venkataraman et al., 2007). Upon recognition of viral RNA, RLRs bind to the adaptor protein MAVS which is localized on mitochondria and peroxisomes (Dixit et al., 2010; Seth et al., 2005). MAVS then recruits downstream signaling mediators which ultimately lead to the activation of the transcription factors IRF3, IRF7, and NF-

κB (Kawai et al., 2005). Activation of the RLR signaling pathway induces a transcriptional response that produces Type I interferons. In *Drosophila*, no orthologs of MAVS, IRF3, or interferons have been identified. While the RLR pathway is absent in flies, one group has drawn a comparison between Dcr2 and the RLR family. They each have a DExD/H-box helicase domain and are closely related (Deddouche et al., 2008). Their common phylogenic clade also includes drh1 which is a dicer protein that mediates antiviral immunity in *Caenorhabditis elegans* (Deddouche et al., 2008; Guo et al., 2013; Lu et al., 2009). All these related proteins are involved in antiviral immunity in their respective species. Thus, the antiviral siRNA pathway in *Drosophila* might have some relationship to the mammalian RLR family.

The relationship between Dcr2 and RLR family proteins has been one aspect of a larger mystery. While RNAi has been established as an antiviral mechanism in plants, fungi, and arthropods, the relative importance of RNAi as an antiviral defense in mammals has been debated (Cullen et al., 2013). One group found that in the absence of Drosha in mammalian cells, SINV titers increased (Shapiro et al., 2014). This effect was independent of Dicer and of type I interferons (Shapiro et al., 2014). The absence of a role for Dicer would suggest a different mechanism from how RNAi antiviral defense is mediated in *Drosophila* and other species. Another study found that mice lacking Dcr1 were more susceptible to VSV infection than wild type mice (Otsuka et al., 2007). The researchers found that the susceptibility was due to a loss of miR24 and miR93 expression, which targeted VSV L transcripts, and that Dcr1 did not produce small RNAs from viral transcripts (Otsuka et al., 2007). In the case of VSV infection, the protection conferred by Dcr1 seems more happenstance instead of being a targeted mechanism directed against the virus. Several studies using embryonic cells do support the idea of RNAi as an antiviral response in mammals. Early studies found that long dsRNA could be substrates for producing siRNA in embryonic cells lines (Billy et al., 2001; Paddison et al., 2001). Later studies also supported the idea that embryonic cells could utilize the RNAi pathway as an antiviral defense. Those studies additionally demonstrated that virus-encoded B2 protein could suppress host production of siRNA (Li et al., 2013; Maillard et al., 2013). However, studies have not extended these findings to prove any significant relevance to differentiated cells. Indeed, others have noted that RNAi seems to function in cell types that have attenuated type I interferon responses (Pare and Sullivan, 2014). In agreement with this hypothesis, another publication showed that RISC is modified via poly-ADP-ribosylation shortly after infection,

inhibiting its silencing function in somatic cells (Seo et al., 2013). In fact, ISG induction seemed to be acutely regulated by RNAi, suggesting an antagonistic relationship between the two (Seo et al., 2013). In support of the idea that RNAi and interferon signaling antagonize each other, a more recent publication took a different approach of introducing *Drosophila* siRNA genes into mammalian cells. Expression of *Drosophila* Dcr2 in HEK293 cells resulted in the production of viral RNAs of 21-nucleotide length, but viral replication was not suppressed (Girardi et al., 2015). Instead, *Drosophila* Dcr2 expression resulted in disruption of interferon signaling (Girardi et al., 2015). Thus, if the RNAi pathway is an antiviral defense mechanism in mammals, it seems to be most relevant in cases where the interferon signaling pathway is attenuated.

While RNAi has been a point of contention, DNA sensing has barely been compared between *Drosophila* and mammals. DNA virus infection is restricted by RNAi in *Drosophila* (Bronkhorst et al., 2012). As discussed, however, RNAi largely does not seem to operate in mammals as an antiviral defense. In contrast, detection of pathogen-derived DNA in the cytoplasm of mammalian cells is mediated by the cGAS/STING pathway (Chow et al., 2015). DNA in the cytoplasm is directly sensed by cGAS, resulting in the production of the cyclic dinucleotide cGAMP (Sun et al., 2013; Wu et al., 2013). Binding of cGAMP to STING, localized to the ER, results in a signaling cascade that activates IRF3. Like in RLR signaling, Type I interferons are produced, leading to the induction of interferon-stimulated genes and the promotion of an antiviral state (Sun et al., 2013). Again, Type I interferons do not exist in *Drosophila*. Whether any orthologous genes in the cGAS/STING pathway exist in fruit flies and whether they function in antiviral defense remains to be reported.

Overall, the main area of overlap between the *Drosophila* immune system and the mammalian innate immune system has been the Toll and TLR pathways. The defined mediators of Toll signaling function similarly to TLR signaling, but further comparisons between the two pathways hinge upon further study of the *Drosophila* system. The same is true for other innate immune pathways because the mammalian system is better defined. We have an increasing knowledge of RLRs, CLRs, NLRs, and the cGAS/STING pathway, but our knowledge of other innate immune systems is lagging. By understanding the immune strategies utilized by fruit flies, the field can move beyond the question of what immune

defenses exist. Having the ability to compare different immune systems will allow us to start tackling the questions of what immune defenses are most effective and why.

Disease Tolerance

While host-pathogen interactions have mainly focused upon disease resistance mechanisms, as described above, alternative strategies exist for a host to mitigate an infection. Instead of applying mechanisms to restrict the growth of a pathogen, an infected host can employ disease tolerance mechanisms. Disease tolerance is the idea of altering homeostatic conditions to improve the health of the host without necessarily reducing the pathogen load. To differentiate disease resistance and disease tolerance one must recognize that the health of the organism is not always directly dependent upon pathogen load. Instead, the health of an organism is dependent upon its ability to mitigate the effects of damage and stress.

The idea of disease tolerance is a budding field that originally stemmed from studies of plant biology (Raberg et al., 2009). In one of the early studies to note disease tolerance, several varieties of winter wheat were compared for their yield, physical characteristics, and chemical composition when grown in the face of infection by the fungus *Puccinia triticina*. One particular variety of wheat, Fulhard, was noted to have comparable levels of infection as other varieties but still had the best grain yield (Caldwell et al., 1934). The observation was confirmed by a separate group that also subjected the Fulhard wheat, along with other varieties of wheat, to *P. triticina* infection (Salmon and Laude, 1932). What was keenly noted by these researchers was that some varieties had reduced fungal infection and resultantly healthier crops (disease resistance). Meanwhile, the Fulhard variety was struck with the highest pathogen load but still managed to yield the most grain (disease tolerance). Although the exact mechanism for disease tolerance was not identified, the hallmarks of disease tolerance were identified, namely the modulation of health without a dependency on changing pathogen load.

Some noteworthy principles about disease tolerance have arisen from plant studies. Plants have been noted for disease tolerance strategies such as increasing photosynthesis, increasing growth, and taking up more nutrients (Redondo-Gomez, 2013). These strategies help to offset the damage caused by a pathogen by increasing nutrient production and availability. Thus, disease tolerance is focused upon

mitigating the effects of tissue damage. Another important lesson about disease tolerance taken from plant studies is that stresses that result in damage can come from biotic and abiotic sources (Redondo-Gomez, 2013). Infection by pathogens such as fungi would fall in the category of biotic stress. Also within the category of biotic stresses would be herbivores, such as insects, that might consume parts of the plant. Interestingly, disease tolerance can be broadened in scope to include mechanisms that deal with abiotic stresses. Abiotic stresses would include environmental stresses such as high/low temperatures, salinity, heavy metal exposure, ultraviolet light damage, and drought. By noting that pathogenic organisms are only one category to induce disease tolerance mechanisms, one is able to better appreciate that disease tolerance is about responding to damage caused by a stress, regardless of whether it is biotic or abiotic.

From plant biology, the concept of disease tolerance has spread into studies in the animal kingdom. Disease tolerance was first noted in the study of *Plasmodium chabaudi* infection of mice (Råberg et al., 2007). Several inbred strains of mice were infected with *P. chabaudi*, and red blood cell count and animal weight were used as indicators of organismal health (Råberg et al., 2007). Pathogen load was measured and correlated to red blood cell count and animal weight. Depending on the mouse strain, the same pathogen load resulted in differing effects on red blood cell count and weight, suggesting that disease tolerance mechanisms varied from one mouse strain to the other (Råberg et al., 2007).

Two other early studies confirming disease tolerance in animals utilized *Drosophila*. In one, a forward genetic screen was performed to find genes important for survival during *Listeria monocytogenes* infection (Ayres et al., 2008). When mutated, one category of identified genes resulted in poor survival without changes in bacterial growth, indicating disease tolerance pathways had been impaired (Ayres et al., 2008). In the second study, infection by *Wolbachia* bacteria altered survival outcomes of flies additionally infected by virus (Teixeira et al., 2008). In the case of flock house virus infection, elimination of *Wolbachia* resulted in poorer survival while viral titers were minimally affected. IIV-6 had the opposite result. When *Wolbachia* was removed from flies by tetracycline treatment, IIV-6 infected flies had improved survival (Teixeira et al., 2008). While the effects of *Wolbachia* on flies can be complicated, the lack of significant change in viral titers would suggest that *Wolbachia* alters disease tolerance pathways that are relevant to virus infection.

Wolbachia affecting disease tolerance such that the host is more susceptible to an additional pathogen is not a unique situation. A similar effect has been observed in mice co-infected with *Legionella pneumophila* and influenza virus. While an infection of either pathogen can be administered to mice at a sublethal dose, the respective sublethal doses becomes lethal in combination (Jamieson et al., 2013). Co-infection did not affect pathogen load in comparison to single infections, suggesting that mortality was a result of a deficiency in disease tolerance. Indeed, Jamieson and colleagues found that administration of amphiregulin, an epithelial growth signal, rescued co-infected mice from mortality, suggesting that increased tissue regrowth is a disease tolerance mechanism (Jamieson et al., 2013). As a proof of concept, Jamieson and colleagues demonstrated that two biotic stressors can be used in combination to reveal disease tolerance mechanisms.

Damage to the host does not necessarily need to be induced by multiple stressors. In some situations, such as *Plasmodium chabaudi* infection, damage can occur directly from one pathogen. *P. chabaudi* lyses red blood cells as part of its life cycle. Seixas and colleagues demonstrated that heme released from damaged red blood cells sensitizes hepatocytes to TNF-mediated cell death, resulting in liver failure and death (Seixas et al., 2009). The group was able to provide mechanistic insight as to how heme-oxygenase-1, produced by hepatocytes, can serve as a disease tolerance mechanism by catabolizing free heme. When mice lacked heme-oxygenase-1, infected mice suffered from increased mortality without any effects on pathogen load (Seixas et al., 2009). Thus, heme-oxygenase-1 was important for reducing circulating heme liberated by *P. chabaudi* infection. Disease tolerance mechanisms can target damage caused directly by a pathogen.

Disease tolerance mechanisms can also target damage caused by the host immune response (immunopathology). A prime example of disease tolerance targeting immunopathology is the situation of sepsis. During sepsis, an overabundance of inflammatory cytokines is produced, leading to multiple organ failure and death. Using mice as an experimental model, one can induce septic shock and subsequent death by injecting a large amount of lipopolysaccharide (LPS) into mice. However, a tolerogenic effect can be induced if the mice are first injected with a sublethal dose of LPS (Dobrovolskaia and Vogel, 2002). In one mechanistic study, LPS tolerance was traced to epigenetic changes that inhibited expression of inflammatory genes (Foster et al., 2007). In this example of disease tolerance, the stress (a

lethal dosage of LPS) is not changed in dosage, and in contrast to a live pathogen, LPS is not directly cytopathic. Epigenetic changes induced by a sublethal LPS dose prevent a cytokine storm and subsequent death. The example of LPS tolerance demonstrates that disease tolerance mechanisms can target damage that is self-inflicted.

Disease tolerance mechanisms are not always separate from disease resistance mechanisms. Sometimes, a cellular pathway can serve as either, depending on the context. Autophagy is a disease resistance mechanism, reducing pathogen load for the benefit of host survival (Levine et al., 2011). In some instances, the pathogen is observed to be destroyed by autophagy (Gomes and Dikic, 2014). However, autophagy can also serve as a disease tolerance mechanism. In *Staphylococcus aureus* infection of mice, α-toxin produced by the bacterium can bind to the protein ADAM10, inducing integrin cleavage that leads to destruction of epithelial and endothelial barrier integrity (Inoshima et al., 2011; Powers et al., 2012). A hypomorph mutation in ATG16L, resulting in reduced autophagic activity, led to an accumulation of ADAM10 and increased mortality when mice were infected with *S. aureus* expressing α-toxin (Maurer et al., 2015). By reducing ADAM10 protein expression, autophagy minimized endothelial barrier destruction and maintained survival of the host (Maurer et al., 2015). Thus, depending on the context, a cellular process can serve as a disease resistance mechanism or a disease tolerance mechanism.

So far, the examples of disease tolerance mechanisms that have been described above seem very specific to the pathogen/stress. However, this may be due to the fact that only a handful of disease tolerance mechanisms in animals have been identified to date. Disease tolerance mechanisms might be more broadly applicable to multiple pathogens. One potential example comes from studies of food supplementation or restriction of infected animals. Depending upon the infection, food restriction versus supplementation can have profound effects upon host survival. In flies infected with *Salmonella typhimurium*, food restriction improved survival of infected flies without an effect on bacterial load, suggesting activation of a disease tolerance mechanism (Ayres and Schneider, 2009). Interestingly, food restriction during *L. monocytogenes* infection resulted in worse survival outcomes for flies because pathogen resistance weakened, again demonstrating that disease tolerance and resistance can be affected by the same stimulus depending on context (Ayres and Schneider, 2009). In the case of mice

however, forced consumption of food, or simply just glucose supplementation, during *L. monocytogenes* infection resulted in poorer survival outcomes (Wang et al., 2016). The decreased survival was not due to changes in bacterial load or increased inflammatory cytokine production, and the result could be replicated simply using an LPS sepsis model as well (Wang et al., 2016). In contrast to bacterial infection, influenza challenge or Poly(I:C) injection (simulating a viral infection) resulted in improved survival outcomes when mice were force fed (Wang et al., 2016). Viral titers were unaffected by dietary supplementation. The dichotomous outcomes of food intake during bacterial versus viral infection in mice suggest that disease tolerance mechanisms can apply to multiple pathogens, albeit with different outcomes. However, multiple underlying molecular mechanisms of dietary restriction/supplementation might exist, and that is important to note. This possibility is suggested by the differing results observed in mice and flies.

Disease tolerance is slowly gaining recognition as an important feature of host-pathogen interactions, but the biggest challenge thus far has been the identification of disease tolerance mechanisms. Genetic tools such as CRISPR/Cas9 are improving the ability to manipulate model organisms such as mice and flies. However, host-pathogen interactions can be complex. Mammalian organisms such as mice have the innate and adaptive arms of the immune system that function coordinately, utilizing cross-talk among many cell types. Pathogens often have strategies to evade the immune system and manipulate the host to their advantage. For disease tolerance models utilizing two pathogens, the complexity is increased. Future work identifying disease tolerance mechanisms could greatly benefit from simple host-pathogen models with easy-to-manipulate genetics.

Anesthesia sensitivity in virus-infected *Drosophila* as a disease tolerance model

Drosophila melanogaster is a genetically tractable model organism that could be a strong candidate to study disease tolerance, as others are already doing (Ayres and Schneider, 2009, 2012; Ayres et al., 2008). One particular line of studies dating back to the 1930s is especially promising as a model for studying disease tolerance. A historical account of these studies follows with justification as to why disease tolerance mechanisms may be applicable.

In the 1930s, a group of French *Drosophila* researchers noticed a stock of flies had an abnormal reaction to anesthesia (L'Heritier, 1948). Normally when working with fruit flies, they must be anesthetized so that they will not escape when flies are being selected for genetic crosses. Flies are exposed to defined gases and will become paralyzed temporarily. Carbon dioxide (CO_2) is often used as an anesthetic, and flies can be placed in a pure CO_2 environment for prolonged periods (hours) without any resulting defects in survival (L'Heritier, 1951). After being returned to a normal air environment, anesthetized flies will regain coordinative function. One particular stock, however, exhibited lasting paralysis. After being anesthetized and returned to a normal air environment, the researchers noticed that the flies would thrash their legs uncontrollably and were incapable of regaining full coordinative function. These flies would shortly die thereafter. Particularly odd was that these anesthesia-sensitive flies did not exhibit any behavioral abnormalities prior to anesthesia exposure.

One feature noticed was that the paralytic phenotype was heritable to some extent (Seecof, 1962). CO_2 sensitive flies can be categorized as stabilized or non-stabilized strains (Brun and Sigot, 1955). Females from stabilized strains passed anesthesia sensitivity to all their progeny when mated to insensitive flies. In the case of males from stabilized strains, progeny sometimes had CO_2 sensitivity, but the frequency was variable. Non-stabilized strains of flies do not always pass on CO_2 anesthesia sensitivity when mated to insensitive flies. Inheritance of the paralytic phenotype did not seem to follow a Mendelian pattern of inheritance, and the CO_2 sensitivity phenotype could be passed to insensitive flies via purified extracts (L'Heritier, 1951). Thus, the researchers concluded that the trait was episomal and potentially a virus. Eventually, they demonstrated that the episomal trait that could be vertically transmitted was a virus, which they named Sigma. Thus, anesthesia, an abiotic stress normally tolerated by fruit flies, becomes life threatening after viral infection, an additional biotic stress. Two non-lethal stresses in combination resulted in paralysis and lethality. This combination of stressors resulting in lethality is reminiscent of other disease tolerance models, such as bacterial and viral co-infections in mice and flies.

Microscopic images of Sigma virus showed bullet-shaped viral particles, a hallmark shape indicative of belonging to the rhabdovirus family (Berkaloff et al., 1965). Additional *Rhabdoviridae* viruses were used to infect flies to test if they also resulted in anesthesia sensitivity. Two fish rhabdoviruses,

spring viraemia of carp and pike fry rhabdovirus, were infectious in *D. melanogaster* and caused CO_2 sensitivity (Bussereau et al., 1975). Chandipura and piry, rhabdoviruses that can infect mammals, could also infect *D. melanogaster* and elicit sensitivity to CO_2 (Bussereau, 1975). Additionally, infection by any strain of VSV caused CO_2 sensitivity in flies (Bussereau, 1973). Additional insects such as mosquitoes were tested for CO_2 anesthesia sensitivity. *Aedes albopictus*, *Toxorhynchites amboinensis*, and *Culex quinquefaciatus* became sensitive to CO_2 anesthesia after infection with rhabdoviruses (Rosen, 1980). *Aedes melanimon*, *Aedes dorsalis*, *Aedes triseriatus*, and *Culex tarsalis* mosquitoes displayed sensitivity to CO_2 anesthesia after infection with the bunyavirus California encephalitis virus (Turell and Hardy, 1980). Wild mosquito populations can also exhibit CO_2 sensitivity (Rosen and Shroyer, 1981). In addition, wild populations of different fly species, *Drosophila affinis* and *Drosophila obscura*, were found to naturally harbor sigma infections adapted to their respective species (Longdon et al., 2011). Many American species of *Drosophila* captured from the wild had a percentage of flies exhibiting sensitivity to CO_2 anesthesia (Williamson, 1961). Thus, the sensitivity to anesthesia is relevant to multiple species of insects, and the phenotype could be generalizable to multiple viruses.

With Sigma infection, virus was detected in the gametes, explaining its heritability. Further study demonstrated that the virus also infected other parts of the host. Interestingly, a correlation was observed such that sensitivity to anesthesia seemed to be related to infection of the thoracic ganglion. The thoracic ganglion is a group of neurons running through the thorax of the animal, akin to a spinal cord for mammals. Infection of the nervous system could help explain the paralytic nature of the phenotype observed. However, the causative effect of anesthesia sensitivity was not proven and remains an open area for further study. Also, the specificity of effects to the particular cell type of neurons hints to a similarity to other disease tolerance models. For example, *Plasmodium* infection and lysis of red blood cells causes hepatocyte toxicity (Seixas et al., 2009). When disease tolerance is lost, the induced damage is often specific to a particular cell type.

Because *Drosophila* are such genetically tractable organisms, a screen was carried out to identify host genetic determinants leading infected animals to become sensitive to anesthesia (Gay, 1978). Five genes were identified (Gay, 1978). Of the genes identified, *ref(2)P* was the best studied, and it was sequenced (Dezelee et al., 1989; Nakamura, 1978; Wyers et al., 1993). Depending on the allele, flies

were more or less refractory to Sigma infection. When virus replication was restricted by a specific *ref(2)P* allele, infected flies were no longer sensitive to anesthesia (Nakamura, 1978). Thus, the relationship between virus replication and anesthesia sensitivity was more clearly delineated. However, the exact function of these identified genes remains unknown.

Unfortunately, further studies were not carried out. An understanding of anesthesia sensitivity at the molecular level remains to be elucidated. As a potential model for disease tolerance, it is of particular interest. A viral pathogen in combination with an abiotic stress (anesthesia) results in paralysis and death. Healthy flies are tolerant of CO_2 anesthesia, but it is unclear how viral infection alters the host such that anesthesia becomes deadly. How is tolerance to anesthesia lost? Combining viral infection and CO_2 anesthesia is potentially an ideal model for studying disease tolerance. CO_2 is an abiotic stress which might be less complicated than an additional, live pathogen. Also, rhabdoviruses are relatively simple viruses, and VSV is particularly an ideal candidate virus to use because it has been extensively studied.

Vesicular stomatitis virus

VSV is a non-segmented, negative-sense, single-stranded RNA virus belonging to the *rhabdoviridae* family. In humans, infection can be asymptomatic, but the infection of livestock such as pigs, horses and cattle can result in pathology indistinguishable from foot-and-mouth disease. Lesions form on the lips, gums, and tongue and affect eating negatively. Lesions on the feet of animals can cause lameness. The virus is believed to be transmitted to livestock by sand flies (Tesh et al., 1987). In addition to these natural hosts for the virus, VSV has been used experimentally in model organisms such as nematodes, fruit flies, and mice (Shelly et al., 2009; Trottier et al., 2005; Wilkins et al., 2005). The breadth of cell types and organisms which VSV can infect has made it a widely-studied prototypic virus for the *Mononegavirales* order to which it belongs. This order of viruses is a burden on human health as viruses such as ebola, measles, mumps, and rabies belong to it. Additionally, VSV can be genetically engineered, which is challenging with other viruses in the same order. Thus, VSV is a genetically tractable virus, representing a larger group that significantly affects humans.

Part of why VSV has been extensively used is because it infects a broad range of cell types. The broad infectivity range would suggest that the cellular receptor used by VSV to attach must be broadly

expressed. The attachment of VSV particles to cells has been proposed to be mediated by VSV G, expressed on the virus particle envelope, interacting with phosphatidylserine (Schlegel et al., 1982, 1983). However, the specificity of phosphatidylserine in mediating cell entry has been debated (Coil and Miller, 2004). The LDL receptor family has also been proposed as mediating viral attachment to cells (Finkelshtein et al., 2013). Another possibility is that the virus nonspecifically interacts with the cell plasma membrane via electrostatic and hydrophobic interactions (Bailey et al., 1984). Regardless of which interactions allow for viral attachment to a cell, additional steps must occur in order for VSV to gain entry into a cell. The viral particle is endocytosed via clathrin-mediated endocytosis (Cureton et al., 2009). However, a clathrin-coated pit is unable to fully form around a VSV particle. After a clathrin-coated pit partially forms, actin helps mediate the internalization of the vesicle (Cureton et al., 2009). Once internalized, the clathrin coat is lost as it traffics through the endosomal pathway.

Inside the endosomal compartment, fusion of the viral envelope to the cellular membrane is dependent upon pH. During trafficking, the endosomal compartment becomes progressively more acidic as it fuses with lysosomes to degrade the contents. VSV G, which functions as noncovalent homotrimers, undergoes a radical conformational change when pH decreases below 6.5 (Bailey et al., 1984; Gaudin et al., 1992; Roche et al., 2006, 2007; Whitt et al., 1991). This conformation change by VSV G mediates viral engagement with the host membrane so that the viral nucleocapsid can be released into the cytoplasm.

The RNA genome of VSV is bound to approximately 1200 copies of VSV N, each of which covers nine nucleotide bases (Green et al., 2000; Thomas et al., 1985). Additionally, 466 copies of VSV P and 50 copies of VSV L are associated with the nucleocapsid (Thomas et al., 1985). In order for VSV L, the RNA-dependent RNA polymerase, to transcribe the virus genome, the RNA genome must be associated with these viral proteins. Naked RNA cannot be transcribed (Banerjee, 1987; Emerson and Yu, 1975). Viral transcription is a necessary first step after entry because of the negative polarity of the viral genome. Unlike positive-stranded viruses, the VSV genome cannot directly serve as an mRNA template that is directly translated by ribosomes. The viral polymerase must use the genomic RNA as a template for transcribing viral mRNA.

VSV shares its gene order in common with all other viruses in the *Rhabdoviridae* family. VSV N, P, M, G, and L are encoded from 3’ to 5’ on the negative strand. While other viruses within the family may encode additional genes in between these five, the organization of these five genes with respect to each other remains unchanged. The genomic organization is particularly important because the level of expression of each gene is dependent upon placement. VSV N is the first gene to be transcribed and capped and methylated at its 5’ end (Abraham et al., 1975). After the addition of a poly-A tail, the mRNA is released. The viral polymerase sometimes dissociates, but more often it proceeds to initiate transcription of the next downstream gene, resulting in a 20-30% reduction in transcription of the downstream gene relative to the gene just transcribed (Iverson and Rose, 1981; Villarreal et al., 1976; Wertz et al., 1998). This reduction in transcription occurs with each successive gene, resulting in VSV N being the most transcribed and VSV L the least. The attenuation of transcription of VSV L after VSV G is particularly drastic compared to the reduction that occurs at other gene junctions although it is unclear why (Ball et al., 1999). The genes are separated by a transcription termination sequence, a spacer sequence, and a promoter. These junctional sequences are the same between each gene. The gene order and abundance of transcripts is vitally important to successful viral replication. When the gene order has been intentionally rearranged in experimental studies, VSV was severely hindered in its replication and pathogenesis (Ball et al., 1999; Wertz et al., 1998).

Transcription of the viral genome leads to the accumulation of mRNA transcripts. The transcripts are translated by the host ribosomal machinery, leading to the accumulation of viral proteins. The binding of VSV N to the nascent transcript switches the viral polymerase from transcribing mRNA to transcribing the antigenome for the purpose of producing more genomic RNA (Patton et al., 1984). VSV P is also instrumental in maintaining the proper folding of VSV N during this process of encapsidating the transcript (Davis et al., 1986; Majumder et al., 2001; Masters and Banerjee, 1988; Masters and Banerjeet, 1988; Peluso, 1988; Peluso and Moyer, 1988). Additionally, the 3’ termini of the genome and anti-genome differ substantially at positions 19-29 and 34-46. These promoter sequences on the genome preference the production of viral mRNA (Li and Pattnaik, 1999; Whelan and Wertz, 1999). On the anti-genome, the sequences at these positions promote the replication of viral genomes, leading to an abundance of

genomes relative to anti-genomes in an infected cell (Finke and Conzelmann, 1997; Simonsen et al., 1979; Soria et al., 1974; Wertz, 1978).

Viral translation is compartmentalized depending upon the gene. In the case of VSV G, mRNA transcripts engage ribosomes at the rough ER. A signal sequence encoded by the nascent polypeptide facilitates translocation into the ER. VSV G is a Type I transmembrane protein where the amino terminus is found in the ER lumen, the carboxy terminus remains in the cytoplasm, and the protein has only one transmembrane domain (Rose and Gallione, 1981; Rose et al., 1980). After the amino terminus passes into the ER, the signal sequence is cleaved (Lingappa et al., 1978). Proper folding, disulfide linkage, and glycosylation are facilitated by the chaperone proteins BiP and calnexin and are required before transport into the Golgi (Doms et al., 1988; Hammond and Helenius, 1994; Machamer and Rose, 1988; Machamer et al., 1990). High mannose oligosaccharides are the first modifications made to VSV G in the ER, and further modifications are made as VSV G traffics through the Golgi (Reading et al., 1978). After proper folding and glycosylation in the ER is complete, VSV G monomers form trimers before passing to the Golgi (Doms et al., 1988). In the Golgi, the high mannose oligosaccharides are modified with N-acetyl glucosamine, galactose, and sialic acid (Reading et al., 1978). In addition to these ectodomain modifications, a fatty acid palmitate is attached to a cysteine in the cytoplasmic domain (Rose et al., 1980; Schmidt and Schlesinger, 1979).

After passing through the Golgi network, VSV G is transported to the plasma membrane. VSV G trimers cluster together at the plasma membrane (Brown and Lyles, 2003a, 2003b). Although these clusters are not associated with lipid rafts, they are enriched in cholesterol and sphingolipids (Luan et al., 1995; Pickl et al., 2001). The plasma membrane is the site where new VSV particles bud. Viral budding sites have been noted to have larger clusters of VSV G compared to VSV G clusters in non-budding areas (Brown and Lyles, 2003b, 2005). The association of VSV G with cholesterol and sphingolipids might be influenced by interactions with the viral nucleocapsid during the budding process (Brown and Lyles, 2005; Swinteck and Lyles, 2008). When VSV G is mutated or deleted, virus budding is reduced. However, VSV G is not essential for viral budding, and viral particles lacking VSV G can be produced (Knipe et al., 1977a; Mebatsion et al., 1996; Robison and Whitt, 2000; Schnell et al., 1997; Takada et al.,

1997). Sequences in the membrane proximal region of the ectodomain have been suggested to help facilitate the membrane curvature needed in virus budding (Robison and Whitt, 2000).

While VSV G is synthesized at the ER membrane and is modified as it traffics through the Golgi, other viral proteins are produced in the cytoplasm. VSV M is found in the soluble fraction of the cytosol, but a small amount is associated with negatively charged phospholipids at the plasma membrane (Flood et al., 2000; Knipe et al., 1977b; Luan and Glaser, 1994; Luan et al., 1995; Ogden et al., 1986; Ohno and Ohtake, 1987; Ono et al., 1987; Ye et al., 1994; Zakowski et al., 1981). Membrane-associated VSV M also forms clusters, but these clusters are distinct from the clusters formed by VSV G (Swinteck and Lyles, 2008). VSV M only binds nucleocapsid in the context of budding (McCreedy and Lyles, 1989; Odenwald et al., 1986; Ohno and Ohtake, 1987; Ono et al., 1987). Once the first copies of VSV M have bound to the nucleocapsid, additional copies of VSV M assemble around the nucleocapsid rapidly (Barge et al., 1993; Lyles and Mckenzie, 1998; Newcomb and Brown, 1981; Newcomb et al., 1982). The nucleocapsid and VSV M complex then buds from the plasma membrane by having VSV M interact with host proteins involved in multivesicular body formation (Harty et al., 1999; Irie et al., 2004a, 2004b; Jayakar et al., 2000).

The process of viral replication, from entry to budding, requires as little as 2 hours. The process is better understood in mammalian cells compared to insect cells. For example, in addition to the processes required for viral replication, VSV is known to inhibit host responses. VSV M inhibits host transcription and mRNA transport out of the nucleus, thereby blocking an IFN response (Ahmed and Lyles, 1998; Ahmed et al., 2003; Black and Lyles, 1992; Dunigan et al., 1986; Ferran and Lucas-Lenard, 1997; Lyles et al., 1996). Host responses to VSV have also been well-characterized in the mammalian system. For example, RIG-I detects VSV and other negative-sense viruses (Kato et al., 2005, 2006). TLRs can also sense VSV, but the cell types expressing these receptors are more restricted in comparison to most cells which express RIG-I (Diebold et al., 2004; Georgel et al., 2007; Lund et al., 2004b; Shi et al., 2011). The insect host response to VSV and related viruses is likely different from the mammalian host response because VSV is known to be cytopathic in mammalian cells but can survive as a persistent infection in *Drosophila* cells. Additionally, sigma virus, which is related to VSV, is transmitted vertically, suggesting that the host must survive with a persistent infection long enough to pass the virus along to offspring. The

interactions of VSV with insect hosts have not been as well-characterized despite the fact that VSV naturally infects sand flies. Additionally, other related viruses such as Chandipura virus and piry virus are transmitted via insect vectors. The opportunity remains to further our knowledge of a group of arthropod-borne viruses that affect public health and agriculture.

VSV is a prototypic virus ideal for such studies in insects. Methods to genetically modify the virus have already been established. In the process, useful recombinant viruses such as ones that express the reporter gene firefly luciferase have already been created (Cureton et al., 2009). Also, the viral genes have been characterized to an extent whereby making further progress is less challenging. For example, mutant VSV M virus has been produced with an inability to inhibit host transcription and mRNA nuclear export (Ahmed and Lyles, 1998; Ahmed et al., 2003; Black and Lyles, 1992; Dunigan et al., 1986; Ferran and Lucas-Lenard, 1997; Lyles et al., 1996). Crystal structures of VSV G are available (Roche et al., 2006, 2007). Thus, studying VSV in insects comes at an opportune moment.

Dissertation Objective

While the mammalian antiviral response has been a major focus of research, the insect response to viral infection has only begun to be studied. *Drosophila melanogaster* is a genetically tractable organism, making it an ideal host organism for scientific study. Historically, identification of genes in *Drosophila* has resulted in major discoveries that can be applied more broadly to other organisms (Anderson et al., 1985a). The insect immune system is also of interest in its own right because significant viral pathogens are transmitted via insect vectors. Additionally, viral pathogens have been proposed to affect bee populations, leading to their decline in recent years (Evans and Schwarz, 2011). These insects play a critical role in agriculture, pollinating crops. Thus, a mandate exists to study insects and their interactions with viral pathogens.

The work in this dissertation will predominantly focus upon VSV. It is a genetically amenable pathogen, and other non-segmented, negative-sense RNA viruses share similar replication strategies. Altogether, these viruses contribute a significant burden upon our lives, and thus VSV is an ideal pathogen to study. As a point of comparison, SINV will occasionally be used. As a non-segmented, positive-sense RNA virus, its replication cycle is different because viral proteins can be directly translated

from its genome. Non-segmented, positive-sense RNA viruses are also a significant burden upon human health as this group includes viruses such as Dengue fever, Rift Valley fever, and Chikungunya viruses. However, limiting the scope of this dissertation research was necessary, and VSV offered plenty of opportunities for new areas of study.

Chapter 2 of this dissertation will focus on studying disease resistance mechanisms of insects. Multiple genetic pathways have been implicated in reducing viral replication in *Drosophila*. Aside from RNAi, these antiviral mechanisms have been studied by a limited number of groups. Thus, evaluating the level of contribution of each antiviral mechanism is crucial. In Chapter 2, we will evaluate the effectiveness of identified antiviral pathways in resisting VSV infection. A secondary objective of Chapter 2 of this dissertation is to further evaluate the similarities shared between insects and mammals in their antiviral response. As described above, *Drosophila* Toll and the mammalian TLR system share similarities. While other antiviral pathways are significantly divergent, they do share some common themes. For example, the insect siRNA pathway has different effector outcomes compared to the mammalian RLR pathway, but they both utilize the strategy of sensing foreign nucleic acids via related helicase domains. The cGAS/STING pathway in mammals senses cytoplasmic DNA, and an orthologous STING gene is present in fruit flies. In Chapter 2, we will evaluate whether *Drosophila* STING (dSTING) similarly has antiviral function.

After evaluating the contributions of various antiviral defenses, Chapter 3 will establish a model for studying disease tolerance. As described above, disease tolerance mechanisms are another strategy infected hosts use to survive. These strategies rely upon mitigating stress and tissue destruction that arise during infection. Currently, too few studies have focused on disease tolerance to be able to draw major conclusions. For example, whether a few disease tolerance mechanisms are broadly applicable to many stresses and pathogens is unclear. Too few disease tolerance models currently exist. Chapter 3 will address this challenge by using VSV infection and CO_2 anesthesia as a method for modeling disease tolerance in *Drosophila*. The goal of Chapter 3 is to understand how VSV infection renders the host unable to tolerate the secondary, abiotic stress of CO_2.

Finally, we will contextualize my results within each chapter by comparing them to what has already been published. Chapter 4 then looks forward to determine significant areas for further research stemming from my data. Specific questions and potential methods for answering them will be described.

Chapter 2

Comparative analysis of antiviral mechanisms

Contributions: STING experiments were done in collaboration with Kate M. Franz, who obtained and verified gene disruption in mutant flies. Invertebrate iridescent virus-6 was obtained and grown by Kate M. Franz. All experiments shown were done by Jonathan Chow.

INTRODUCTION

At the beginning of my dissertation research, multiple publications had indicated that the siRNA pathway can restrict viral replication (Bronkhorst and van Rij, 2014). A few reports also suggested that autophagy and Jak/STAT signaling can function as an antiviral defense (Dostert et al., 2005; Moy et al., 2013; Nakamoto et al., 2012; Shelly et al., 2009). Finally, the Toll and IMD signaling pathways were also implicated in antiviral immunity in addition to their roles in anti-bacterial immunity (Costa et al., 2009; Zambon et al., 2005).

Although the siRNA pathway had been well-established to mediate antiviral immunity, two additional RNAi pathways exist in *Drosophila*. The miRNA pathway recognizes endogenously produced small RNAs and uses them to modulate endogenous gene expression. The piRNA pathway had been found to restrict transposons in the reproductive system from translocating and disrupting endogenous gene expression. The prevailing notion is that the pathways work distinctly from each. However, endogenous sources of siRNA have been identified (Czech et al., 2008). Additionally, accessory RNAi proteins such as R2D2 and loquacious have been implicated to function in multiple RNAi pathways (Czech et al., 2008; Marques et al., 2010). Whether these RNAi pathways can function redundantly as antiviral immune defenses is unclear.

Additionally, autophagy, Jak/STAT signaling, Toll signaling, and IMD signaling as antiviral defenses have thus far been poorly characterized. Many questions remain to be answered. How are viruses recognized? What genes are involved in the downstream signaling? How do these defense mechanisms restrict viral infection? Additionally, these immune defenses are compelling to study because parallel immune defenses are well-characterized in mammals. Autophagy restricts infections in mammals (Gomes and Dikic, 2014). Jak and STAT proteins mediate signaling in mammals to upregulate interferon stimulated genes, thus promoting an antiviral state. The mammalian Toll-like receptors recognize a variety of pathogen-associated molecular patterns, resulting in inflammation. If we had enough information to begin comparing a *Drosophila* defense mechanism to its mammalian counterpart, how similar would they be? In order to begin answering these questions, we first needed an experimental assay to interrogate each of these defense mechanisms.

To begin studying these antiviral defense mechanisms in *Drosophila*, we selected a virus that can infect mammals and insects, vesicular stomatitis virus (VSV). VSV is a well-characterized virus used extensively in mammalian and *Drosophila* studies. We hoped that using VSV as a pathogen would result in greater chances of making biological discoveries in *Drosophila* that would be comparable to the mammalian innate immune system. Genetically modified versions of VSV have been engineered to study the virus and host immune responses. We began our studies using one such recombinant version of VSV, VSV-firefly luciferase (VSV-ffLuc). This virus has a firefly luciferase gene in addition to the genes normally encoded. Production of firefly luciferase can serve as a proxy for how well the virus is able to replicate.

To interrogate these proposed antiviral defense mechanisms, we employed a gene silencing strategy in a *Drosophila* cell line. We found that the siRNA pathway restricted VSV infection, and the miRNA and piRNA pathways cannot serve as redundant antiviral mechanisms. Autophagy, Jak/STAT signaling, Toll signaling, and IMD signaling are not involved in restricting VSV infection. Finally, we identified a *Drosophila* ortholog of mammalian STING later in my dissertation research. Mammalian STING is involved in resisting viral infection. We investigated if *Drosophila* STING (dSTING) also mediates antiviral immunity in infected flies. Interestingly, we find dSTING promotes survival of flies infected with viruses with RNA or DNA genomes.

MATERIALS AND METHODS

Viruses

Sindbis virus (SINV) and vesicular stomatitis virus (VSV) stocks were produced by infecting BHK cells. Original VSV stocks were obtained from Sean P. Whelan. BHK cells were grown in complete Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. BHK cells were grown to 90% confluence in T175 tissue-culture treated flasks (Corning 353112). Roughly $2X10^7$ cells were in a flask. Media was removed from flasks and cells were infected with virus diluted in serum-free DMEM for VSV stocks and with 3% serum DMEM for SINV. Cells were infected at MOI 0.1. Virus was adsorbed in 5 mL culture volume for 1 hour at 37°C with intermittent rocking. After incubation, virus media was removed and cells were grown in 15 mL of

complete DMEM at 37°C. Cells would exhibit cytopathic effects at 24 hour post-infection with SINV and VSV-firefly luciferase (VSV-ffLuc). At which point, culturing media was collected and cell debris was pelleted by spinning at 1500 rpm for 10 minutes. Supernatant was passed through a 0.4 um syringe filter.

To concentrate VSV, filtered virus supernatant was spun at 21,000 rpm for 90 minutes at 4°C in Ty50.2 Ti rotor. Supernatant was removed and virus was resuspended by incubating virus pellet in NTE buffer (0.1 M sodium chloride, 1 mM EDTA, and 0.01 M Tris, pH 7.4) overnight at 4°C. Fifteen percent sucrose (w/v) was dissolved in NTE buffer and chilled on ice. Sucrose solution was aliquoted into 10.4 mL polycarbonate tube (Beckman Coulter 355603), and resuspended virus was gently layered on top. Virus was pelleted through the sucrose cushion by spinning at 47,000 rpm for 1 hour at 4°C using Beckman rotor Type 70.1Ti. Pelleted virus was slowly resuspended by incubating pellets in NTE buffer overnight at 4°C. Virus titers were measured by plaque assay and stocks of virus were stored at -80°C.

To concentrate SINV, virus was first precipitated by increasing sodium chloride concentration to 0.5 M and adding 10% (w/v) polyethylene glycol. Virus supernatant was rocked at 4°C for at least 2 hours. Virus was then pelleted by spinning at 15,000 g for 15 minutes in Ty50.2 Ti rotor. Pellet was allowed to slowly resuspend in TNE buffer (0.1 M sodium chloride, 1 mM EDTA, 0.05 M Tris, pH 7.4) overnight at 4°C. Resuspended virus was layered over ice-cold TNE buffer containing 15% sucrose in a tube. Virus on sucrose cushion was spun at 40,000 rpm in a 70.1 Ti rotor for 2 hours. Pelleted virus was resuspended slowly by incubating overnight at 4°C with TNE buffer. Virus titers were measured by plaque assay, and virus stocks were stored at -80°C.

Invertebrate iridescent virus 6 (IIV-6) virus stocks were made by Kate M. Franz by infecting S2 cells and releasing virus from cells via multiple freeze-thaw cycles. Virus was stored at -80°C.

Production of double-stranded RNA

Long, double-stranded RNA (dsRNA) was produced by polymerase chain reaction (PCR) and *in vitro* transcription. To find unique gene sequences, the SnapDragon tool (http://www.flyrnai.org/snapdragon) was used, and specific forward and reverse primers were selected flanking these regions. Searches were carried out using gene names and default search constraints. Amplified sequences were predicted to be at least 300 base pairs in length. GFP and Ago2 primer

sequences were obtained from alternate sources. GFP primers were designed by Lorri Marek, and Ago2 primers were previously published (Cernilogar et al., 2011). The T7 polymerase promoter sequence (5'-TAATACGACTCACTATAGG-3') was added to the 5' end of oligonucleotides, and primers were ordered from Integrated DNA Technologies. Table 2.1 lists targeted genes, primer sequences, and expected PCR product sizes.

The designed oligonucleotides were used to amplify targeted sequences. S2 cell genomic DNA was used as a template. Tsg DNA polymerase (Lamda Biotech) and its accompanying buffer reagents were used for PCR. Magnesium chloride was used at a final concentration of 1 mM, and deoxynucleotide triphosphates were used at 0.2 mM final. The Tsg DNA polymerase enzyme was added at 2.5 units/reaction in 50 uL total reaction volumes. Amplified sequences were run on 1% agarose and predicted-size bands were purified using the MinElute gel extraction kit (Qiagen).

MEGAscript T7 transcription kit (Thermo Fisher Scientific) was used for *in vitro* transcription of RNA. Reaction mixes were made according to recommendations of the manufacturer for 20 uL reaction volumes. Transcription reactions were incubated at 37°C for at least 16 hours. One uL of DNAse provided by manufacturer was added to each reaction and then incubated for another 30 minutes at 37°C to degrade template DNA. To purify dsRNA by ethanol precipitation, sodium acetate (0.3 M final) was added to each reaction for a total 100 uL volume. Three hundred uL of 100% ethanol was added to each reaction (75% ethanol final), and the RNA was allowed to precipitate at -20°C for at least 1 hour. RNA was pelleted by spinning reactions at 4°C at >13,000 g for 30 minutes. Pellets were washed with ice cold 75% ethanol and briefly spun at >13,000 g for 10 minutes. Pelleted RNA was resuspended in DEPC-treated water.

***In vitro* VSV-luciferase reporter infections of S2 cells**

Gene knockdown was induced in S2 cells in order to measure for increased production of a virus reporter gene, luciferase. S2 cells were grown in complete Schneider's media (10% heat-inactivated fetal bovine serum, 100U/mL penicillin/streptomycin). Cells were counted, pelleted, and resuspended in serum-free Schneider's media at $2X10^6$ cells/mL. Cells were plated in 1 mL volumes with a total of 10 ug of dsRNA. In the case of simultaneous gene knockdowns, 5 ug of each dsRNA was used. Table 2.1 lists all dsRNA used for in vitro gene knockdown experiments. Cells were incubated with dsRNA under

Tabel 2.1 Double-stranded RNA used for targeted gene knockdown. Labels in data figures are listed in the table along with corresponding genes being targeted for knockdown. Primer sequences used for amplifying sequence target are listed. All sequences are written from 5' to 3' orientation. Note that the T7 polymerase promoter sequence was added to all primer sequences (not shown in table). Expected product sizes do not account for the added length of T7 sequences flanking both ends of PCR products. *dsGFP primers were obtained from Lorri Marek. **dsAgo2 primer sequences were previously published (Cernilogar et al., 2011).

Figure Label	Target Gene	Forward Primer	Reverse Primer	Size (bp)
dsGFP*	GFP	AGCAGCACGACTTCTTCA	ATGCCGTTCTTCTGCTTGTC	247
dsDcr2	Dcr2	ACATTCCCAAAACGCTCAAC	ATCGGCTATCACCTTGTTGG	323
dsAgo2**	Ago2	GCTGCAATACTTCCAGCAC A	CTCGGCCTTCTGCTTAATTG	463
dsR2D2	R2D2	GCATTGAGGTAGTGCAGCA A	TAGTTGTGTCGGTCGCAGAG	340
dsDcr1	Dcr1	CGGAACACGATTATTTGCCT	CGCAACACGGTGACAATATC	551
dsAgo1	Ago1	ATGATGGAGGTACGAGGAC G	TATACTGGAGTCTTGCCGGG	419
dsPiwi	Piwi	TTTGACATTGCGAAGAGCA C	TGACGAACTTGTTGCGAGAC	467
dsAgo3	Ago3	TTTAATGCTATGCTGCGACG	ACGCCCACTTATCTTGTTGG	574
dsAub	Aub	CTAAAAGTTATCGCGCCTC G	TAGCGCTCTGGCAAGGTAAT	578
dsToll7	Toll-7	GCCATACAAAATCGCACCTT	TGGCGTTAAAGTCCAGCTCT	475
dsAtg5	Atg5	TCCGAGCATCTGAGCTATC C	CGCTTCTTTTAAATGCTGGC	316
dsHop	Hopscotch	GCAACTGCAAGGGTAAGAG C	GTTGAACACACGGATTGTGC	417
dsSTAT92E	STAT92E	AAGCTGCTTGCCCAAAACT A	GTCGACGATAAAGGCAGAG C	402
dsDIF	DIF	AAGTCCCAATTTTGTGCAG G	CCTTGTGGCGGTATGCTTAT	450
dsDorsal	Dorsal	TGAACAGCAAGTCCATGAG C	GCCTGAGAGTTCACCTCGAC	384
dsRelish	Relish	CGCAAACTTATCGAGCACA A	ACCTGTATCGTCTGGATGGC	308
dsBasket	Basket	CGCAAAGGAACTTGGAAGA G	AAAATGACCTCTGGAGCCCT	376
dsJun	Jun	ACTCACCGGATCTGTCATC C	CTAGCCAGGTCGACGTTCTC	526
dsKayak	Kayak	GCAACGCGAATACCTCAAA T	GCTTGAGATCCAAGGGTGAA	492

serum starvation conditions for one hour at room temperature. One mL of Schneider's media containing 2X concentration of serum and penicillin/streptomycin was then added to each well. Cells were incubated at room temperature for 4 days to allow gene knock down to proceed. Cells from each knockdown condition were then counted, pelleted, and resuspended in fresh complete Schneider's medium at $0.5X10^6$ cells/mL. Cells were plated in 100 uL volumes (50,000 cells/well) in triplicate in a 96-well flat-bottom tissue culture plate. VSV firefly luciferase (VSV-ffLuc) was diluted in Schneider's complete medium and added to cell cultures at 10 uL/well for an MOI 1. At 2 or 3 days post-infection (dpi), cells were pelleted and media was removed. Cells were lysed with 50uL/well Bright-Glo luciferase reagent (Promega) and luminescence was measured on a plate reader.

***In vivo Drosophila* infections**

Flies carrying a P-element, y^1w^{67c23}; P{EPgy2}$CG1667^{EY06491}$ (Bloomington 16729), were verified by qPCR to have disrupted gene expression of *Drosophila* STING (dSTING) by Kate M. Franz. Mutant flies were compared to isogenic stock, y^1w^{67c23} (Bloomington 6599). $Dcr2^{KO}$ flies refer to $Dcr2^{L811fsX}$ loss of function mutants. Flies were reared at 25°C in a 12-hour light cycle incubator. Adult flies used for infections were collected and infected within 10 days of eclosion. IIV-6 infected flies were incubated at 25°C. SINV infected flies were incubated at 29°C and monitored for survival. See Chapter 3 Materials and Methods section for plaque assay method to measure virus titers.

For virus infections, defined volumes of virus stocks were injected into the thorax using the Nanoject II (Drummond). Glass capillaries were melted using a needle puller to produce injection needles. Injection needles were backfilled with mineral oil to prevent injection volume fluctuations. For Sindbis infections, virus was diluted to 10^{11} plaque forming units (PFU)/mL in TNE buffer. IIV-6 was used at undiluted stock concentration. Virus was injected into the thorax of animals at 9.2 nL/fly.

For bacterial infections, a septic infection was induced by injury of the thorax with a minutien pin (Fine Science Tools). A single colony of *Escherichia coli* was picked from a Luria broth (LB) agar plate. The colony was grown overnight as a 10 mL LB culture in a 37°C shaker. A single colony of *Enterococcus faecalis* was picked and grown in 10 mL of brain-heart infusion media overnight in a 37°C shaker. Flies were infected with *E. coli* or *E. faecalis* by dipping the pin in overnight bacterial cultures and pricking flies in the thorax.

RESULTS

S2 cells use the siRNA pathway as an antiviral defense against VSV

Many genetic pathways have been identified as having an antiviral function in *Drosophila*. Most of these pathways have not been sufficiently investigated. Because the siRNA pathway has been the best characterized, it was used initially in order to develop an experimental system to interrogate other potential antiviral pathways.

S2 cells are a genetically tractable cell line derived from *Drosophila* embryos (Schneider, 1972). They have been used extensively in high-throughput genetic screens because RNAi gene knockdown is very efficient. For this reason, S2 cells were selected to identify genetic pathways that restrict VSV infection. To do this, S2 cells were bathed in serum-free media containing dsRNA targeting a specific gene for 1 hour. During this time, the cells internalized the nucleic acids. S2 cells were then incubated in complete Schneider's medium for 4 days to induce gene knockdown. Cells were plated and infected with VSV-ffLuc at a multiplicity of infection (MOI) 1. Luciferase activity was measured 2 and 3 days post-infection (dpi).

When genes from the siRNA pathway were knocked down, luciferase activity increased, suggesting that VSV infection was not restricted. At 2 dpi, Dcr2, Ago2, and R2D2 gene knockdowns led to greater than 1.5-fold increase in luciferase activity compared to a control, GFP knockdown ($p<0.05$ t-test, Figure 2.1A). At 3 dpi, luciferase signals were greater than 3.5-fold higher in Dcr2, Ago2, and R2D2 knockdowns compared to GFP (Figure 2.1B). In comparison, knockdown of genes in the miRNA pathway did not suggest a role in antiviral defense. Luciferase activity at 2 dpi was slightly elevated for Dcr1 knockdown compared to GFP knockdown whereas Ago1 knockdown was slightly lower than GFP knockdown (Figure 2.1A). However by 3 dpi, Dcr1 and Ago1 knockdowns did not result in elevated luciferase activity compared to GFP knockdown (Figure 2.1B). Collectively, these data demonstrate that the siRNA pathway restricts VSV infection but the miRNA pathway does not.

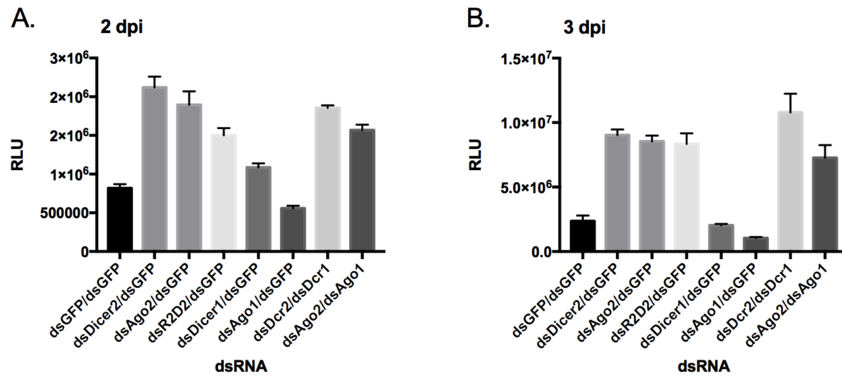

Figure 2.1 The siRNA but not the miRNA restricts VSV infection. S2 cells were incubated with 5 ug of each dsRNA to induce targeted gene knockdown. Four days later, cells were infected with VSV-ffLuc at MOI 1. At 2 (A) and 3 (B) dpi, luciferase activity was measured on a plate reader. Error bars represent standard error. n=3

The possibility exists that the siRNA pathway is the main antiviral defense, and the miRNA pathway could potentially play a redundant role only in the situation when the siRNA pathway is not functioning properly. To test this possibility, both Dicer genes or both Ago genes were simultaneously knocked down. When luciferase activity was measured at 2 and 3 dpi, double knockdowns did not result in a statistically significant increase compared to single knockdown of Dcr2 or Ago2 (t-test, Figure 2.1A, B). These results suggest that even in the absence of an antiviral response by the siRNA pathway, the miRNA pathway cannot take on an antiviral function.

The piRNA pathway does not restrict VSV infection

No evidence suggests that the miRNA pathway can function as an antiviral defense in *Drosophila*. However, the piRNA pathway restricts the movement of transposable elements in the *Drosophila* reproductive tract (Aravin et al., 2007). Because the piRNA pathway can restrict the proliferation and movement of foreign genomic elements, we tested the hypothesis that the piRNA pathway could restrict VSV infection. We focused our studies on three argonaute-family genes in the piRNA pathway: *piwi*, *ago3*, and *aub*. Knockdown of these three genes individually did not produce a consistent elevation in luciferase signals at 2 and 3 dpi (Figure 2.2A, B). In contrast, knockdown of

R2D2, a gene in the siRNA pathway, resulted in elevated luciferase activity 2 and 3 dpi, consistent with previous results (Figure 2.2A, B).

Knocking down genes in the piRNA pathway did not affect the expression of the viral reporter gene, luciferase. Since the siRNA restricts VSV infection in S2 cells, we wanted to test the possibility that the piRNA pathway could play a redundant role when the siRNA pathway is ineffective. To test this, we knocked down R2D2 simultaneously with one of the piRNA genes. When piwi, Ago3, or aubergine were knocked down in conjunction with R2D2, no additive effects were seen in luciferase activity at 2 and 3 dpi (Figure 2.2A, B). Thus, the piRNA pathway does not restrict VSV infection in S2 cells.

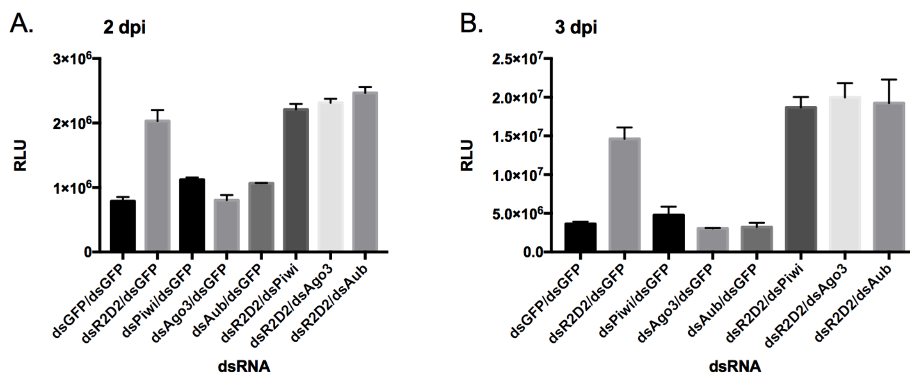

Figure 2.2 The piRNA pathway does not restrict VSV infection. S2 cells were incubated with 5 ug of each dsRNA as indicated to knock down gene expression. Four days later, cells were infected with VSV-ffLuc at MOI 1. Luciferase activity was measured 2 (A) and 3 (B) dpi. Error bars represent standard error. n=3

Autophagy does not restrict VSV infection in S2 cells

In a genome-wide screens, Toll-7 and components of the autophagy pathway were identified as having antiviral functions (Moy and Cherry, 2013). Because these findings were partly based on experiments done with VSV in S2 cells, we wanted to confirm these results in the hopes of further elucidating the genetic components of this antiviral pathway. We knocked down expression of Toll-7 and Atg5 in S2 cells and then infected them with VSV-ffLuc. As expected, Dcr2 knockdown resulted in increased luciferase activity in comparison to control GFP knockdown at 2 and 3 dpi (Figure 2.3A, B).

However, Toll-7 and Atg5 knockdowns did not result in increased luciferase activity 2 and 3 dpi in comparison to GFP controls (Figure 2.3A, B). These results suggest that relative to the RNAi pathway, Toll-7 and Atg5 do no contribute substantially to antiviral defense.

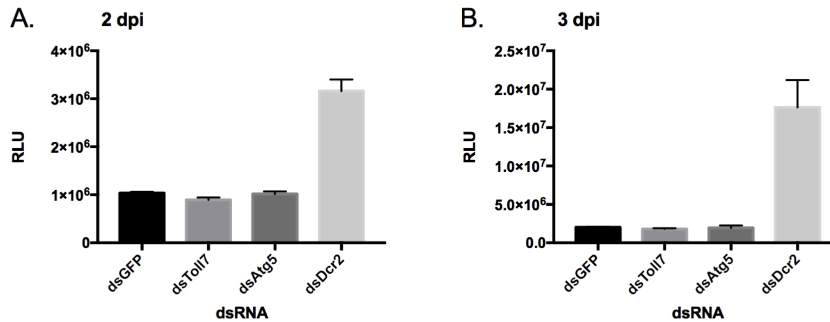

Figure 2.3 Toll-7 and Atg5 do not have antiviral activity. S2 cells were treated with 10 ug dsRNA to target knockdown of indicated genes. Four days following knockdown, cells were infected with VSV-ffLuc at MOI 1. At 2 (A) and 3 (B) dpi, luciferase activity was measured. Error bars represent standard error. n=3

Toll, IMD, and Jak/STAT signaling do not restrict VSV infection

The Toll, IMD, and Jak/STAT pathways have been reported to have antiviral functions in *Drosophila*. MAPK signaling is initiated downstream of IMD signaling (Silverman et al., 2003; Sluss et al., 1996). Additionally, MAPK signaling is initiated downstream of TLR signaling in mammals (Silverman and Maniatis, 2001). To test if any of these signaling pathways could restrict VSV infection, genes from each were targeted for knockdown in S2 cells. Hopscotch and STAT92E are the only Jak and STAT in *Drosophila*, respectively. After 2 or 3 dpi, knockdown of either gene did not result in increased luciferase activity compared to control GFP knockdown (Figure 2.4A, B). DIF and dorsal are homologs of the NFκB protein downstream of the Toll receptor. Knocking down either gene did not produce consistent, elevated luciferase activity at 2 and 3 dpi (Figure 2.4A, B). The IMD pathway signals to another NFκB-like protein, relish. Again, knocking down relish expression did not result in a significant increase in luciferase activity compared to control GFP knockdown at 2 and 3 dpi (Figure 2.4A, B). Finally, basket and Jun are orthologs of mammalian JNK and Jun genes, respectively. Kayak is an ortholog of Fos. In comparison to

control GFP knockdown, none of these genes involved in MAPK signaling resulted in increased luciferase activity 2 and 3 dpi when knocked down (Figure 2.4A, B). Although published literature suggests that Toll, IMD, and Jak/STAT signaling could be antiviral, none of these pathways restrict VSV infection under the experimental conditions examined in this study.

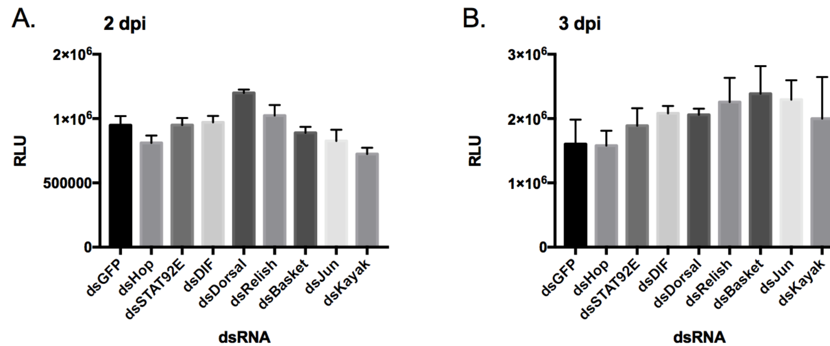

Figure 2.4 Toll, IMD, and Jak/STAT pathways are not involved in restricting VSV infection. S2 cells were incubated with 10 ug of dsRNA for 4 days to induce targeted gene knockdown. Cells were then infected with VSV-ffLuc at MOI 1. At 2 (A) and 3 (B) dpi, cells were measured for luciferase activity. Error bars represent standard error. n=3

Drosophila STING restricts viral infection *in vivo*

In mammals, the possibility of RNAi functioning as an antiviral defense has been a contentious idea (Cullen et al., 2013). If mammalian RNAi truly does have antiviral properties, it is only in very specific contexts, such as when the interferon pathway is ablated (Maillard et al., 2016). Numerous pattern recognition receptors that bind nucleic acids have been identified in mammals, but RNAi is the only immune defense demonstrated to recognize foreign nucleic acids in *Drosophila*. In comparison to mammalian antiviral defenses, RNAi is very different. Mammalian immune defenses have been characterized to induce a transcriptional host response. In comparison, the mechanism of action of RNAi is to prevent translation of viral transcripts. In this respect, RNAi is most similar to the mechanism of action of the mammalian protein kinase R (PKR), but PKR acts directly on ribosomal machinery in order to block viral protein translation. Theoretically, the Jak/STAT pathway could function similarly to the mammalian system, where it mediates downstream signaling from interferon receptors. This could result in the induction of an antiviral state, thus inhibiting the spread of viral infection. However, no genes

orthologous to mammalian interferons have been discovered in *Drosophila*. If a system similar to interferon signaling exists in *Drosophila*, our data (Figure 2.4) and the work of others would suggest that it is more context specific, which contrasts the wide applicability of the mammalian interferon system (Kemp et al., 2013). Thus, whether arthropods and mammals share any similar antiviral mechanisms remains unknown.

In mammals, STING is known to mediate an interferon response after detection of foreign nucleic acids (Chow et al., 2015). While interferons do not exist in *Drosophila*, an ortholog of STING exists in flies. Whether *Drosophila* STING (dSTING) has any antiviral functions is unknown. Conveniently, a stock of flies containing a transposable element in the dSTING locus is available from the Bloomington Drosophila Stock Center. After acquisition, the stock of flies was verified to have disrupted expression of dSTING by qPCR analysis (K. M. Franz, unpublished data).

Since mammalian STING has been implicated in the detection of foreign DNA, we first challenged $STING^{KO}$ flies with a DNA virus. $STING^{KO}$ flies were infected with invertebrate iridescent virus 6 (IIV-6). As controls, $dSTING^{KO}$ flies were compared to isogenic stocks (WT) and $Dcr2^{KO}$ flies. All three genotypes were infected or mock treated. Infected $Dcr2^{KO}$ and $STING^{KO}$ flies died with similar kinetics (Figure 2.5). In comparison, the median time to death of infected WT flies was delayed 5 days (Figure 2.5). While all infected flies were dead by 16 dpi, most mock treated flies survived past 23 day (Figure 2.5). This experiment would suggest that Dcr2 and dSTING are equally important in mediating survival during IIV-6 infection.

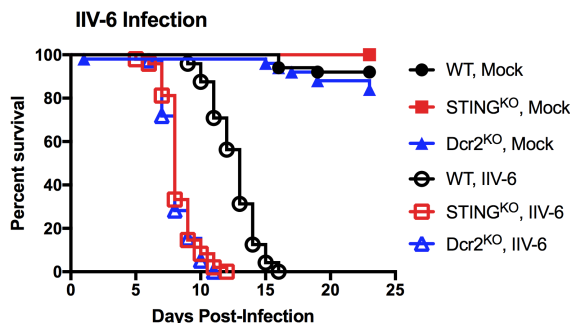

Figure 2.5 dSTING promotes survival during IIV-6 infection. Flies were injected in the thorax with IIV-6 or were mock treated. After infection, flies were incubated at 25°C and monitored for survival daily. n>35 for each experimental group.

In addition to DNA viruses, mammalian STING restricts RNA virus infection (KMF, unpublished data). Although the mechanism of action is undefined, we wanted to determine if dSTING could function similarly to restrict RNA virus infection. SINV was chosen because it is a positive-sense, single stranded RNA virus that readily infects *Drosophila*. WT and $STING^{KO}$ flies were infected with SINV or were mock treated. After infection, flies were kept at 29°C and monitored for survival. $STING^{KO}$ flies died significantly faster than infected WT flies and mock treatment controls ($p<0.0001$ log-rank test, Figure 2.6). Thus, dSTING also mediates survival of flies during SINV infection.

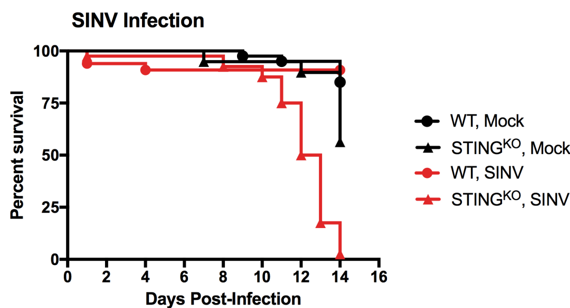

Figure 2.6 dSTING mediates survival during SINV infection. WT and $STING^{KO}$ flies were injected in the thorax with 10^6 PFU of SINV or were mock treated. After infection, flies were incubated at 29°C and monitored for survival. n>30 for each experimental group.

Although wild type expression of dSTING is important in mediating survival during viral infection, the function of dSTING is still unknown. Survival data by itself is unable to explain how dSTING sustains viability during infection. If dSTING functions similarly to its mammalian counterpart, one would expect viral replication to be restricted. Since SINV titers can be easily measured, we decided to titrate virus from infected flies. $STING^{KO}$ flies took nearly 2 weeks for 50% to succumb to SINV infection (Figure 2.6). If dSTING was involved in restricting virus replication, we hypothesized that $STING^{KO}$ flies would exhibit elevated viral titers compared to WT flies even before survival defects begin to develop. SINV-infected flies were collected 1, 3, 5, and 7 dpi and measured for infectious viral particles. No significant differences in viral titers were observed 1 dpi in $STING^{KO}$ compared to WT flies (t-test, Figure 2.7). However, by 3 dpi and onward, $STING^{KO}$ flies had approximately 100-fold higher viral titers compared to

WT flies ($p<0.05$, multiple t-tests, Figure 2.7). The increased SINV titers in STING^{KO} flies during the first week of infection suggest that dSTING is involved in restricting viral replication.

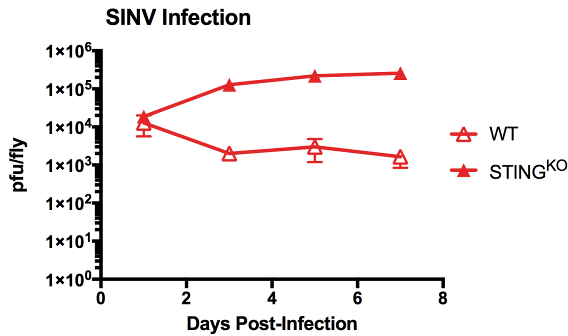

Figure 2.7 dSTING restricts SINV replication. WT and STING^{KO} flies were injected in the thorax with 10^6 PFU/fly of SINV. During infection, flies were incubated at 29°C. At 1, 3, 5, and 7 dpi, flies were collected, and viral titers were measured by plaque assay. Error bars represent standard error. n=3

Despite the similarity of dSTING to its mammalian counterpart, dSTING has thus far been uncharacterized. We had established an antiviral role for dSTING, but the mechanism of how it effectively mediates viral resistance is unknown. Its promiscuous role in both DNA and RNA virus infections was particularly intriguing, but the limits of dSTING in immunity had yet to be tested. One could hypothesize that dSTING is generally involved in immunity and not specific to viral infections. To test the hypothesis, dSTING mutant flies were infected with gram-negative and gram-positive bacteria (Figure 2.8). The Imd signaling pathway resists infection by the gram-negative bacterium *Escherichia coli*, and a loss-of-function mutant of *kenny* (Key^{KO}) renders flies susceptible to infection. Most infected animals die within 3 days, but infected wild-type and STING^{KO} flies survive for longer than a week ($p<0.0001$, log-rank test, Figure 2.8A). Resistance to the gram-positive bacterium *Enteroccocus faecalis* is mediated by the Toll pathway, and loss-of-function mutants for *Drosophila* MyD88 (dMyD88^{KO}) succumb to infection. Nearly all dMyD88^{KO} flies die within 1 day of infection with *E. faecalis,* but approximately 80% of wild-type and dSTING^{KO} remain alive at 1 dpi in comparison ($p<0.0001$, log-rank test, Figure 2.8B). The ability of dSTING^{KO} animals to maintain bacterial resistance suggests that

dSTING is not a gene with general immune function. Instead, dSTING specifically restricts viral infections.

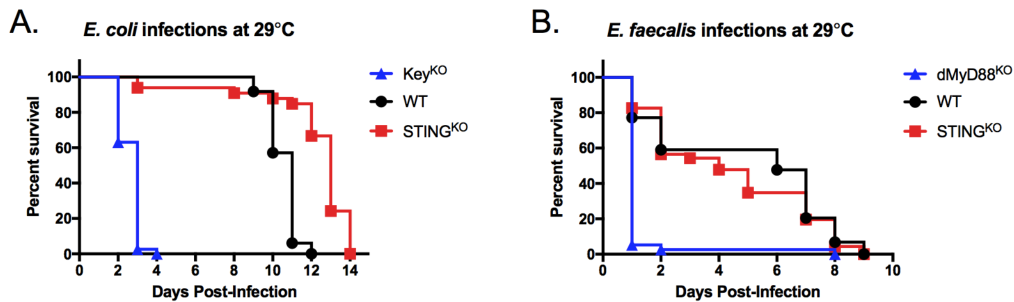

Figure 2.8 dSTING does not restrict bacterial infections. (A) Wild-type, dSTING^{KO}, and Key^{KO} flies were infected with *E. coli* via thoracic injury with a minutien pin swabbed in bacterial culture. Animals were monitored for survival daily after infection. (B) Wild-type, dSTING^{KO}, and dMyD88^{KO} flies were infected with *E. faecalis* via thoracic injury with a minutien pin swabbed in bacterial culture. Animals were monitored for survival daily after infection. n>30 for each experimental group.

DISCUSSION

In this chapter, we have briefly surveyed the antiviral defenses that were identified prior to the start of my dissertation work to determine their relative roles in VSV infection. Using the *Drosophila* S2 cell line, we found that only one of the three RNAi pathways, namely the siRNA pathway, is able to mediate antiviral immunity against VSV (Figures 2.1-2.2). Autophagy, Jak/STAT signaling, Toll signaling, and IMD signaling do not restrict VSV infection (Figures 2.3-2.4). We then discovered *in vivo* an ortholog of mammalian STING, dSTING, that mediates survival of flies infected by viruses but not bacteria (Figures 2.5-2.8). Thus, we find that antiviral defenses are selectively utilized by the insect host, depending on the virus.

The conclusions of my work are largely based on experiments using an *in vitro* reporter assay. Because these studies were very limited in scope, my conclusions come with several caveats to consider. First, potential antiviral defense pathways were interrogated by inducing gene knockdown. Gene knockdown has been noted to be highly efficient in S2 cells (Marques and Imler, 2016). However,

the dsRNA used for these studies were not verified to induce knockdown. Thus in the situation where a gene is truly antiviral, a lack of change in luciferase activity could be due to the fact that gene expression was unaffected. In addition to inefficient gene targeting, off-target effects of gene silencing could also be a potential problem. Because of these potential issues, we took the approach of targeting multiple genes in a pathway to reduce the chance of inefficient knockdown and off-target silencing.

A second major caveat of my *in vitro* studies is that only VSV was used. The antiviral pathways thus far identified (Toll, IMD, Jak/STAT, autophagy) might not function against all viral infections. Indeed, others have reported that the Jak/STAT pathway interferes with a select group of viruses, not including VSV (Kemp et al., 2013). Thus, my *in vitro* work only applies to VSV and antiviral defenses beside RNAi might have more significance for other viruses.

A third caveat of my *in vitro* assay is that it does not take into account the possibility that gene knockdown could affect cell viability. Although cells were counted between the steps of gene silencing and virus infection in order to normalize the cells used for the assay, the time length of virus infection (2 and 3 days) was fairly long. If a gene were involved in virus restriction, a knockdown would produce more luciferase. However, luciferase expression could have been offset by cell death. In such a situation, luciferase signal might not have significantly increased, resulting in a false negative.

Despite all these caveats, my results do confirm what others have published. Three genes of the siRNA pathway (Dcr2, Ago2, and R2D2) relieved restriction of VSV infection when knocked down (Figure 2.1). Flies with mutations in Dcr2, Ago2, and R2D2 have been found to succumb to VSV infection (Wang et al., 2006). Additionally, disruption of Dcr2 expression *in vivo* resulted in survival defects when flies were infected with IIV-6 (Figure 2.5). My results were further corroborated by published results showing IIV-6 infection in multiple mutant flies (Kemp et al., 2013). Kemp and colleagues demonstrated a susceptibility to IIV-6 infection using flies with the same Dcr2 mutation and also with flies with another Dcr2 mutation. Additionally, mutations in Ago2 and R2D2 resulted in animals with similar susceptibility to IIV-6 infection as the Dcr2 mutants (Kemp et al., 2013). Further supporting the role of RNAi in antiviral immunity, the siRNA pathway has been found to restrict broad classes of viruses differentiated by genomic make-up (RNA and DNA) and by particle structure (enveloped and

non-enveloped) (Bronkhorst and van Rij, 2014). Altogether, my data supports the assertion that the siRNA pathway is a robust antiviral defense mechanism that mediates immunity against many viruses.

In addition to RNAi, autophagy has been proposed to be an immune defense against VSV infection in *Drosophila* (Shelly et al., 2009). The study undertaken by Shelly and colleagues was interesting because it took an approach similar to our luciferase assay, utilizing RNAi to knock down genes in S2 cells. While we used VSV-ffLuc to report on viral replication activity in S2 cells, Shelly and colleagues utilized VSV-GFP to quantify GFP expression by immunofluorescent microscopy. In a subsequent study by the same lab, Toll-7 was identified to control the induction of autophagy (Nakamoto et al., 2012). However, our work came to different conclusions. Knockdown of Atg5 or Toll-7 did not result in increased VSV replication (Figure 2.3). The contradictory results could perhaps be reconciled by the difference in readout timing. The results of Shelly and colleagues were based on data collected 20 or 24 hours post-infection (Shelly et al., 2009). In contrast, my data was collected at 2 and 3 dpi (Figure 2.3). If both of our results were correct, it would suggest that autophagy plays a minimal role in antiviral immunity since any differences resulting from autophagy deficiency observed early in the infection are no longer present in subsequent days. An alternative interpretation is that disruption of autophagy alters the kinetics of viral entry. In this alternative model, disruption of autophagy might be offset by increased activity of endocytic pathways, leading to increased uptake of VSV. More VSV inside cells would then lead more viral protein production early in the infection. This is a nuanced interpretation of the data that would suggest autophagy does not restrict viral replication directly. Instead, a lack of autophagy would shift the cellular state to be more permissive to infection. It is noteworthy to point out that the authors of the autophagy studies did not produce evidence of viral proteins inside autophagosomes. Thus, the studies lack the most definitive evidence that autophagy can inhibit replication of VSV.

As another point of reference, a second group has recently published work investigating the role of autophagy during VSV infection (Lamiable et al., 2016a). In their studies, Lamiable and colleagues found that deletion of Atg7 in flies resulted in increased mortality starting 10 days after VSV infection, in comparison to mock treatment. The mortality late in infection would go against my model that autophagy deficiency allows for increased viral entry. One might expect mortality and increased viral replication earlier in the course of infection. However, *in vivo* studies have numerous additional factors to consider

in comparison to infection of cell cultures. Route of virus entry into the organism, cellular tropism of the virus, and cellular cross-talk are some of the factors that can drastically affect the course of infection. Interestingly, Toll-7 mutant flies did not display survival defects or increased viral RNA (Lamiable et al., 2016a), which agrees with our data (Figure 2.3). Ultimately, the role of autophagy in VSV infection, and more broadly in other viral infections, remains unclear. To prove autophagy as an antiviral defense would require careful analysis of multiple viruses infecting multiple genetic mutant hosts. Even if autophagy is proven to be an antiviral defense, the upstream signaling events that initiate autophagy remain to be defined.

In regards to Jak/STAT signaling, several viruses have been used to test if it serves as an antiviral defense. In one study, VSV, SINV, IIV-6, flock house virus, *Drosophila* X virus, *Drosophila* C virus, and cricket paralysis virus were used to infect hopscotch mutant flies (Kemp et al., 2013). *Drosophila* X virus and IIV-6 resulted in minor decreases in survival but no changes in viral titers (Kemp et al., 2013). VSV, SINV, and flock house virus infections were unaffected by mutating hopscotch, which is in accordance with our data (Figure 2.4). However, cricket paralysis virus and *Drosophila* C virus infections had significant effects on survival and viral titers in hopscotch mutants, confirming previous results (Dostert et al., 2005). The survival defects of hopscotch mutants during *Drosophila* C virus and cricket paralysis virus infections suggests that the Jak/STAT pathway is selectively important for *Dicistroviridae* since both viruses belong to this family. While no defects in survival were observed in hopscotch mutant flies when infected with flock house viruses, one study found that the virus induces Jak/STAT signaling (Dostert et al., 2005). This observation raises the possibility that the viruses seemingly unaffected by the Jak/STAT pathway might have virulence mechanisms that inhibit Jak/STAT signaling, further complicating the interpretation of data. For example, VSV M blocks type I interferon production by inhibiting host transcription and nuclear export (Ferran and Lucas-Lenard, 1997; Her et al., 1997). Whether transcriptional immune responses are inhibited by viruses in *Drosophila* remains to be demonstrated.

Other transcriptional responses (Toll and IMD) also do not seem to restrict VSV infection (Figure 2.4). However, previous work demonstrated a role for Toll signaling in Drosophila X virus infection *in vivo* (Zambon et al., 2005). IMD mutant flies were found to be more susceptible to cricket paralysis virus

(Costa et al., 2009). In comparison to the body of literature published on the antiviral effects of RNAi, supportive data of antiviral transcriptional responses is small. Thus, the relative importance of transcriptional responses such as Toll, IMD, and Jak/STAT signaling are most likely virus-dependent.

The role of dSTING marks a new frontier in *Drosophila* antiviral immunity. We found that flies mutant for dSTING were susceptible to IIV-6 and SINV infection (Figures 2.5-2.7). While a transcriptional response downstream of mammalian STING has been well characterized, K. M. Franz has noted that the C-terminus of mammalian STING, which mediates downstream transcriptional responses, is not present on dSTING (unpublished data). Whether dSTING mediates transcriptional responses upon viral infection is currently unknown. Intriguingly, mutation of dSTING results in survival kinetics comparable to $Dcr2^{KO}$ during IIV-6 infection (Figure 2.5). Dcr2 has been previously implicated in regulating the expression of the virally-induced host gene vago (Deddouche et al., 2008). At this point, we have no mechanism for how dSTING mediates antiviral immunity, but we speculate that dSTING might be working in the same pathway as Dcr2. Since Dcr2 but not R2D2 is involved in the transcriptional induction of *vago* during *Drosophila* C virus infection (Deddouche et al., 2008), this difference could help differentiate whether dSTING has a transcriptional function, an RNAi function, or both. By combining dSTING and RNAi mutations, one could carry out epistasis studies to determine if dSTING operates in the same pathway or is an independent antiviral defense mechanism.

While most of the data presented in this chapter have been negative, the results from work on dSTING point to more unresolved areas of antiviral immunity in *Drosophila*. The siRNA response to viral infection has proven to be robust, but unanswered questions still exist. While established immune pathways such as Toll and IMD seem to be less involved in viral infection responses, more work needs to be done. In Chapter 4, we will further detail what the most compelling questions to be answered about *Drosophila* antiviral immunity are.

Chapter 3

Disruption of neuronal function by combinatorial stresses

The substance of this chapter was previously published.

Chow, J., Márka, Z., Bartos, I., Márka, S., and Kagan, J. C. (2017). Environmental Stress Causes Lethal Neuro-Trauma during Asymptomatic Viral Infections. Cell Host Microbe. 22, 48-60.e5.

Contributions:

Jonathan Chow designed and performed all *Drosophila* studies. Jonathan Chow and Zsuzsa Márka set up video recordings for FlyWalker analysis and performed mosquito infections. Imre Bartos created, updated, and customized FlyWalker software. Jonathan C. Kagan and Szabolcs Márka helped design and plan research.

INTRODUCTION

Pathogens kill because they cause cellular stresses that exceed what is viable. Sometimes, as in asymptomatic infections, no changes in host health are observable. However, considerable effort by the host may be required to maintain this asymptomatic state, suggesting that stresses on the host may be difficult to detect. These changes in cellular homeostasis are not without significance, as asymptomatic infections can render the host sensitive to a variety of secondary environmental stresses. Secondary stresses can come in the form of a subsequent microbial encounter, such as bacterial infections that lead to the death of influenza virus infected patients (Rynda-Apple et al., 2015). While neither of these infections alone is lethal, influenza virus alters lung homeostasis such that subsequent bacterial encounters incite massive tissue damage and death. Non-infectious stresses (*e.g.* environmental chemicals) can also provoke disease symptoms in an infected individual. An example of this principle can be found from studies of subclinical hepatitis C virus infections, which result in life-threatening liver pathology when combined with alcohol consumption (Novo-Veleiro et al., 2016). While we have an increasing understanding of how virulent pathogens elicit homeostatic disruptions that cause disease directly, our knowledge of how asymptomatic infections poise a host for sensitivities to secondary stress is limited.

Despite our lack of understanding of how infections provoke sensitivity to secondary stress, it is widely assumed that these sensitivities are the result of active pathogen replication in a specific tissue (*e.g.* influenza infection). Thus, under conditions where pathogen replication is limited, there should be no sensitivity to a secondary stress. However, there are reports that viral infections cause sensitivities to the environment, even under conditions of minimal pathogen replication, such as during Epstein-Barr virus latency (Takeda et al., 2014). These observations suggest that stress sensitivity may not necessarily be the consequence of tissue damage associated with pathogen replication, but rather may result from an activity encoded by a viral protein, even in the absence of replication. Little is known of how viral proteins, independent of pathogen replication, can cause disease. In this study, we sought to address how viral infection influences sensitivities to the environment, using a new experimental model.

To understand how viral infections could provoke sensitivity to a secondary stress, we sought an experimental system that would allow us to dissect mechanisms within the host and pathogen. The fruit fly *Drosophila melanogaster* is an ideal model organism to study due to its genetic tractability and its ability to be infected by a variety of human pathogens (Schneider et al., 2007). This host has been used extensively to identify pathways that determine the outcome of virulent infections (Buchon et al., 2014), yet the use of the fruit fly to study asymptomatic infections is more limited. The rhabdovirus sigma naturally infects fruit flies, yet infected animals exhibit no apparent symptoms of disease. Studies dating to the 1930s reported that sigma virus causes flies to become sensitive to carbon dioxide (CO_2) anesthesia (L'Heritier and Teissier, 1937, 1938a, 1938b), in that infected flies die upon exposure to CO_2. Sigma virus infection therefore alters some aspects of fly physiology that renders these organisms susceptible to an otherwise non-lethal stress of CO_2 anesthesia. Using this experimental model, we sought to identify mechanisms underlying infection-associated disease susceptibility.

MATERIALS AND METHODS

Antibodies and plasmids

Mouse anti-GFP (JL-8, Clontech) was diluted 1:2000 for Western blotting. Rabbit anti-Beta Actin (4967, Cell Signaling) was used at 1:2000 dilution. Mouse anti-VSV G (V5507, Sigma) was diluted 1:10,000. For microscopy, mouse anti-GFP (ab1218, Abcam) at 1:1000 dilution and rabbit anti-GFP (632592, Clontech) at 1:200 dilution were used depending on which other primary antibodies were used. Anti-luciferase antibody (ab21176, Abcam) was diluted 1:1000, and anti-horseradish peroxidase (123-005-021, Jackson Immunoresearch) was diluted 1:200. DRAQ5 (Thermofisher) was diluted 1:2000.

Using Phusion polymerase (New England Biolabs), VSV genes were amplified from the pVSV1(+)GFP plasmid (gift of Sean P. Whelan) with restriction sites added to the 5' and 3' ends and consensus Kozak sequence (GCCGCCACC) added before the translation start codons. For VSV G, L, M, N, and P, 5' and 3' restriction sites were respectively used as follows: NotI/BamHI, KpnI/NotI, KpnI/XbaI, KpnI/XbaI, and KpnI/BamHI. Restriction enzyme digested DNA was ligated into corresponding restriction sites of the pUASP vector plasmid (Drosophila Genomics Resource Center).

VSV G wild type and mutant genes were cloned into the pValium10-moe vector (Transgenic RNAi Project). VSV G^{G124E} and G^{D137L} were made by site directed mutagenesis. Kozak sequence was added to the translational start site and restriction enzyme sites EcoRI and BglII were respectively added to the 5' and 3' ends.

Animal strains

Anopheles gambiae G3 eggs were obtained from MR4 (contributed by Mark Q. Benedict). Mosquito were reared at 24°C with a 12-hour light cycle. Larvae were grown in distilled water and fed pulverized fish meal. Eclosed adults were then transferred to cages using netting and a light suction vacuum. Adults were fed 10% sucrose diluted in distilled water. Sheep blood was given to females to maintain stocks. For experiments, animals were infected within three days of eclosion and were not blood-fed. Both males and females were used for experiments.

Flies were fed a standard cornmeal medium made by the Norbert Perrimon laboratory. Flies were incubated at 25°C with a 12-hour light cycle. Unless otherwise noted, flies were anesthetized with CO_2 for collection and breeding. For ease of maintenance, only adult males were used for studies although CO_2 sensitivity was observed in females as well. Flies were infected for experiments within 10 days of eclosion. Fly genotypes used in experiments are listed in Table 3.1.

pUASP and pValium10 plasmids were injected into embryos to make transgenic flies by Rainbow Transgenic Inc. (Camarillo, CA). pUASP vectors were injected into a W^{1118} fly background. pValium10 plasmids were injected into flies containing P{nos-phiC31/int.NLS}X, P{CaryP}AttP40 on the X chromosome and 25C6 respectively. Transformants were balanced using yv, wg^{Gla-1}/CyO flies.

Dicer2^{L811fsX} and *Ago2*^{414} mutant flies were a gift from Richard Carthew. *Heat shock-Gal4*, *Actin5C-Gal4*, *Breathless-Gal4*, and *UAS-GFP* were gifts from Norbert Perrimon. *Elav-Gal4* (8765), *Repo-Gal4* (7415), *r4-Gal4* (33832), *UAS-Dicer2* (25756), TubP-$Gal80^{ts}$ (7018) and *UAS-Apoliner* (32123) were obtained from Bloomington fly stocks. *UAS-Ago2-IR* (100356) was obtained from the Vienna Drosophila Resource Center.

Table 3.1 Genotypes of flies used. Nomenclature used in this chapter refers to the genotypes specified in this table.

Name	Genotype
Wild type (WT)	w^{1118}; ;
Dcr2^{KO}	w*; Dcr2^{L811fsX};
Ago2^{KO}	w^{*}; ; Ago2^{414}
Dcr2^{KO}, Glia>GFP	w*; Dcr2^{L811fsX}, UAS-GFP/Dcr2^{L811fsX}; Repo-Gal4/+
Dcr2^{KO}, Neuron>GFP	w*; Dcr2^{L811fsX}, Elav-Gal4/Dcr2^{L811fsX}, UAS-GFP;
Dcr2^{KO}, Trachea>GFP	w*; Dcr2^{L811fsX}, Breathless-Gal4/Dcr2^{L811fsX}, UAS-GFP;
Dcr2^{KO}, Control	UAS-Dcr2, W^{1118}; Dcr2^{L811fsX}/Dcr2^{L811fsX};
Dcr2^{KO}, Glia>Dcr2	UAS-Dcr2, W^{1118}; Dcr2^{L811fsX}/Dcr2^{L811fsX}; Repo-Gal4/+
Dcr2^{KO}, Neuron>Dcr2	UAS-Dcr2, W^{1118}; Elav-Gal, Dcr2^{L811fsX}/Dcr2^{L811fsX};
Control>Ago2 RNAi	w*; UAS-Ago2-IR/+;
Actin>Ago2 RNAi	w*; Actin5C-Gal4/UAS-Ago2-IR;
Neuron>Ago2 RNAi	w*; Elav-Gal4/UAS-Ago2-IR;
Glia>Ago2 RNAi	w*; UAS-Ago2-IR/+; Repo-Gal4/+
Neuron + Glia>Ago2 RNAi	w*; Elav-Gal4/UAS-Ago2-IR; Repo-Gal4/+
Heat Shock>VSV G	w*; Heat Shock-Gal4/+; UAS-VSV G/+
Heat Shock>VSV M	w*; Heat Shock-Gal4/+; UAS-VSV M/+
Heat Shock>VSV P	w*; Heat Shock-Gal4/UAS-VSV P;
Heat Shock> VSV N	w*; Heat Shock-Gal4/UAS-VSV N;
Heat Shock> VSV L	w*; Heat Shock-Gal4/+; UAS-VSV L/+
Glia>VSV G	w*; ; Repo-Gal4/UAS-VSV G
Control>VSV G	w*; ; UAS-VSV G/+
Glia>Control	w*; ; Repo-Gal4/+
Fat body>Control	w*; ; R4-Gal4/+
Fat body>VSV G	w*; ; R4-Gal4/UAS-VSV G
TS Control>VSV G	w*; ; +/UAS-VSV G, TubP-Gal80^{ts}
TS Glia>VSV G	w*; ; Repo-Gal4 /UAS-VSV G, TubP-Gal80^{ts}
TS Fat body>VSV G	w*; ; R4-Gal4/UAS-VSV G, TubP-Gal80^{ts}
Heat shock>VSV G^{WT}	w*; Heat shock-Gal4/UAS-VSV G^{WT};
Heat shock>VSV G^{G124E}	w*; Heat shock-Gal4/UAS-VSV G^{G124E};
Heat shock>VSV G^{D137L}	w*; Heat shock-Gal4/UAS-VSV G^{D137L};
TS Glia>VSV G^{WT}	w*; UAS-VSV G^{WT}/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>VSV G^{G124E}	w*; UAS-VSV G^{G124E}/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>VSV G^{D137L}	w*; UAS-VSV G^{D137L}/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>Apoliner, VSV G^{WT}	w*; UAS-VSV G^{WT}/+; Repo-Gal4, TubP-Gal80^{ts}, UAS-Apoliner/+
TS Glia>Apoliner, VSV G^{G124E}	w*; UAS-VSV G^{G124E}/+; Repo-Gal4, TubP-Gal80^{ts}, UAS-Apoliner/+
TS Glia>Apoliner, VSV G^{D137L}	w*; UAS-VSV G^{D137L}/+; Repo-Gal4, TubP-Gal80^{ts}, UAS-Apoliner/+
TS Glia>GFP	w*; UAS-GFP/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>GFP, VSV G^{WT}	w*; UAS-GFP, UAS-VSV G^{WT}/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>GFP, VSV G^{G124}	w*; UAS-GFP, UAS-VSV G^{G124E}/+; Repo-Gal4, TubP-Gal80^{ts}/+
TS Glia>GFP, VSV G^{D137L}	w*; UAS-GFP, UAS-VSV G^{D137L}/+; Repo-Gal4, TubP-Gal80^{ts}/+

Infections and CO_2 recovery assay

Virus stocks were grown in BsrT7 cells and purified to a concentration greater than 10^{11} PFU/mL via centrifugation and pelleting through a sucrose cushion. Indiana strain VSV, VSV-GFP, and VSV-Luc were gifts from Sean P. Whelan. Sindbis-GFP was a gift from Raul Andino. For infection, flies were anesthetized using CO_2. A minutien pin (Fine Science Tools) was swabbed in the concentrated stock of virus, and flies were pricked on one side of the thorax. Flies were returned to a normal air environment in vials containing standard cornmeal fly media. Survival was measured 3 hours later, and non-viable flies were excluded from the experiment. Alternatively, flies or *Anopheles gambiae* were injected with virus using Nanoject II (Drummond) in the thorax. Mosquitoes which did not survive 24 hours after injection were removed from the experiment.

For CO_2 recovery assay, JWatcher software was used to quantify the recovery rate of anesthetized flies. Recovery was defined by the ability of flies to stand upright on their legs. Simultaneously with the start of the timer, a blowgun (Genesee Scientific) was used to fill fly vials with pure CO_2 for 30 seconds. Flies were then transferred into fresh food vials and laid out evenly along the vial wall for observation. Flies were observed for a maximum of 15 minutes. The same procedure was used for N_2 anesthesia, except anesthesia lasted for 60 seconds to accommodate for the longer time required for flies to become anesthetized. More than 20 flies were used for each experimental condition for recovery assays and survival data.

Behavioral assays

Flies were assessed for behavioral abnormalities using the startle induced negative geotaxis assay. The method used has been previously described (Barone and Bohmann, 2013). Briefly, flies were collected using CO_2 anesthesia and infected with VSV or mock treated. For each experimental condition ≥15 flies were transferred to fresh food vials and were incubated at 29°C. Two and three days later, flies were transferred to two empty vials taped together. Flies were acclimated to the environment for thirty minutes before negative geotaxis assay began. Flies were startled by tapping vials against the bench top for 10 seconds while being filmed. The films were used to calculate what percentage crawled at least 2 cm above the bottom of the vial 10 seconds after startling ended. After a 30 second rest, the assay was repeated 14 more times. The 15 replicates were averaged for an n=1.

Dcr2^{KO} flies were tested for gait dysfunction using FlyWalker analysis (Mendes et al., 2013). Flies were infected with VSV by consistently pricking one side of the thorax. Infections were incubated at 29°C. At 2 and 3 dpi, flies were placed in a chamber and filmed to detect light scatter of frustrated total internal reflection caused by footsteps contacting the surface of illuminated optical glass. FlyWalker software was used to measure multiple gait parameters.

Plaque assay

Flies were homogenized in serum-free DMEM (Life Technologies) and filtered through 0.45um filter spin columns (Millipore). Ten-fold dilutions were made of each sample and plated onto confluent Vero cells for VSV and BHK cells for SINV in 6-well plates. After a 1 hour incubation at 37°C with gentle shaking every 10 minutes, inoculum was removed and Minimal Essential Media (MEM) (ThermoFisher 11700077) supplemented with 0.12% sodium bicarbonate and 25mM HEPES, pH 6.7 was added onto cells. MEM contained 0.25% agarose for VSV plaque assays and 1% agarose for SINV plaque assays. After 18 hours of incubation Vero cells were fixed using buffered formalin (Sigma HT501128-4L), and BHK cells were fixed after 48 hours. Overlay was removed and cells were stained with 10% ethanol containing 0.1% crystal violet to quantify plaques.

Microscopy

Using two pairs of fine tweezers, brains were dissected from adult flies in PBS. Within a 15 minute span, brains were collected into a wire mesh container soaked in PBS and then were fixed for 20 minutes in PBS containing 4% paraformaldehyde. Sindbis-GFP infected fly brains were washed three times in PBS and then mounted onto slides using Prolong Gold antifade reagent if no other additional staining was necessary (Life Technologies). Otherwise, SINV-GFP dissections were treated the same as VSV-infected brains. Dissected, VSV infected brains were washed three times in PBS. Brains were then blocked in blocking buffer (PBS containing 0.5% bovine serum albumin and 0.2% Triton X-100) for 30 minutes. After three washes in wash buffer (PBS containing 0.2% Triton X-100), brains were stained with primary antibodies overnight at 4°C. After three 10-minute washes, brains were incubated in Alexafluor secondary antibodies (Life Technologies) diluted to 1:600 for 1.5 hours at room temperature. DRAQ5 staining was added for an additional 30-minute incubation. Brains were washed three more times at 10 minute intervals before being mounted to glass slides using Prolong Gold antifade reagent. Imaging was

done on a conventional fluorescent microscope or a Zeiss axiovert spinning disk confocal microscope, and ImageJ software was used for image analysis.

Quantitative real-time PCR

Flies were collected at 3/condition and homogenized in 1mL of RNAbee (Tel-Test, Inc., Cs-501B). To purify RNA, 200uL chloroform was added to each tube and shaken vigorously for 30 seconds. After a 15 minute incubation on ice, tubes were spun at >13,000g for 15 minutes at 4°C. Aqueous phase was collected and transferred to a fresh tube. An equal volume of isopropanol was added, and tube was shaken. RNA was allowed to precipitate at room temperature for 15 minutes before being spun again at >13,000g for 15 minutes at 4°C. RNA pellet was washed with 70% ethanol and spun in same conditions. Pellet was allowed to dry briefly before being dissolved in DEPC-treated water.

cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad 1708890) per manufacturer instructions. Generated cDNA was used for quantitative PCR. Reactions were prepared as per manufacturer instructions for iQ SYBR Green Supermix (Bio-Rad 1708882). Primers are listed in Table 3.2. Samples were run alongside a serially diluted standard derived from Dcr2^{KO} flies infected with VSV for 3 days at 29°C. PCR was run on the CFX384 Real-Time System (Bio-Rad).

Table 3.2 Primers used for quantitative real-time PCR. Oligonucleotide sequences are listed in 5' to 3' direction.

Primer	Sequence
VSV G Forward	CATTATTGCCCGTCAAGCTCAG
VSV G Reverse	CCGTCTGCTTGAATAGCCTTGTG
VSV L Forward	TGGTGGAACCGATATGCAAC
VSV L Reverse	ATGAGGGAATTGTGGGACTAAAGG
VSV M Forward	ATGAAGAGGACACTAGCATGGAG
VSV M Reverse	ATTCGGATCATAGGTGTCCATCTCG
VSV N Forward	GCAAATGAGGATCCAGTGG
VSV N Reverse	CAGGGCTTTCAAGGATAC
VSV P Forward	TCAGGCGGTAGGAGAGATAGATGAG
VSV P Reverse	CTAGTATGCTCTTCCACTCCGTCC
RpL32 Forward	CGATATGCTAAGCTGTCGCAC
RpL32 Reverse	CGATGTTGGGCATCAGATACTGTC

Statistics

Statistical details can be found in the results, figures, and figure legends. P-values <0.05 were considered statistically significant. All survival data was tested for statistical significance using the Gehan-Breslow-Wilcoxon test. Acute anesthesia recovery assay results were tested for statistical significance using the log-rank test. For each condition in survival and acute recovery assays, at least 20 flies were used. In acute recovery assay graphs, each data point represents one fly.

For viral titer measurements and qPCR data, the unpaired t-test was used to test for statistical significance. For viral titer data, 3 samples of 5-10 flies were collected for each condition for one experiment unless otherwise noted. Data from three experiments are shown in aggregate. For qPCR data, one sample for each condition was collected, run in triplicate, and averaged. Averages from three experiments are shown in aggregate.

Behavioral assays (negative geotaxis and FlyWalker analysis) utilized two-way ANOVA to test for statistical significance. For one negative geotaxis experiment, each condition had three cohorts of >15 animals/vial. Flies from each cohort were startled and observed 15 times. The 15 replicates were averaged for an n=1. For FlyWalker studies, n refers to the number of animals recorded for each condition. Mean and standard error are represented on graphs.

All error bars represent standard error. Prism software was used for statistical analysis.

RESULTS

Vesicular stomatitis virus infection elicits a rapid sensitivity to CO_2 anesthesia

Vesicular stomatitis virus (VSV) is the best-studied rhabdovirus. A study from the early 1970s suggested that, like sigma virus, VSV-infected *Drosophila* are sensitive to CO_2 (Bussereau, 1973). To confirm these findings, we induced a systemic infection by pricking flies in the thorax using a pin that had been swabbed in liquid stock of virus containing 10^{11} plaque forming units (PFU)/mL. This method resulted in the delivery of several hundred PFU of virus into each fly, as assessed by plaque assay immediately following infection (Figure 3.1A). When infected flies were incubated for 7 days at 29°C and then anesthetized with CO_2 for 30 seconds, only 30% of VSV-infected flies remained viable the next day. Uninfected flies and flies not exposed to CO_2 did not display this pronounced loss in viability after CO_2

anesthesia (Figure 3.1B). Thus, VSV-infection of *Drosophila* provides a model to study infection-induced sensitivities to environmental stress.

In considering how VSV could elicit a life-threatening sensitivity to CO_2, the length of time between CO_2 exposure and death assessment (1 day) yielded multiple explanations. For example, CO_2 exposure could have rendered flies temporarily immunodeficient (Helenius et al., 2009), which would result in enhanced viral replication and death over the subsequent 24 hours. Alternatively, CO_2 exposure could result in some form of acute trauma, independent of subsequent viral replication. These models can be distinguished kinetically, as the former model depends on enhanced viral replication after CO_2 exposure. If correct, several hours would likely need to pass before any phenotypes associated with CO_2 sensitivity would be revealed. In contrast, the latter model of acute trauma may manifest itself in a short period of time. We therefore examined the earliest possible phenotype associated with CO_2 exposure: the ability of flies to wake and stand on their legs. To recover from CO_2 anesthesia, as defined by returning to an upright position on their legs, uninfected flies required 3.7 minutes (median recovery time) (Figure 3.1C). In contrast, VSV-infected flies exhibited a delayed recovery from anesthesia with a 6.2 minute median recovery time (Figure 3.1C). Thus, in addition to the long-term consequence of CO_2 exposure (lethality), an acute sensitivity to this stress is evident. The speed by which this phenotype is revealed following CO_2 anesthesia (minutes) eliminates the possibility that an increase in viral replication is responsible. We therefore conclude that CO_2 causes some acute change in the physiology of VSV-infected flies that leads to death.

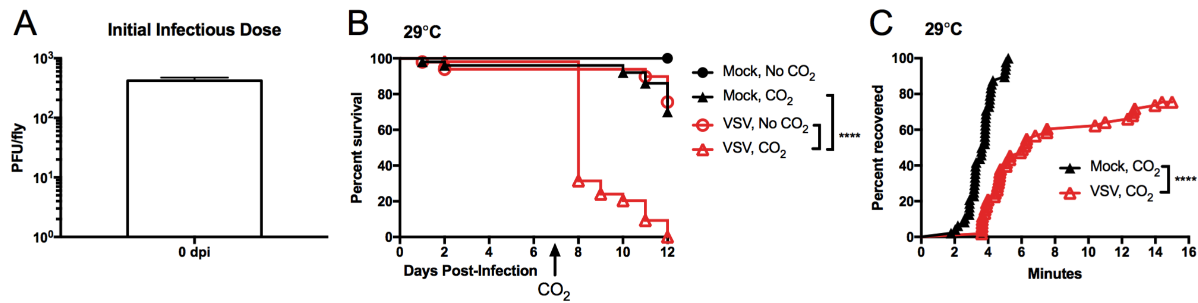

Figure 3.1 Vesicular stomatitis virus infection sensitizes *Drosophila melanogaster* to carbon dioxide anesthesia. (A) *Drosophila melanogaster* were infected using standard method of pricking the thorax with a pin dipped in VSV. Flies were immediately collected following infection and homogenates were titrated on Vero cells to measure plaque forming units (PFU) (n=3). (B, C) Adult flies were pricked with a sterile pin or infected with VSV. Survival was monitored during the course of infection (B), and acute recovery to 30 seconds of CO_2 anesthesia was monitored 7 dpi (C). One experiment representative of three is shown. ****$p<0.0001$

To determine if VSV could elicit similar disease phenotypes in other hosts, we performed infections of the clinically significant insect vector *Anopheles gambiae* (Figure 3.2). Similar to *Drosophila*, the combination of VSV infection and CO_2 anesthesia elicited a significant delay in recovery from CO_2 anesthesia ($p<0.0001$, Figure 3.2A), with life-threatening consequences observed 24 hours later (Figure 3.2B). VSV-induced CO_2 sensitivity may therefore be a common feature of infections with this pathogen. In contrast to other models of infection-induced sensitivities to subsequent stress, which are revealed over the course of several days (Jamieson et al., 2013), the speed of disease manifestation in VSV-infected flies provides a unique model to understand acute sensitivities to infection-induced stress.

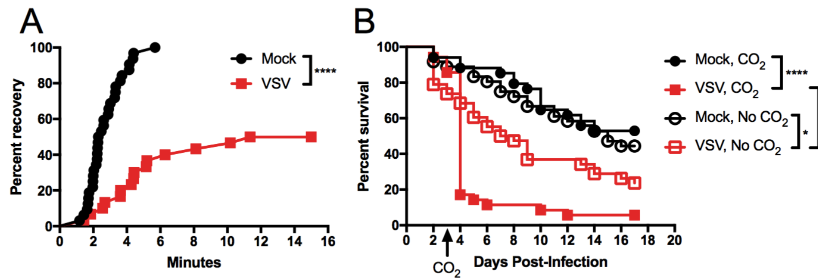

Figure 3.2 *Anopheles gambiae* also develop CO_2 anesthesia sensitivity after VSV infection. *Anopheles gambiae* mosquitos were injected with buffer or VSV (10^7 PFU) in the thorax. After 3 days, animals were anesthetized for 30 seconds with CO_2. Acute recovery after anesthesia (A) and survival (B) during the course of infection were monitored. One of three representative experiments is shown. *p<0.05; ****p<0.0001

The fly innate immune system regulates CO_2 sensitivity during asymptomatic VSV infection.

To better understand how VSV causes CO_2 sensitivity, we sought methods to modulate the productivity of the viral infection. This was addressed in two ways. First, we took advantage of the fact that infected flies incubated at 21°C are more resistant to VSV infection than those incubated at 29°C. Consequently, flies incubated at 21°C have decreased viral titers than their counterparts incubated at 29°C (p<0.0001, Figure 3.3A). A second means of altering infection productivity is to manipulate the antiviral RNA interference (RNAi) pathway in flies. Using a loss of function mutant of Dicer-2 ($Dcr2^{KO}$), we observed more than a 100-fold increase in the production of infectious viral particles compared to wild type (WT) flies (p<0.0001, Figure 3.3B). Thus, temperature changes and RNAi manipulation can be used to assess the influence of viral replication on CO_2 sensitivity. In contrast to infections at 29°C, VSV-infected WT flies incubated at the lower temperature of 21°C displayed no immediate sensitivity to CO_2 anesthesia (Figure 3.3C). Under these conditions, no lethality was observed in VSV-infected flies 24 hours after CO_2 exposure (Figure 3.3C). These data suggest that limiting VSV replication renders the host insensitive to CO_2. If this prediction is correct, then flies lacking the antiviral RNAi pathway should be sensitive to CO_2, even at 21°C. This hypothesis was tested by performing VSV infections of $Dcr2^{KO}$ flies

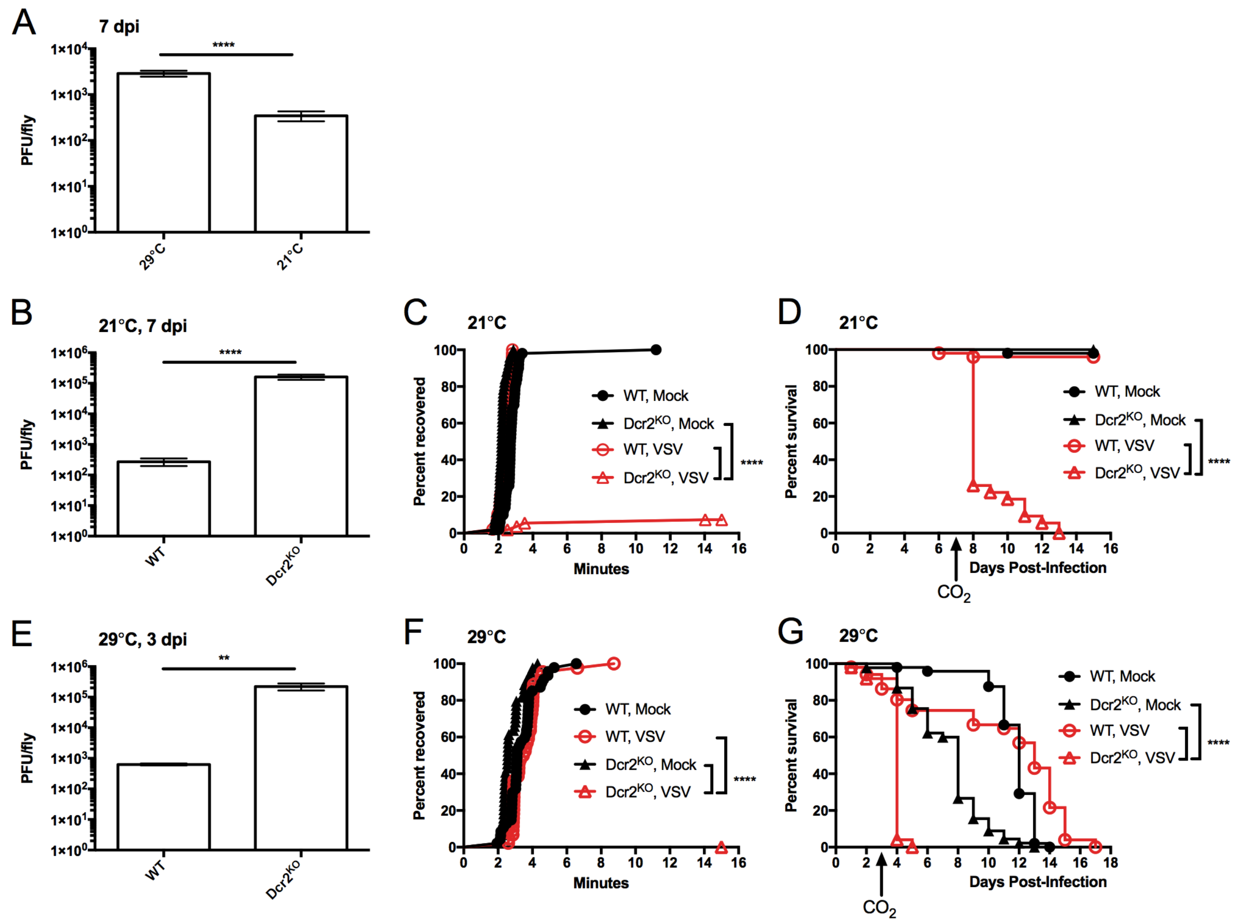

Figure 3.3 Parameters affecting viral load modulate CO_2 sensitivity. (A) VSV-infected WT flies were incubated at 29°C or 21°C. At 7 dpi, flies were collected to measure PFU. (n=3) (B) VSV titers were compared between 21°C infections of WT and $Dcr2^{KO}$ flies collected 7 dpi. (n=3) (C, D) WT and $Dcr2^{KO}$ flies were infected with VSV or received mock treatment. Acute recovery from CO_2 anesthesia was monitored 7 dpi (C), and survival was tracked during the course of infection (D). One experiment representative of three is shown. (E) VSV titers were compared between 29°C infections of WT and $Dcr2^{KO}$ flies collected 3 dpi (n=3). (F, G) VSV infected and mock treated WT and $Dcr2^{KO}$ flies were incubated at 29°C. At 3 dpi, flies were subjected to CO_2 anesthesia and monitored for acute recovery (F). Flies were monitored for survival during infection (G). One experiment representative of three is shown. **$p<0.01$; ****$p<0.0001$

at 21°C. VSV- infected Dcr2^{KO} flies were highly sensitive to CO_2 (Figure 3.3C). Indeed, less than 5% of Dcr2^{KO} flies recovered from CO_2 anesthesia, and these flies exhibited a precipitous drop in survival in the day following treatment (Figure 3.3D). Thus, two means of modulating viral replication (temperature and RNAi inactivation) resulted in differential sensitivity to CO_2 exposure. When high temperature was combined with Dcr2 deficiency, a severe sensitivity to CO_2 was observed. At 29°C, more than a 100-fold increase in viral titers could be observed in Dcr2^{KO} flies compared to WT flies ($p<0.01$, Figure 3.3E). Under these conditions, acute and lethal CO_2 sensitivity were observed in Dcr2^{KO} flies 3 days post-infection (dpi) (Figure 3.3F, G). Taken together, these data establish that the productivity of viral infection positively correlates with CO_2 sensitivity.

The observed sensitivity to CO_2 was most severe in Dcr2^{KO} flies incubated at 29°C. This sensitivity to CO_2 anesthesia was evident at 3 dpi, whereas anesthesia applied 1 or 2 dpi had no effects on survival (Figure 3.4A). Thus, a clear division in CO_2 sensitivity exists between 2 and 3 dpi within Dcr2^{KO} flies. We utilized this distinct threshold to determine if Dcr2^{KO} flies displayed symptoms of disease before and after onset of CO_2 sensitivity, without administration of CO_2 anesthesia. The startle-induced negative geotaxis assay assesses climbing behavior after flies have been tapped down to the bottom of a vial (Barone and Bohmann, 2013). We first determined if uninfected Dcr2^{KO} had any climbing deficiencies compared to WT flies. Uninfected Dcr2^{KO} were not defective for climbing activity, and actually performed better than WT flies after being incubated at 29°C for 2 and 3 days (two-way ANOVA, Figure 3.4B). We proceeded to test VSV-infected Dcr2^{KO} flies for climbing activity 2 and 3 dpi at 29°C and compared them to mock treated flies. No significant differences were observed between infected and uninfected flies, and no changes in climbing activity were observed from 2 dpi to 3 dpi (two-way ANOVA, Figure 3.4C). These results suggest that, prior to CO_2 anesthesia, VSV infection in *Drosophila* is asymptomatic.

To complement these behavioral analyses, an optical recording method and FlyWalker software were used to rigorously test the possibility that VSV infection alone alters coordinative function (Mendes et al., 2013). The experimental setup detects refracted light from footsteps of flies as they walk across a lighted platform. The data is recorded as videos at 300 frames per second and further processed to measure parameters such as speed and leg positioning in relation to the body. We compared mock and VSV-infected Dcr2^{KO} flies at the crucial time points of 2 and 3 dpi, when infections change from

anesthesia insensitivity to sensitivity. In comparison to mock treatment at 2 and 3 dpi, VSV infections resulted in no changes in footstep distances, gait of animals, and walking speed (Figure 3.4D-I). These behavioral analyses therefore support the idea that VSV causes asymptomatic infections in *Drosophila*, and that CO_2 treatment converts these infections into a life-threatening risk to the host.

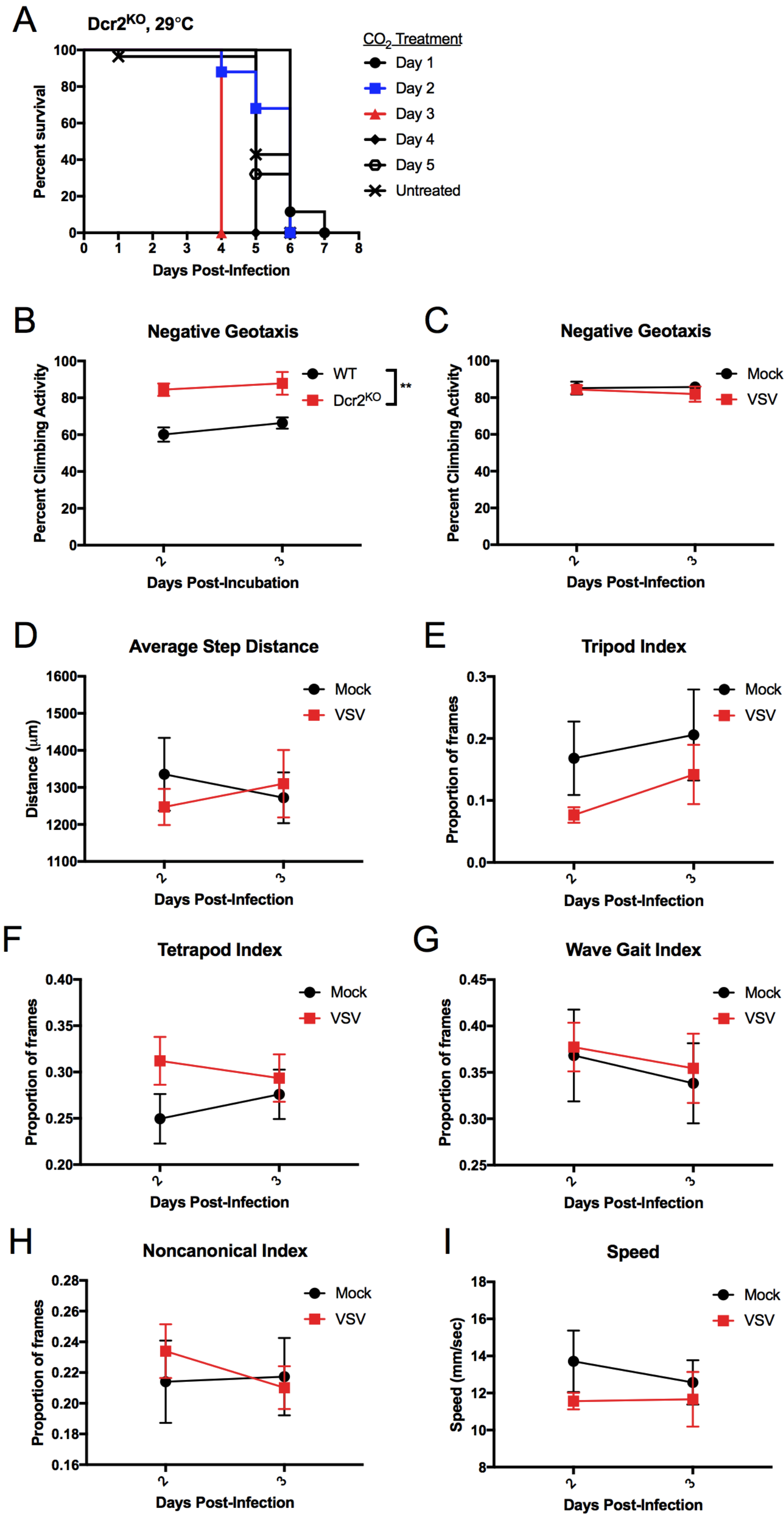

Figure 3.4 Climbing behavior and gait of animals are unaffected by VSV infection.

Figure 3.4 (Continued) (A) $Dcr2^{KO}$ flies were infected with VSV and kept at 29°C. Flies were anesthetized at indicated days with CO_2 for 30 seconds or left untreated. Survival was monitored. (B) WT and $Dcr2^{KO}$ flies were pricked on the thorax and then incubated at 29°C. On days 2 and 3, flies were assessed for their ability to climb up the vial after being startled. No statistical significance was observed between the two time points assessed (two-way ANOVA). A statistically significant difference in climbing activity was observed across the two genotypes (**$p<0.01$, two-way ANOVA, n=3). (C) $Dcr2^{KO}$ flies were infected with VSV or mock treated and incubated at 29°C. Flies were assessed for climbing activity after being startled. No statistically significant differences were observed between infected and mock-treated flies nor between days 2 and 3 post-infection (two-way ANOVA, n=3). (D-I) $Dcr2^{KO}$ flies were all pricked on the same side of the thorax and were either infected with VSV or mock treated. Flies were incubated at 29°C and tracked longitudinally 2 and 3 dpi. Video recordings of fly gait behavior were analyzed using FlyWalker software. No statistical differences were observed between mock (n=8) and VSV (n=11) infected groups at each time point for average step distance (D), tripod index (E), tetrapod index (F), wave gait index (G), noncanonical index (H), and speed (I) (t-test).

An inability to tolerate a state of hypercapnia underlies VSV-induced CO_2 sensitivity

Having established the contribution of viral infection to the sensitivity to an environmental stress, we sought to understand the specific role of CO_2 in these phenotypes. CO_2 anesthesia has two simultaneous effects. Blowing pure CO_2 into a fly vial creates a hypercapnic environment and simultaneously depletes oxygen, creating a hypoxic environment. To differentiate between the effects of hypercapnia and hypoxia, nitrogen gas (N_2) was used as an alternative anesthetic. Blowing pure N_2 into the fly vial reduces oxygen levels without increasing CO_2 levels. Thus, N_2 induces hypoxia but not hypercapnia, whereas CO_2 induces both. The comparison of N_2 and CO_2 anesthesia therefore allows us to distinguish the role of hypoxia from hypercapnia in infection-induced sensitivity to stress. As we have observed, VSV-infected WT flies that were incubated at 21°C recovered from CO_2 anesthesia without

delay (Figure 3.5A). In contrast, $Dcr2^{KO}$ flies that were infected with VSV were highly sensitive to CO_2, with greater than 90% of flies being unable to recover from this anesthesia ($p<0.0001$, Figure 3.5A). Under these conditions, the median recovery time shifted from 2.5 minutes (WT) to >15 minutes ($Dcr2^{KO}$). When flies were anesthetized with N_2, the median recovery times for WT and $Dcr2^{KO}$ flies differed by 1 minute. While statistically significant ($p<0.0001$), this difference was modest (Figure 3.5A). In addition, a 70% drop in survival the day following anesthesia was only observed for $Dcr2^{KO}$ flies treated with CO_2 (Figure 3.5B). $Dcr2^{KO}$ flies treated with N_2 lived for several more days before finally succumbing to infection (Figure 3.5B) (Galiana-Arnoux et al., 2006). Because flies displayed little acute or long-term sensitivity to N_2 anesthesia, hypoxia is not likely responsible for the disease phenotypes associated with infection. We therefore conclude that the disease associated with CO_2 sensitivity results from infected flies being unable to tolerate a state of hypercapnia.

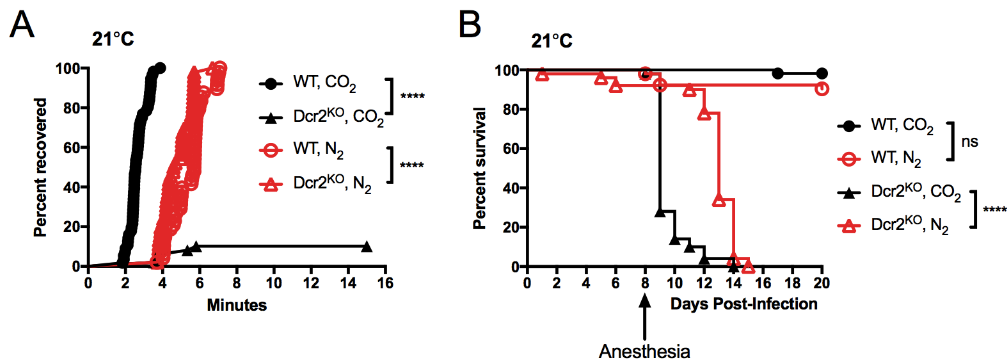

Figure 3.5 Anesthesia sensitivity is specific to hypercapnia. WT and $Dcr2^{KO}$ flies were infected with VSV and kept at 21°C. At 8 dpi, flies were anesthetized with CO_2 or N_2. Acute recovery (A) and survival (B) are shown. One experiment representative of three is shown. ****$p<0.0001$; ns, not significant

VSV must infect glial cells to elicit CO_2 sensitivity

By modulating infection temperature and host immune defenses, a correlation was observed, suggesting that high viral titers on the day of CO_2 administration causes lethality (Figure 3.3). To determine if high viral titers at the onset of infection are required for CO_2-induced lethality, we used an injector to deliver precise volumes into the thorax of flies with concentrations of VSV that spanned 1000-fold (Figure 3.6). Results 2 dpi provided the most insightful data. The group with the lowest infectious dose was lower in titer compared to the other groups at 2 dpi ($p<0.01$, multiple t-tests). Most of these flies were not CO_2 sensitive. Groups receiving higher virus doses had higher viral titers 2 dpi and were mostly CO_2 sensitive. Despite having high viral titers, some flies did not display CO_2 sensitivity (Figure 3.6). By 3 dpi, virtually all flies, regardless of infectious dose, were CO_2 sensitive. These results indicate that the initial dose of infectious virus influences disease progression, but viral titers in whole flies cannot fully explain CO_2 sensitivity. We therefore considered the possibility that VSV must infect a specific region in the fly in order to elicit CO_2 sensitivity.

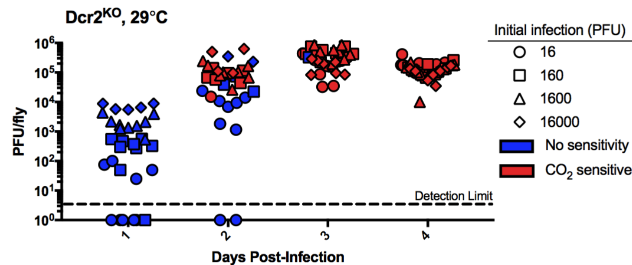

Figure 3.6 Viral titers cannot fully explain CO_2 sensitivity. Nanoject II was used to inject 9.2nL volumes of VSV into the thorax of Dcr2^{KO} flies at specified PFU. Infections were incubated at 29°C and anesthetized with 30 seconds of CO_2 at 1, 2, 3, and 4 dpi. Flies were observed for 5 minutes to determine which had delayed CO_2 anesthesia recovery before being collected to measure virus titers.

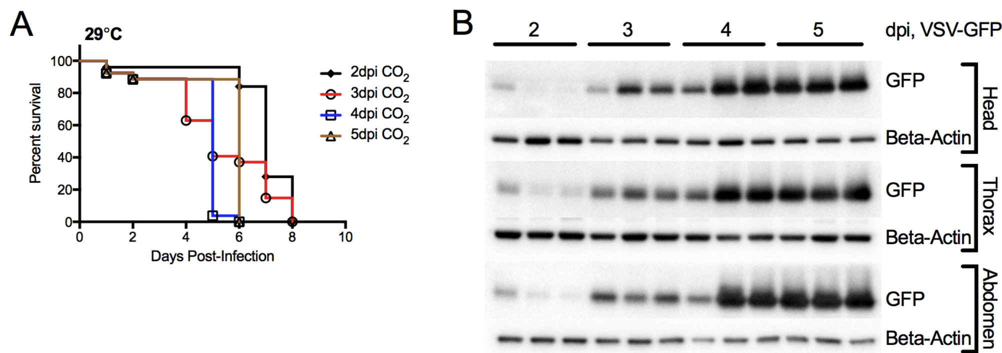

Figure 3.7 CO_2 sensitivity kinetics do not correlate with virus spread to a specific body segment. (A) Ago2^{KO} flies were infected with VSV-GFP via pricking of the thorax. Groups of infected flies were anesthetized with CO_2 on the indicated days post-infection and survival was recorded. One of three experiments is shown. (B) Ago2^{KO} flies were infected with VSV-GFP via pricking of the thorax. At indicated times post-infection, flies were segmented as heads, thoraxes, and abdomens, homogenized, and blotted for GFP protein expression. Each lane represents a single fly. One of three representative experiments is shown.

To identify sites with infected flies that support VSV replication, we monitored viral protein production in specific anatomical locations over time. A recombinant VSV-green fluorescent protein (GFP) reporter virus was used to infect Argonaute 2 (Ago2^{KO}) mutants, another RNAi gene required for antiviral defense (van Rij et al., 2006). At 29°C, Ago2^{KO} flies began showing signs of CO_2 sensitivity as early as 3 dpi, as assessed by a decrease in survival 24 hours after CO_2 anesthesia (Figure 3.7A). About 30% of animals were CO_2-sensitive 3 dpi, and by 4 dpi more than 90% of flies were CO_2-sensitive (Figure 3.7A). Using the GFP reporter to track VSV within infected flies, we assayed the three body segments (head, thorax and abdomen) for GFP expression via Western blotting. We found that the timing of CO_2 sensitivity onset correlated with strong GFP expression (Figure 3.7B). However, GFP protein did not preferentially accumulate in one body segment (Figure 3.7B), suggesting simultaneous virus spread throughout the body of the fly. To identify specific tissues affecting CO_2 sensitivity, we focused on the nervous system because many rhabdoviruses are neurotropic and CO_2 anesthesia acts directly on the nervous system (Nicolas and Sillans, 1989). Fluorescent microscopy confirmed that VSV could be detected in the brains

of CO_2 sensitive flies (Figure 3.8). To gain further clarity of the cell types infected, we used the Gal4/UAS overexpression system to drive expression of GFP in specific cell types of $Dcr2^{KO}$ flies (Brand and Perrimon, 1993). Flies were infected with a VSV-luciferase (VSV-Luc) reporter virus and explanted brains were stained with a luciferase-specific antibody. While VSV-Luc colocalized with GFP expressed by glia and neurons, no colocalization was observed with tracheal cells (Figure 3.9). Thus, VSV infects the two cell types of the nervous system, neurons and glia.

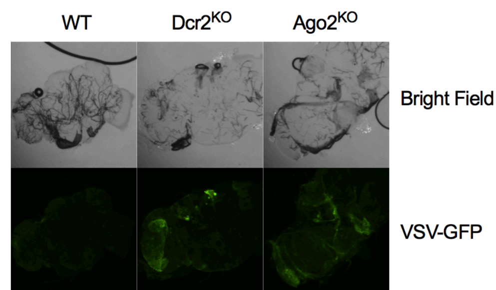

Figure 3.8 Fluorescent imaging detects virus in brains. WT and RNAi knockout flies were infected with VSV-GFP and incubated at 21°C for 11 days. Brains were dissected and stained for GFP expression. One of three representative experiments is shown.

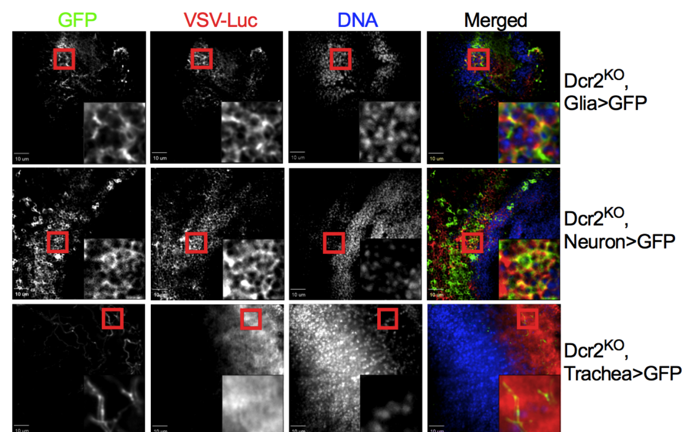

Figure 3.9 VSV infects neurons and glial cells. Dcr2^{KO} flies expressing GFP under the control of a glial, neuronal, or tracheal specific Gal4 were infected with VSV-Luc. After 11 days at 21°C, brains were dissected and stained for GFP and luciferase expression. DRAQ5 staining was used to detect nuclear DNA. Confocal images were taken with a 40X oil objective lens. Insets are 4X magnifications of selected areas.

We reasoned that if CO_2 sensitivity required infection of the nervous system, then preventing viral replication in the nervous system should render flies resistant to CO_2. To test this idea, Dcr2^{KO} flies were genetically altered such that Dcr2 function was rescued in either glia or neurons. Under these conditions, at 21°C, these cell types should restrict viral replication. In contrast, all other cells in the fly should support viral replication. When we restored Dcr2 function in neurons, 20% of neuronal Dcr2 rescue flies recovered within 5 minutes of anesthesia, whereas 10% of Dcr2 knockout flies recovered ($p<0.05$, Figure 3.10A). Restoring Dcr2 function to glial cells resulted in 70% of flies recovering within 5 minutes of CO_2 removal ($p<0.0001$, Figure 3.10A). In the day following CO_2 anesthesia, over 80% of Dcr2 knockouts were deceased, whereas over 80% flies with Dcr2 function rescued in glia or neurons remained viable (Figure 3.10B). VSV titers in flies were high for the three genotypes, indicating that VSV was still capable of replicating in other cell types despite being restricted from neurons or glia (Figure 3.10C). These data indicate that VSV replication in the nervous system is required to elicit sensitivity to CO_2.

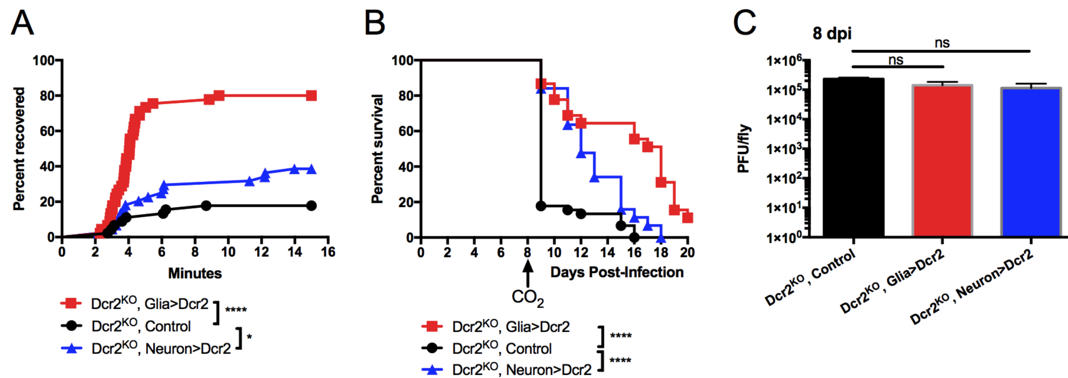

Figure 3.10 Inhibition of VSV infection in the nervous system inhibits CO_2 sensitivity. (A, B) $Dcr2^{KO}$ flies were bred to express functional Dcr2 in neurons or glia. Flies were infected with VSV and kept at 21°C. Flies were anesthetized with CO_2 at 8 dpi and kinetics of acute recovery were recorded (A). Survival throughout infection was tracked daily (B). One experiment representative of three is shown. (C) VSV titers were measured 8 dpi from animals incubated at 21°C (n=3). *$p<0.05$; ****$p<0.0001$; ns, not significant

To determine if VSV replication in the nervous system is sufficient to elicit CO_2 sensitivity, we inactivated the antiviral RNAi pathway in glial cells or neurons. This was accomplished by overexpressing a hairpin RNA to knock down expression of Ago2. Knocking down Ago2 in specific cell types renders these cells susceptible to VSV infection, while the remaining host tissues are resistant to infection at 21°C. Flies with Ago2 knocked down specifically in neurons behaved like WT flies, recovering from CO_2 anesthesia within 5 minutes (Figure 3.11A). Although a statistically significant difference was observed between WT and neuronal knockdown groups ($p<0.001$), the median recovery times were negligible (2.52 and 2.354 minutes, respectively). Knockdown of Ago2 in glia caused a delay in recovery, and a combination knockdown of Ago2 in glia and neurons resulted in recovery kinetics comparable to flies with Ago2 knocked down throughout the entire body (Figure 3.11A). Compared to control flies expressing Ago2, survival was decreased following CO_2 anesthesia for flies with Ago2 knocked down ubiquitously and in neurons and glia ($p<0.0001$, Figure 3.11B). When viral titers were measured at the time point when flies were anesthetized with CO_2, low levels of infectious virus were detected in WT flies and flies with

Ago2 knocked down in neurons (Figure 3.11C). In contrast, viral titers were increased in flies with Ago2 knocked down in glia and were further increased in flies with Ago2 knocked down in neurons and glia ($p<0.001$, Figure 3.11C). These collective data support a model whereby VSV infection of neurons and glia is sufficient to cause complete sensitivity of flies to CO_2 and that relative to neurons, infection of glia is more important in causing CO_2 sensitivity.

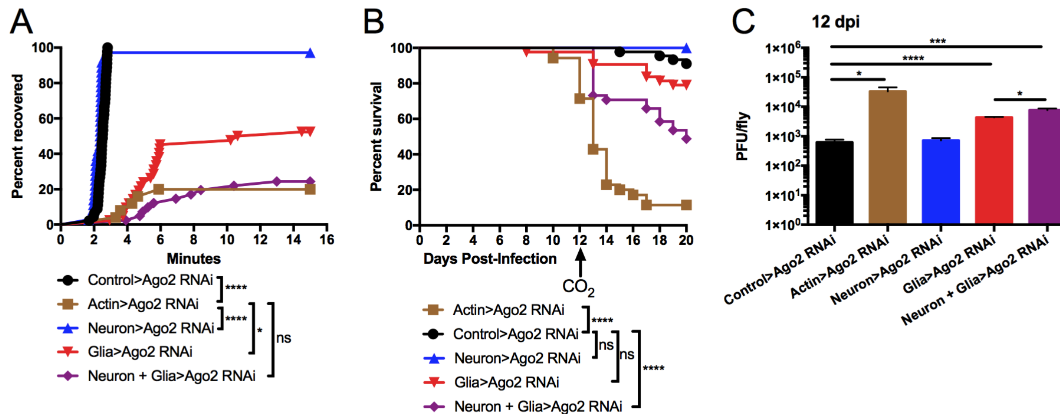

Figure 3.11 VSV infection of glial is sufficient to cause CO_2 sensitivity. (A, B) Ago2 expression was knocked down ubiquitously or in specific cell types. VSV infected flies were incubated at 21°C. At 12 dpi, flies were assessed for acute recovery from CO_2 anesthesia (A) and survival (B) throughout the experiment. One experiment representative of three is shown. (C) Infected animals kept at 21°C for 12 days were homogenized to measure VSV titers (n=3). *$p<0.05$; ***$p<0.001$; ****$p<0.0001$; ns, not significant

Not all neurotropic viruses elicit CO_2 sensitivity

During infections, symptoms of disease can result from stress on a vital tissue in the host. Sensitivity to CO_2 anesthesia was caused by VSV infection of the nervous system. Thus, perturbation of the nervous system could be the root cause of CO_2 sensitivity. VSV, the inducer of nervous system perturbation, could potentially be replaced by a similar stressor. To determine the relative significance of nervous system perturbation, we considered whether another neurotropic pathogen would elicit a similar inability to tolerate CO_2. To address this possibility, flies were infected with a recombinant togavirus, Sindbis virus (SINV) encoding a GFP reporter gene. After 3 dpi, virally expressed GFP was detected in

brains dissected from infected $Dcr2^{KO}$ animals but not in WT animals (Figure 3.12A). Confocal imaging of infected $Dcr2^{KO}$ brains detected virus in neurons and additional cell types in the brain (Figure 3.12B). $Dcr2^{KO}$ flies exhibited a 1000-fold increase in viral titers compared to WT flies ($p<0.01$, Figure 3.12C). Thus, like VSV, SINV replication is restricted by the RNAi pathway and can disseminate to the brain. However, $Dcr2^{KO}$ flies did not exhibit any signs of CO_2 sensitivity when infected with SINV (Figure 3.12D, E). Median recovery time of SINV infected $Dcr2^{KO}$ flies was within 1 minute of all other groups (Figure 3.12D). Additionally, SINV-infected $Dcr2^{KO}$ flies did not exhibit increased lethality 24 hours after anesthesia (Figure 3.12E). Thus, despite SINV also infecting the nervous system, CO_2 sensitivity is specific to rhabdoviruses such as VSV and sigma (L'Heritier, 1948). Thus, CO_2 sensitivity is unlikely to be the result of general perturbation of the nervous system. Instead, CO_2 sensitivity is pathogen specific.

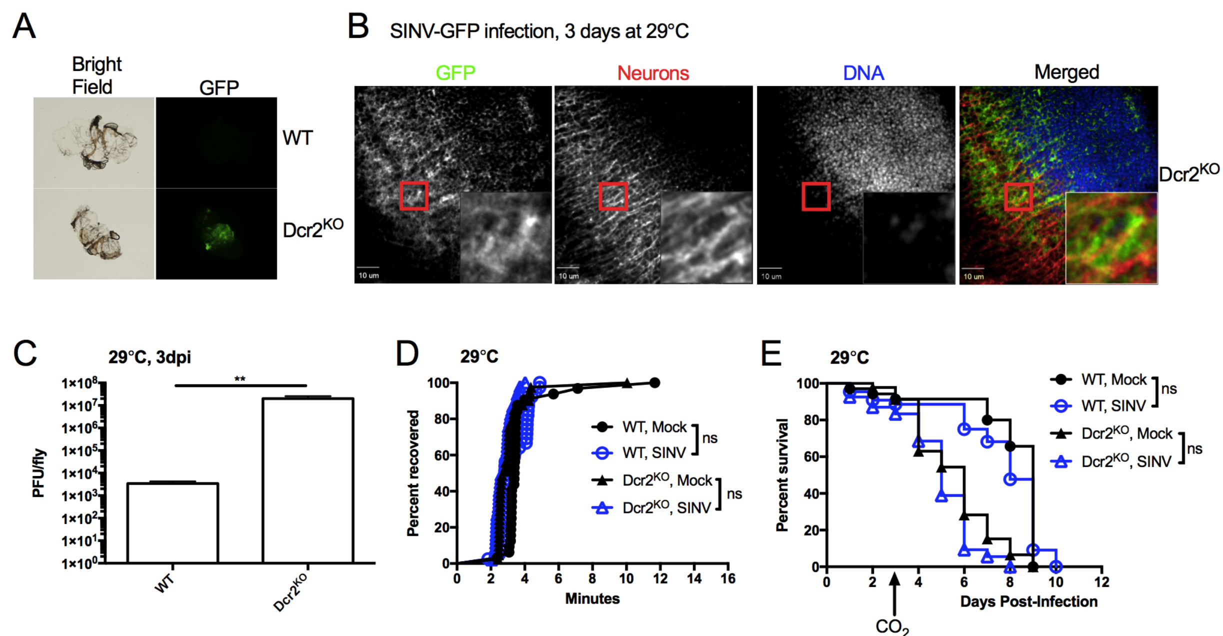

Figure 3.12 Sindbis virus infection does not cause CO_2 sensitivity. (A) Flies were pricked in the thorax with a pin swabbed in a concentrated stock of reporter virus, SINV-GFP (10^{11} PFU/mL), and infected flies were incubated at 29°C. At 3 dpi, brains were dissected and imaged for viral GFP expression. Representative dissections from one of three experiments are shown. (B) $Dcr2^{KO}$ flies were infected with SINV-GFP for 3 days at 29°C. Explanted brains were fixed and stained with antibodies against GFP and HRP to detect virus and neurons, respectively. DRAQ5 staining was used to detect nuclear DNA. Insets are 4X magnifications of selected areas. (C) Flies were injected in the thorax with 10^6 PFU of SINV. After 3 days at 29°C, viral titers were measured from infected flies (n=3). (D, E) Flies were injected with buffer or 10^6 PFU of SINV and incubated at 29°C. At 3 dpi, flies were subjected to CO_2 anesthesia and acute recovery (D) and survival (E) were assessed. One of three representative experiments is shown. **$p<0.01$; ns, not significant

A single viral protein, independent of infection, causes a lethal sensitivity to CO_2

The specificity of CO_2 sensitivity to VSV could be explained by a unique pathology associated with the VSV infectious cycle, but it could also be explained by the actions of a protein that is common to rhabdoviruses, independent of infection. This latter idea is unusual, as models of virus-induced disease sensitivity are thought to result from a productive viral infection that alters cellular homeostasis (Jamieson et al., 2013; Novo-Veleiro et al., 2016). It should therefore be impossible to dissociate VSV infection from sensitivity to CO_2. The simplicity of the VSV genome provides an opportunity to test this hypothesis: that a single viral protein may cause disease sensitivity. Transgenic lines of flies were therefore generated, each of which encode a single gene from the VSV genome. Because they were left untagged, detection of protein production via western analysis would not provide consistent comparisons of each protein. Instead, we used qPCR analysis to confirm transcription of the viral transgenes driven by heat shock-Gal4. After 8 days of incubation at 29°C, gene specific mRNAs were quantified. This analysis revealed that VSV G and VSV L transgenes were expressed at comparable levels. In contrast, the other three VSV transgenes are expressed at levels that differ from that of VSV G and VSV L ($p<0.05$, Figure 3.13A). Western analysis of VSV G and VSV M demonstrated that these proteins could be detected in transgenic and infected flies, but transgene expression was low compared to viral infection (Figure 3.13B). Despite low expression relative to viral infection, VSV G expressing transgenic flies exhibited delayed acute recovery from CO_2 anesthesia, and these flies had a 70% decrease in survival 24 hours later (Figure 3.13C, D). In contrast, expression of the L, M, N, and P genes from VSV in transgenic flies did not induce characteristic acute or lethal phenotypes upon exposure to CO_2 (Figure 3.13C, D). Expression of L is perhaps the best comparison to G since no significant difference in mRNA expression was found by qPCR ($p=0.4666$, Figure 3.13A). A single viral protein (VSV G) is therefore sufficient to induce the same sensitivity to CO_2 that is observed following VSV infection.

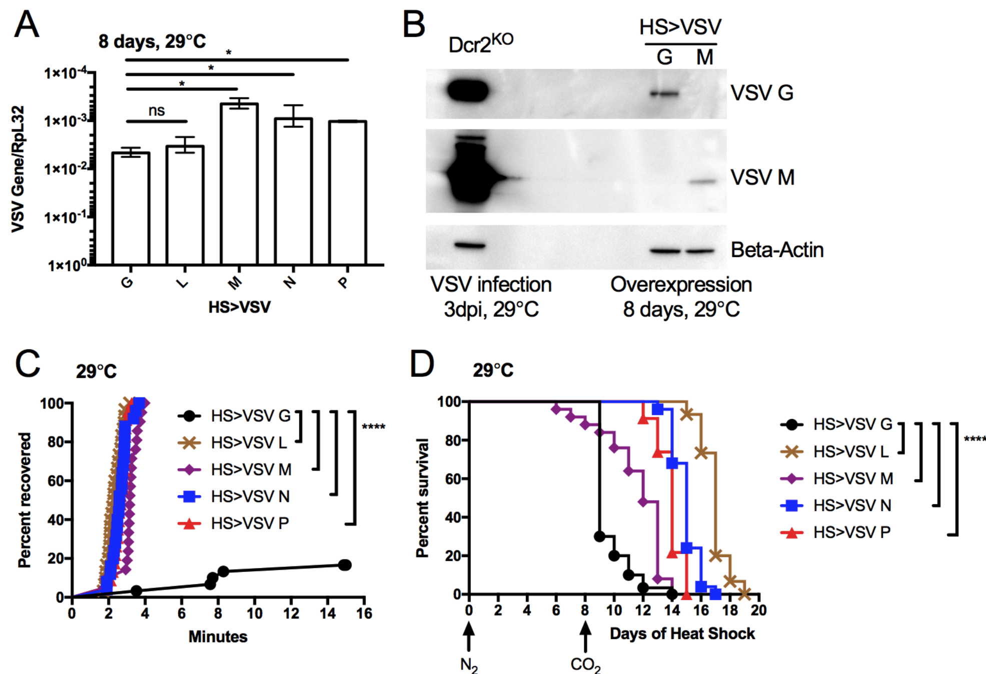

Figure 3.13 VSV G expression alone is sufficient to elicit CO_2 sensitivity. Flies were reared at 18°C. Flies were collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. (A) VSV transgene expression was driven by a heat shock-Gal4 (HS). After 8 days at 29°C, RNA was purified from flies and gene-specific mRNA was measured by qPCR (n=3). (B) Dcr2^{KO} were infected with VSV and incubated for 3 days at 29°C. VSV G and M transgene expression was driven by HS by incubating flies at 29°C for 8 days. Protein lysates of samples were western blotted for VSV G and M expression. Beta-actin was used as a loading control. (C, D) Flies were incubated at 29°C for 8 days to drive expression of VSV genes. Flies were subjected to CO_2 anesthesia to assess acute recovery (C) and then were returned to 29°C to continue monitoring survival (D). One experiment representative of three is shown. *p<0.05; ****p<0.0001; ns, not significant

VSV infection of glial cells is necessary and sufficient to induce CO_2 sensitivity (Figure 3.10, 3.11). To determine if VSV G transgene expression recapitulated these attributes of infection-induced CO_2 sensitivity, we expressed VSV G in glial cells. VSV G protein was readily produced in glia (Figure 3.14A), and expression of VSV G in glia was sufficient to induce acute recovery delays and death following CO_2 anesthesia (Figure 3.14B, C). To determine the specificity of these phenotypes, VSV G was also expressed in the fat body (Figure 3.14A). Fat body expression of VSV G did not result in acute recovery delays to CO_2 or survival defects (Figure 3.14B, C). Thus, CO_2 sensitivity specifically arises due to expression of VSV G in glial cells.

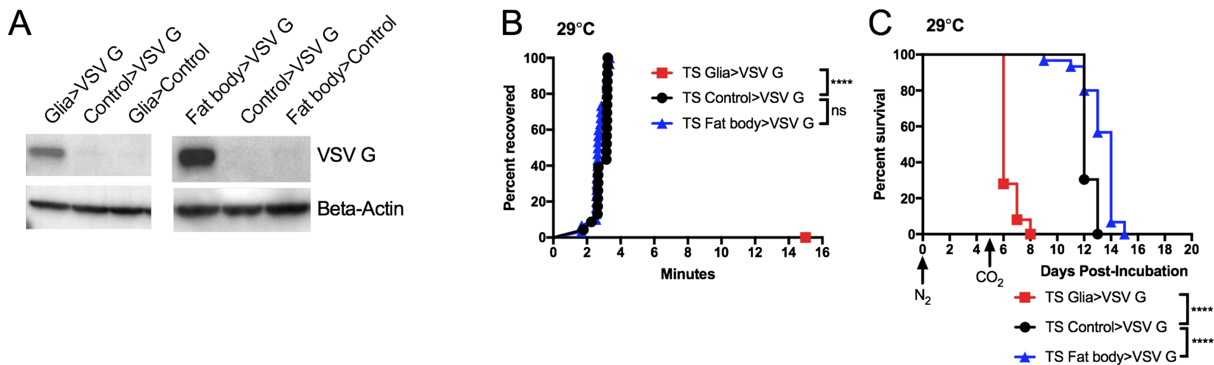

Figure 3.14 VSV G expression in glial cells is sufficient to induce CO_2 sensitivity. Flies were reared at 18°C and collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. (A) Glia and fat body-specific Gal4 transgenes drove VSV G expression. Flies were compared to ones containing only a Gal4 driver or VSV G transgene as controls. One of three representative experiments is shown for each blot. (B, C) A temperature sensitive transcriptional inhibitory protein (TS) repressed VSV G expression in glial and fat body cells until flies were incubated at 29°C. After 5 days at 29°C, flies were subjected to CO_2 anesthesia. Acute recovery to anesthesia (B) was assessed and survival (C) during the course of heat shock was monitored. One experiment representative of three is shown. ****$p<0.0001$; ns, not significant

pH-dependent fusogenic activity of VSV G promotes glia-neuron fusion and acute neuro-trauma

To understand how VSV G results in CO_2 sensitivity, we returned to the observation that CO_2 anesthesia causes hypercapnia. Hypercapnia is defined as an increase in CO_2, which results in acidification of the exposed tissues and/or fluids. This property was of interest because VSV G causes cell-cell fusion (syncytia formation) at acidic pH (Florkiewicz and Rose, 1984; Riedel et al., 1984). CO_2 can have an acidifying effect whereas N_2 anesthesia does not. We therefore hypothesized that the acute sensitivity to CO_2 was the result of syncytia formation induced by VSV G. To test this hypothesis functionally, we compared wild type VSV G (G^{WT}), which has a fusion threshold at a pH of 6.3, to two fusion defective VSV G mutants. These mutants were chosen because they encode full-length proteins that display no defect in folding or transport to the plasma membrane (Fredericksen and Whitt, 1995, 1996). A G→E mutation at amino acid position 124 (VSV G^{G124E}) causes fusion to occur at a pH of 5.5 or lower (Fredericksen and Whitt, 1995). A milder mutation, VSV G^{D137L} allows fusion at pH of 5.7 or lower (Fredericksen and Whitt, 1996). Transgenic flies were engineered to express VSV G^{WT} or the mutant alleles as a single copy from the same recombinogenic site on chromosome 2. The alleles were expressed at comparable levels using a heat shock-Gal4 (Figure 3.15A). We then tested whether VSV G fusion activity correlated with CO_2 sensitivity. As expected, VSV G^{WT} flies displayed acute recovery and survival defects in response to CO_2 anesthesia (Figure 3.15B, C). While VSV G^{D137L} mutants were also CO_2 sensitive, VSV G^{G124E} expressing flies did not display any CO_2 sensitivity (Figure 3.15B, C). The fusogenic activity of VSV G is therefore responsible for CO_2 sensitivity, and the difference observed between the two mutants suggests that CO_2 anesthesia acidifies pH between 5.7 and 5.5.

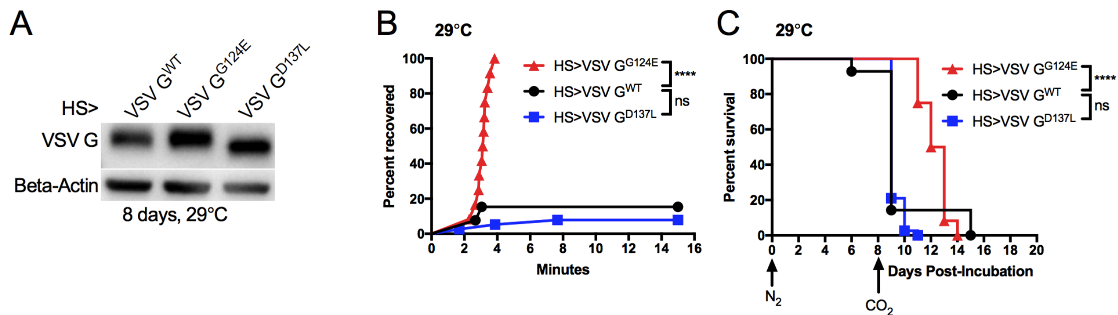

Figure 3.15 Modulating the VSV G fusion activity threshold abrogates CO_2 sensitivity. Flies were reared at 18°C and collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. (A) Expression of WT and fusogenic mutants of VSV G were induced via 29°C incubation for 8 days. Flies were collected and VSV G expression was detected by Western blotting. One of three representative experiments is shown. (B, C) Flies were heat shocked at 29°C for 8 days to induce expression of VSV G^{WT}, G^{G124E}, or G^{D137L} before being assessed for acute recovery to CO_2 anesthesia (B). Flies were then returned to the 29°C incubator to continue measuring viability (C). One experiment representative of three is shown. ****$p<0.0001$; ns, not significant

As infection of glial cells is critical in causing recovery delays and death following CO_2 anesthesia, we examined the influence of the fusion-defective VSV G mutants in these cells. VSV G^{WT}, G^{G124E}, and G^{D137L} expression were induced in glia for 5 days. While G^{WT} and G^{D137L} were sufficient to induce CO_2 sensitivity, the G^{G124E} fusion mutant expressed in glia could not cause sensitivity to CO_2 (Figure 3.16A, B). N_2 anesthesia, which does not have acidifying effects, did not result in acute recovery delays and precipitous death within a day following anesthesia (Figure 3.16A, B). Thus, expression of a fusion competent VSV G is sufficient to recapitulate the hallmarks of infection-induced CO_2 sensitivity, in that expression in glia is sufficient and the disease is not elicited by N_2 anesthesia.

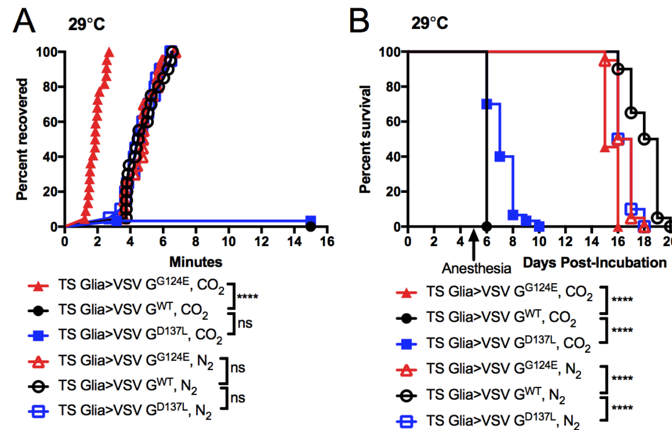

Figure 3.16 Neuro-trauma is mediated by a combination of acidifying CO_2 anesthesia and fusogenic VSV G expressed by glial cells. Flies were reared at 18°C. Flies were collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. Flies were incubated for 5 days at 29°C to induce VSV G expression. Flies were subjected to 30 seconds of CO_2 or 60 seconds of N_2 to assess recovery rate (A). Flies were then returned to 29°C incubation to continue tracking survival (B). One experiment representative of three is shown. ****p<0.0001; ns, not significant

Our functional analysis indicates that upon CO_2 treatment, VSV G is likely influencing some aspect of glial cell physiology that is detrimental to host viability. One explanation is that CO_2-induced membrane fusion initiates changes in glial physiology that result in apoptotic cell death. Alternatively, CO_2 induced fusion could cause immediate, non-regulated trauma by disrupting glial cell function. To distinguish these possibilities, the apoptosis reporter transgene, Apoliner, was utilized (Bardet et al., 2008). Apoliner encodes a red fluorescent protein connected to GFP via an amino acid sequence that can be cleaved by caspase enzymes that mediate apoptosis. VSV G and Apoliner were expressed in glial cells to induce CO_2 sensitivity. Flies were exposed to 30 seconds of CO_2 anesthesia and collected immediately, 3 hours later, and 6 hours later. Apoptosis was assayed by western analysis for size shifts in GFP. No changes were observed in cleaved Apoliner abundance for VSV G^{WT}, G^{G124E}, and G^{D137L} over the course of the six hours (Figure 3.17). As acute recovery phenotypes are evident within minutes of CO_2 exposure, yet Apoliner cleavage was not observed over the time course, apoptosis of glia cannot account for the disease phenotypes associated with CO_2 exposure.

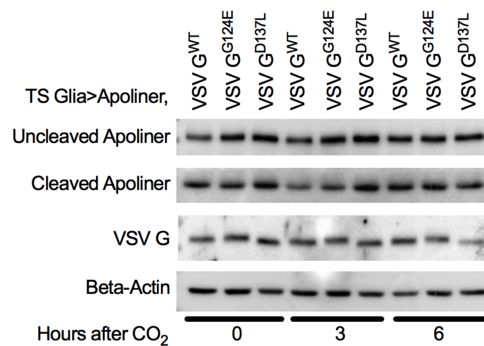

Figure 3.17 Apoptosis cannot account for neuro-trauma. Flies were reared at 18°C. Flies were collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. Apoliner and VSV G variants were expressed in glial cells by incubating flies at 29°C for 7 days. Flies were anesthetized with CO_2 for 30 seconds and collected immediately, 3 hours later, or 6 hours later. Western blotting of fly protein lysates was used to assess Apoliner cleavage by GFP blotting. One experiment representative of three is shown.

As glia and neurons are tightly associated and glial cells support neuronal function (Freeman, 2015), another possibility is that glia and neurons fuse upon exposure to CO_2. This event would be expected to acutely interfere with nervous system operation. To determine if there was any evidence of these fusion events in flies, the glia-specific Gal4 driver was used to co-express GFP and VSV G. A horseradish peroxidase (HRP) antibody was used to label neurons in dissected brains (Jan and Jan, 1982). When VSV G^{WT} or G^{D137L} were expressed, colocalization of glial and neuronal labeling was observed in CO_2-treated flies suggesting fusion between neurons and glia (Figure 3.18). When no VSV G was expressed and when VSV G^{G124E} was expressed, the neuronal and glial markers were closely associated, but colocalization was not observed (Figure 3.18). This microscopy analysis suggests that CO_2 anesthesia induces fusion events between neurons and glia, resulting in immediate neuro-trauma. These data therefore provide a molecular explanation for how VSV infection causes sensitivity to CO_2 and provide an example of how a single viral protein, independent of productive infection, can precipitate susceptibility to environmental stress.

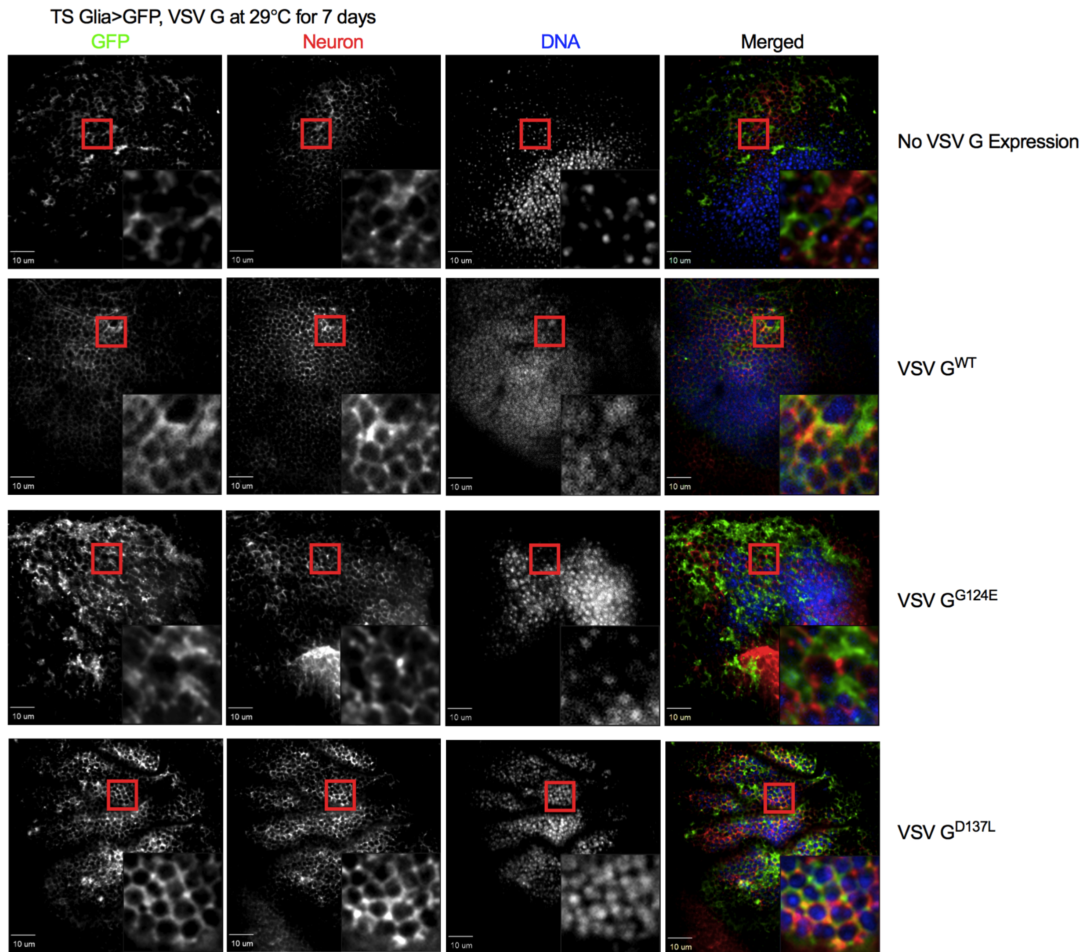

Figure 3.18 VSV G mediates fusion of glia to neurons upon CO_2 exposure. Flies were reared at 18°C. Flies were collected after eclosion by anesthetizing with N_2 and sorting them on an ice-cold glass dish. VSV G and GFP expression was induced in glial cells by placing flies at 29°C for 7 days. Flies were anesthetized with CO_2, and dissected brains were stained with GFP and HRP antibodies to stain for glia and neurons, respectively. DRAQ5 staining was used to stain for DNA. Confocal imaging was done using a 40X oil objective lens. Insets are 4X magnifications of selected areas.

DISCUSSION

In this chapter, we explored how an asymptomatic viral infection can be rendered lethal upon exposure to an environmental stress. The most notable feature of this disease state is that it is not elicited by either infection or the stress. Only the combination of infection and stress causes disease. This

experimental model therefore provided a unique perspective into the means by which asymptomatic infections can convert to symptomatic infections, through the exposure to a secondary stress to the host. Moreover, the speed which CO_2 associated symptoms are observed (minutes) and the specificity of disease to VSV strongly supports the conclusion that a novel mechanism of disease tolerance has been uncovered. This conclusion does not, however, preclude the discussion of resistance mechanisms in disease progression, as our analysis supports the need for viral spread to the nervous system for VSV G to be expressed in glia. Thus, while the spread of the virus through the body is largely controlled by resistance mechanisms (*e.g.* RNAi), the mere presence of virus in the nervous system is not sufficient to cause lethality. Only upon exposure to an environmental stress is disease observed. For this reason, we propose that insufficient resistance mechanisms render the host highly dependent on context-dependent tolerance mechanisms to survive.

While our infections with VSV resulted in sensitivity to a secondary stress, the opposite outcome is also possible. For example, chronic infection with herpesviruses renders mice resistant to *Listeria* and *Yersinia* infections (Barton et al., 2007). Presumably, the viral infection raises basal interferon gamma expression, priming the immune system for resistance to subsequent bacterial infections. These host-pathogen relationships become increasingly more complex when considering that organisms outside of controlled experimental conditions are faced with many infectious challenges over the course of life. As others have noted, the history of previous immune challenges can affect outcomes to the next stress (Foxman and Iwasaki, 2011; MacDuff et al., 2015; Reese et al., 2016; Stelekati et al., 2014). Thus, we risk losing important information if we focus only on the direct, destructive effects of a pathogen in isolation.

Our analysis of the host and viral regulators of CO_2 sensitivity revealed several determinants of disease progression. We found that productive viral infection is necessary to allow for sensitivity to CO_2 anesthesia to arise, as revealed by manipulations of temperature and the RNAi pathway. We defined glial cells as the principle cell type that must be infected to render *Drosophila* sensitive to CO_2, as restricting VSV replication from these cells prevented disease development. Under these conditions, VSV was capable of replicating in other cell types in the fly. These data therefore establish that even for a broadly tropic infection, catastrophic consequences on viability stem from changes to a specific tissue. A deeper

understanding of pathogenicity will require studies that focus on crucial cell types and mechanistic changes that affect their unique function in the host.

Our mechanistic analysis of how VSV induces CO_2 sensitivity revealed that we can completely dissociate infection from the onset of disease, in that transgenic flies expressing only VSV G sufficiently reproduce the phenotype. Application of CO_2 acidifies the nervous system, inducing VSV G to mediate cell-cell fusion. Using two VSV G mutants allowed us to approximate the pH acidification induced by CO_2 anesthesia between 5.5 and 5.7. SINV fusion is also pH dependent, but it has been noted to require an optimal pH between 5.3 and 5.5. This observation may explain why SINV does not cause CO_2 sensitivity (Mann et al., 1983).

Our results highlight how single viral proteins, not active pathogen replication, can alter the host in ways that create sensitivities to the environment. These ideas may be most important when considering latent viral infections, such as those caused by herpesviruses, where latency associated mRNAs and proteins have been reported to elicit physiological responses in the host (Bhattacharjee et al., 2016; DiMaio et al., 2014). Examples of latency associated changes in the host include Kaposi's sarcoma-associated herpesvirus (KSHV), where viral replication does not occur, but angiogenesis of the endothelium is induced through unclear mechanisms (DiMaio et al., 2014). In addition, Epstein Bar virus encodes proteins that promote lymphoma development, even under conditions of latency (Bhattacharjee et al., 2016). Whether these or other latent viral infections also cause sensitivities to secondary environmental stresses is unclear, but our findings provide a mandate to consider such possibilities in experimental and clinical settings.

Chapter 4

Future Directions and Concluding Remarks

In this dissertation, we have explored disease resistance mechanisms relevant to viral infections and the loss of disease tolerance in *Drosophila melanogaster*. In Chapter 2, we found that the siRNA pathway is the primary immune defense against viral pathogens. This conclusion is supported by several publications that have demonstrated the antiviral function of the siRNA pathway for a variety of viruses (Bronkhorst and van Rij, 2014). In contrast, other reported antiviral defense systems seem to be less relevant to VSV infection. *In vivo* work suggests that dSTING also functions as an antiviral defense. However, its mechanism of action and other interacting genes remains to be elucidated. In Chapter 3, we infected flies with VSV to study how tolerance to CO_2 anesthesia is lost. VSV G is the sole viral gene responsible for the phenotype, and CO_2 sensitivity is mediated through the fusion activity of VSV G. CO_2 anesthesia likely results in acidification in the nervous system, activating the fusion activity of VSV and leading to syncytia formation of neurons and glia. As a result, flies remain paralyzed after CO_2 anesthesia and die shortly afterwards.

The conclusions of my work open several opportunities for further study. They fall into one of three major areas: antiviral mechanisms, syncytia formation, and hypercapnic tolerance. In the category of antiviral mechanisms, an opportunity exists to elucidate the dSTING antiviral pathway and also to further study the function of the RNAi pathway. Syncytia formation is a feature common to multiple viruses, and it might be a feature that can be utilized to our advantage. Finally, hypercapnic tolerance is a subject that is particularly interesting for those who study stroke in mammals. Further discussion of each of these categories follows.

Antiviral mechanisms

One of the most exciting discoveries of my work was that a STING ortholog in *Drosophila* contributed to resistance to viral infection. We identified a fly with an insertion in the dSTING locus that disrupted gene expression. These flies were more susceptible to SINV and IIV-6 infections compared to wild-type flies (Figure 2.5-2.7). Although we have established that dSTING can function in antiviral immunity, many questions have yet to be answered.

Given our knowledge of mammalian STING and how it is activated by cyclic dinucleotides produced by cGAS, dSTING is unlikely to mediate antiviral immunity alone. Genes with similar protein

domains as cGAS exist in *Drosophila*. Testing these genes for antiviral activity would help determine if dSTING functions similar to its mammalian counterparts. One simple way of testing these potential cGAS orthologs would be to mutate them using the CRISPR/Cas9 system. If any of these genes are involved in antiviral immunity, flies infected with viruses such as SINV or IIV-6 should have accelerated death compared to wild-type flies. Similar to dSTING mutant flies, SINV titers should also be elevated compared to wild-type flies. An alternative approach using RNAi-mediated gene knockdown could also be used to silence expression of these candidate antiviral genes. One advantage of *Drosophila* is that the Gal4/UAS system provides the opportunity to drive gene expression (Cas9 and guide RNA) or gene knockdown (siRNA) with precision. These gene disruption strategies can be applied temporally or in a tissue-specific manner.

If a cGAS ortholog also functions in flies, the next likely hypothesis to propose would be that a cyclic dinucleotide mediates a signal to dSTING. Testing for a role for cyclic dinucleotides *in vivo* could be challenging because the molecules would need to be delivered intracellularly to cells that would be susceptible to virus. An easier start in testing this hypothesis would be to infect cells in tissue culture. S2 cells could potentially be adapted to mammalian methods of delivering cyclic dinucleotides (Gao et al., 2013; Sun et al., 2013). To test if cyclic dinucleotides could function in antiviral immunity, they could be delivered to S2 cells before exposure to virus. These cells would need to be compared to control treatments where cyclic dinucleotides were not delivered to cells prior to viral infection. At various time points after infection, the supernatant would be measured for viral titers. Alternatively, cells could be assayed by western analysis for viral protein expression.

With a potential cGAS in flies and antiviral activity induced by delivery of cyclic dinucleotides, one can begin to relate everything together in a signaling pathway. For example, one could knock down or knock out expression of the candidate *Drosophila* cGAS and then attempt to rescue antiviral activity by delivering cyclic dinucleotides in cell culture. In this scenario, antiviral activity should be functional since the production of cyclic dinucleotides would be downstream of *Drosophila* cGAS. However, delivery of cyclic dinucleotides into dSTING mutant cells would have no antiviral effect. Since dSTING would not be present, signaling downstream of cyclic dinucleotides could not occur. This set of experiments would demonstrate that the dSTING signaling pathway functions similarly to its mammalian counterpart.

If indeed a cGAS ortholog exists and cyclic dinucleotides restrict viral infection, a transcriptional response to viral infection likely exists. Thus far, transcriptional responses to viral infection seem to be limited to select viruses in *Drosophila*. We were unable to detect antiviral functions for the Jak/STAT signaling pathway, at least in the context of VSV infection (Figure 2.4). However, others have found Jak/STAT signaling to be limited to *Drosophila* C virus, cricket paralysis virus, and *Drosophila* X virus (Dostert et al., 2005; Kemp et al., 2013). Determining a dSTING-dependent transcriptional response would be very exciting. This could be done potentially by using wild type and dSTING-defective S2 cells. After infection, these cells could be collected and extracted RNA could be sequenced. By comparing the two groups of cells, one could determine which genes are regulated by dSTING. Whether this dSTING transcriptional pathway has any overlap with known signaling pathways like the Toll, IMD, or Jak/STAT signaling pathways would be of interest. At this point, any suggestion of genes induced or repressed by dSTING would be completely speculative.

Of course, dSTING could function in a completely unpredictable way, with no transcriptional response activated downstream. In this model, a role for *Drosophila* cGAS and cyclic dinucleotides is likely unnecessary. Unpublished work by K. M. Franz suggests that mammalian STING has antiviral activity distinct from its transcriptional effects. In addition to initiating an antiviral state via type I interferons, mammalian STING seems to be capable of inhibiting the translation of viral transcripts independent of a transcriptional response (K. M. F., unpublished results). In mammals, downstream transcriptional responses are dependent upon the C-terminus of STING, and this C-terminus is unnecessary for its viral translation inhibition activity (K. M. F., unpublished results). The C-terminal sequence involved in mammalian STING transcriptional responses is not present in dSTING.

One could hypothesize that the antiviral activity of dSTING is mediated by inhibition of viral translation. One could take the same approach as K. M. Franz and fractionate polysomes. Highly translated mRNA is often associated with multiple ribosomes whereas less translated transcripts might only be bound to one ribosome or none at all. One could infect wild-type cells or cells with disrupted dSTING expression and fractionate the cytosol to separate mRNA depending on the number of ribosomes bound to it. After fractionation, one could measure the presence of viral transcripts in each of the fractions to see if viral transcripts are associated with polysomes. If dSTING restricts viral translation,

one would expect wild-type cells to have viral transcripts associated with lighter fractions with only one or two ribosomes bound. In dSTING mutant cells, the viral transcripts would be preferentially found in heavier fractions with more ribosomes bound to each transcript. If dSTING is involved in viral translation inhibition, *Drosophila* would be an ideal model organism for identifying genetic regulators of this activity.

Our results suggesting that dSTING mutants die from IIV-6 infection with similar kinetics to Dcr2 mutants was intriguing (Figure 2.5) and raise the possibility that dSTING functions in the same pathway as Dcr2. This would account for the overlapping survival kinetics of the two mutant flies. While a function for dSTING in RNAi would be unsupported by published literature, another possibility is that dSTING and Dcr2 regulate a transcriptional response together. Some data suggests that Dcr2 regulates the expression of the antiviral gene *vago*, independent of Ago2 and R2D2 (Deddouche et al., 2008). Assuming Dcr2 has two independent effector functions, RNAi and transcriptional regulation, dSTING would need to be upstream in the pathway in order to account for the overlapping survival curves shown in Figure 2.5. A preliminary experiment would first test whether dSTING also regulates the expression of *vago*. In order to do this experiment, one would first need to test if dSTING restricts *Drosophila* C virus infection since this virus has been demonstrated to induce *vago* expression (Deddouche et al., 2008). If dSTING mutants die faster than wild type flies during *Drosophila* C virus infection, one could conduct quantitative real-time PCR analysis of infected flies to determine if dSTING mutants have decreased *vago* expression compared to wild-type infected flies. If this is indeed the case, one could proceed to more comprehensive analyses such as high-throughput RNA sequencing to determine if Dcr2 and dSTING regulate the same genes after *Drosophila* C virus infection. RNA sequencing would also determine if generation of siRNA is dependent upon dSTING. From there, one might want to understand at a mechanistic level how Dcr2 and dSTING cooperate together to regulate transcription. One possibility to test is that they may physically associate with each other in a signaling complex. Immunoprecipitation studies could help test this model for antiviral activity.

Independent of dSTING, Dcr2 and other regulators of RNAi are still of interest for further studies. We found extremely low expression of VSV genes in transgenic overexpression flies compared to VSV infection of Dcr2 mutant animals (Figure 3.13B). While the low expression could be due to an artifact of the system, a more interesting possibility is that these viral genes have sequence-intrinsic characteristics

that are recognized as foreign by the host RNAi machinery. A straightforward method of testing this hypothesis is to drive individual VSV genes using heat shock-Gal4 in a wild-type or Dcr2-deficient background. One would expect expression of VSV genes to increase in Dcr2 deficient animals if indeed the viral transgenes have sequence-intrinsic features that are recognized by the siRNA pathway. If VSV genes in the overexpression system are Dcr2-dependent, the next task would be to identify the sequence substrates for the siRNA pathway. In a Dcr2-sufficient background, VSV genes could be driven by heat shock-Gal4. High throughput RNA sequencing could be used to identify 21-nucleotide sequences bound to Ago2. One could look in each transgenic fly for common sequences within the siRNA or in the flanking regions of the generated siRNA. One could also look for other patterns such as the placement of acidic or basic residues. These data potentially have a significant impact because the selection of viral RNA sequences for silencing is still unclear. Although complementary viral siRNA mapping to the same locations on the genome and anti-genome have been detected in infected cells, no evidence has demonstrated that these sequences bind to each other to produce dsRNA as a substrate for Dcr2 (Mueller et al., 2010). The opportunity exists to determine how viral sequences are selected for silencing.

The siRNA pathway seems to confer the broadest protection against viral infections in flies. We found no evidence to support the hypothesis that the miRNA or piRNA pathways could serve redundant functions when the siRNA is non-functional (Figures 2.1, 2.2). At least in the case of the piRNA pathway, the system might not be active in most somatic cells. However, further examination of possible functional redundancy is worth pursuing. Sigma virus, which is related to VSV, is transmitted vertically from parent to offspring (Seecof, 1962). Whether VSV can be transmitted vertically in fruit flies is unknown. Moreover, other arthropod-borne rhabdoviruses, such as Chandipura and Piry viruses, have not been tested for their potential to be transmitted vertically. Given that viral transmission would need to occur via the gametes where the piRNA system is known to be active, the piRNA system could potentially restrict viral infection in the reproductive system. This would be the most likely place where the piRNA pathway could demonstrate antiviral function. Indeed, virus-derived small RNA within the size range of piRNA have been detected in flies and mosquitoes, although their biological significance have yet to be evaluated (Bronkhorst and van Rij, 2014). To further use VSV as a prototypic pathogen in exploring piRNA pathway function, one would first need to determine if VSV could be transmitted vertically. This could be simply

done in wild-type and in siRNA genetic mutants. Adults could be infected with VSV-GFP and the reproductive organs can be dissected to identify GFP signal via microscopy. If the virus is capable of reaching the reproductive tract, the next experiment would be to test if the virus can be transmitted to offspring. Infected flies could be mated, and viral titers could be measured from the offspring to verify transmission. Whether virus reaches the reproductive system in the offspring would also be of interest in order to determine if this mode of VSV transmission is stable. Of course, using VSV for these experiments is not necessary, although the availability of reagents would make the strategy easier to pursue compared to other viruses. Whether the piRNA pathway has any antiviral role could also be resolved using sigma virus. One benefit of using sigma virus would be that vertical transmission is already known to occur. Various cell types can have different antiviral mechanisms. For example, most mammalian cells use RLR signaling to detect foreign RNA, but plasmacytoid dendritic cells instead rely upon TLR7. Cell-type specific antiviral mechanisms could produce context-dependent responses that are more desirable for the host, and studying antiviral immunity in the *Drosophila* reproductive tract could serve as a potential model for this concept.

Chapter 2 supports the idea that VSV is mainly restricted by the siRNA pathway. Comparison of my data to published literature on antiviral mechanisms in insects would suggest that additional antiviral mechanisms can be relevant but is pathogen-dependent. Why other viruses such as *Drosophila* C virus induce a transcriptional response is unclear. One possibility is that the siRNA pathway serves as the main antiviral response and other antiviral mechanisms can serve as additional barriers to limit pathology. Indeed, identified viruses that elicit transcriptional responses have RNAi evasion mechanisms. One method of testing this model of a stratified immune defense would be to compare transcriptional responses via RNA sequencing of infected animals with immune deficiencies. If the various antiviral defenses function in series instead of in parallel, one would expect transcriptional responses to be initiated in siRNA mutants since RNAi no longer sufficiently resists infection. In an RNAi-sufficient host, these transcriptional responses would be absent. VSV could be used for such experiments but also other viruses as well. The findings would help integrate the known antiviral mechanisms into a cohesive model, explaining why multiple resistance mechanisms exists and why they are selectively used depending on the viral pathogen.

Another feature of VSV infection that we observed was that it is never fully cleared but instead lasts as a persistent infection in *Drosophila* cells (Wyers et al., 1980) and *in vivo* (Figure 3.3A). Viral persistence seems to be a feature for Nora virus, *Drosophila* C virus, and sigma virus as well (Huszar and Imler, 2008). Whether viral persistence is advantageous and is deliberately chosen by the host is unclear, but persistent infection does not induce cytopathic effects. Although RNAi is normally considered a cell-intrinsic antiviral response, some evidence suggests that gene silencing can spread to uninfected cells (Goic et al., 2013; Saleh et al., 2009). A persistent infection could enable this systemic response by providing a source of siRNA. One might argue that a persistent infection might be more energetically burdensome for the host since immune pathways cannot be turned off without consequence. Considering immune responses can be damaging to the host if overactive, perhaps a persistent infection is a host compromise that has benefits and costs. Testing the significance of viral persistence is challenging because a method of inducing the complete elimination of virus would be necessary. At this point, the required knowledge and tools do not exist, but understanding viral persistence in *Drosophila* could further inform how antiviral immunity works in arthropods.

Syncytia formation by VSV G

A major finding of Chapter 3 is that pathogenicity can be induced by combining VSV G expression and the abiotic stress of CO_2 anesthesia. The fusion activity of VSV G is induced by low pH, and CO_2 has the ability to acidify an aqueous environment once incorporated. Paramyxoviruses are known to induce syncytia formation and its contribution to pathology has been well appreciated (Aguilar et al., 2016). Although the process of fusion is different compared to rhabdoviruses, the overall argument of finding therapeutics targeting viral fusion is strengthened by our data shown in Chapter 3. While VSV is not unique in causing syncytia formation, the pervasiveness of syncytia as a pathological effect has yet to be fully appreciated.

For example, HIV envelope glycoprotein mediates the fusion of infected cells to uninfected CD4 T cells (Garg et al., 2012). This phenomenon helps account for the massive loss of CD4 T cells even though only a small percentage of them are infected with HIV. However, the extent to which this contributes to a chronic disease, even when antiretroviral therapies are administered, is unclear.

Determining the contribution of cell-cell fusion to disease progression could have a significant impact on treatment. HIV therapeutics targeting inhibition of viral particle fusion already exists. However, these are not broadly employed due to their cost relative to other effective treatment options. If syncytia formation is a major contributor to CD4 T cell decline, there would be a mandate to more broadly employ the use of fusion inhibitors and further develop cost-effective therapies.

In the case of HIV infection, the impact of syncytia formation could be assessed by the use of transgenic mice. As demonstrated in Chapter 3, the expression of a single viral gene can have pathological effects. A similar approach could be taken by overexpressing the HIV *env* gene in humanized mice. Mice expressing human CD4 and CCR5 are already available (Browning et al., 1997), and they would be susceptible to cell-cell fusion if the HIV glycoprotein was expressed by CD4 T cells. If the viral gene was placed under an inducible promoter, such as one dependent upon tetracycline, one could potentially modulate the strength of gene expression. However, as observed with VSV G, small amounts of glycoprotein can still have pathological repercussions (Figure 3.13B). These humanized mice expressing high or low amounts of the HIV glycoprotein in their CD4 T cell compartment could be measured for CD4 T cell count and observed for short and long-term pathology. Because therapies already exist to target HIV fusion, this line of inquiry could have immediate impact upon patient treatment strategies.

Other viral infections have not had as many therapeutics developed. In the case of rabies virus, more treatment options are needed. Because of the long incubation period of rabies virus, the infected person might not seek treatment until symptoms finally develop. At which point, the infection is difficult to treat. If more therapeutics are to be developed, a better understanding of rabies virus pathogenesis is required. Although the threat of rabies infections has been recognized for many centuries, the development of pathology in the nervous system is still unclear. Cell death cannot fully explain nervous system dysfunction (Jackson, 2003). While some of the pathology has been linked to the viral glycoprotein, the mechanism by which pathology develops has not been fully explored (Lafon, 2011). Thus far, the possibility that syncytia formation by rabies glycoprotein leads to neuronal dysfunction has not been examined. Unfortunately, fruit flies are unlikely to be a good model organism to study rabies glycoprotein because VSV pseudotyped with rabies glycoprotein is not infectious in fruit flies (unpublished

work). Instead, one could use mice as a host. One could potentially engineer a rabies virus that cannot complete its replication cycle. For example, the genome could be altered such that a mutation in the matrix protein inhibits particle formation and budding. This mutation could be combined with a mutation in the glycoprotein that loses the capacity to mediate fusion. The fusion mutant could be compared to one that has a functional glycoprotein but is also replication incompetent. To make such viruses, the host cell would need to express functional versions of these genes separate from the viral genome to complement the mutations. Thus, infectious virus particles could be produced. Once inside the host cell, the virus could produce viral proteins but cannot produce more infectious virus particles and spread to other cells. Using these replication defective virus particles, one could infect mice and look for behavioral abnormalities, gross pathology, and syncytia formation. While one might expect some pathology to develop from the fusion defective virus, only the fusion competent virus would induce syncytia formation. As a result, the fusion competent virus would be expected to have more severe pathology and behavioral defects. If the fusogenic activity is a major contributor of pathology in rabies virus infection, development of therapeutics that target this viral activity could have two major benefits for an infected person: inhibition of virus spread and inhibition of syncytia formation. For an infection that has little recourse once symptoms develop, this would be a major advancement. Viral fusogenic activity could provide an opportunity to treat infection.

If glycoprotein-mediated fusion is a critical aspect of rabies infection, one could genetically screen for host factors involved in its synthesis. Rhabdovirus glycoprotein production is heavily dependent upon host machinery, such as proteins involved in folding, glycosylation, and transport. These host genes could be potential therapeutic targets to treat rabies infection. Of note, such a strategy might not necessarily incite a pro-inflammatory response which could be destructive for the nervous system. To screen for host factors involved in rabies glycoprotein production, one could use a siRNA library or CRISPR/Cas9 guide RNA library. As a readout, one could stain cells for surface expression of the glycoprotein using high-throughput microscopy. This approach would be narrower in scope compared to a screen with live virus which generated gene candidates involved in many possible aspects of infection (Wallis et al., 2015). This approach would allow easier characterization of candidate gene function. Also, there would be fewer safety concerns since live virus is unnecessary, and the screen could be done with easily cultured cells

as long as they can be transfected efficiently. In our screening approach, cell death could help further eliminate candidate genes that affect viability. To enrich for genes that are specific to viral glycoprotein production, a counter screen should be performed. One could screen for genes affecting another type I transmembrane protein such as CD4. Any genes commonly regulating CD4 and rabies glycoprotein would be eliminated as potential therapeutic targets. Thus, studying the regulation of rhabdovirus glycoprotein production could identify therapeutic targets that ameliorate pathology associated with infection.

Viral fusogenic activity can also be used to our advantage for therapeutic purposes. VSV and other viruses have been of interest because they have been observed to be oncolytic. For this reason, VSV is being developed as a therapeutic agent to target the death of cancer cells (Lichty et al., 2004). VSV has been noted to be preferentially tropic for tumor cells over healthy cells due to the insensitivity of tumor cells for interferons (Stojdl et al., 2000, 2003). Additionally, the extracellular environment of tumors has been found to be acidic (Griffiths, 1991). The acidic tumor microenvironment is the result of metabolic changes that occur in tumor cells, and the acidity fosters beneficial effects for the tumor such as immune suppression, extracellular matrix changes, and metastasis (Kato et al., 2013). Our data demonstrates that VSV G can induce fusion rapidly upon exposure to CO_2. Presumably, this is due to a shift in pH. One could hypothesize that the tropism of VSV for tumor cells is partially dependent upon the acidic microenvironment.

In the attempt to generate an oncolytic VSV for therapeutic purposes, the selectivity of the virus for tumor cells has been an aspect requiring improvement (Bergman et al., 2003). Because the tumor microenvironment is acidic, VSV G fusogenic hypomorphs could further improve selectivity of the virus for tumor cells. One could potentially engineer a virus with a mutant G that would be endocytosed and degraded in the endolysosomal pathway of normal healthy cells before reaching its fusion threshold. However, this mutant would be capable of fusion with tumor cells at the plasma membrane due to the lack of degradative enzymes and acidic extracellular environment, leading to productive infection and cell killing. To test this idea for improving virus selectivity, one could start with the mutants used in Chapter 3: VSV G^{G124E} and G^{D137L}. These mutants begin fusion at a lower pH than wild-type G. In a mouse solid tumor model, one would hypothesize that VSV G^{G124E} and G^{D137L} would be better than wild-type at

targeting the tumor and less of the virus would be infecting off-target cells. Thus, two parameters one could study in this experiment would be the rate of tumor reduction over time and also the viral titer found in various organs of the animal, separate from the tumor. This first experiment would provide proof of concept that a lower pH fusion threshold of VSV G improves the selectivity of the virus for tumor cells.

If the concept proves true, one might want to then further improve the pH threshold for VSV G fusion. One approach that could be taken is a random mutagenesis screen of VSV G. After creating a library of mutant VSV G, one could encode them in plasmids and express them in a mammalian cell line. The transfected cells can then be briefly subjected to cell culture media at a defined pH. To determine which mutants fuse at the desired pH, one would look for syncytia formation in the cell cultures. Inherent in this screening strategy is the elimination of candidates that do not express VSV G correctly at the cell surface and elimination of candidates that no longer fuse at any pH. Also, by testing a series of pH, one could determine the range at which a mutant VSV G is capable of fusion. Such a screening approach could potentially be done in a high-throughput manner, utilizing high-throughput microscopy to identify mutants forming syncytia. Candidate VSV G mutants can then be used to generate viruses for infection of tumor animal models.

Hypercapnic tolerance

Chapter 3 highlights an interesting feature of fruit flies, the ability to tolerate a hypercapnic environment. Hypercapnic tolerance is likely an attribute of fruit flies because the animals must function in low oxygen environments where air circulation is limited. Such an environment might be encountered during the larval stage as the animals eat and crawl through soft, rotting fruits. In the laboratory, fruit flies are anesthetized with pure CO_2, which is beyond physiological levels. The high CO_2 levels cause the dorsal vessel, the equivalent of a heart in insects, to cease pumping haemolymph (Sillans and Biston, 1979). Furthermore, synapses at neuro-muscular junctions stop responding to glutamate, resulting in the paralysis observed during CO_2 anesthesia (Kato et al., 2013). The current model for how CO_2 paralyzes insects is that it forms hydrate crystals, interfering with protein function at the plasma membrane (Badre et al., 2005; Sillans and Biston, 1979). The cell types and proteins affected are not entirely clear,

particularly in adult-stage insects. Thus, understanding how insects survive extreme hypercapnic environments requires further research.

In order to further clarify how insects tolerate CO_2, our transgenic flies overexpressing VSV G can be utilized. Syncytia formation can be used as a method to track the site of action of CO_2 since syncytia require CO_2 to form. While syncytia formation was detected in the brains of flies (Figure 3.18), the data does not definitively prove that the main site, or only site, of action is in the brain. In fact, CO_2 sensitivity in sigma virus-infected flies was first hypothesized to be localized to the thoracic ganglion (L'Heritier, 1948). The experiments supporting this hypothesis required the head of flies to be exposed to gases separately from the rest of the body. When heads of flies were exposed to CO_2, no sensitivity was observed. Meanwhile, CO_2 exposure to the thorax resulted in paralytic behavior. However, the trachea that ramify the fly body and facilitate gas exchange have spiracles in the thorax and abdomen. Thus, it is possible that CO_2 exposure to only the head of a fly does not facilitate delivery of the gas to the nervous system. Thus, the anatomical locations impacted by CO_2 exposure are still unclear. In order to gain a clearer understanding of which tissues are affected by hypercapnia, more thorough histological studies need to be done. For example, the thoracic ganglion should be dissected from VSV G-expressing flies to search for syncytia formation. Syncytia should be quantified in the brain and thoracic ganglion, and the location of syncytia formation should be noted in order to identify sites regularly affected. Neuromuscular junctions might also be an area of interest. These studies were not carried out in this dissertation research due to the challenging nature of this work. However, such experiments are feasible.

Additional genetic studies can provide further clarification of the cells exposed to hypercapnic conditions. CO_2 sensitivity was dependent on VSV G expression by glial cells, but the Gal4 promoter used drives expression all glial cells. Glia in the central nervous system can be classified into several major subsets: astrocytes, cortex glia, and ensheathing glia (Freeman, 2015). Glial cells also function in the peripheral nervous system. Transgenic Gal4 flies have been produced to drive expression in various glial subsets. These Gal4 lines could be coupled to transgenic flies encoding VSV G under the UAS promoter. By exposing these flies to CO_2 anesthesia, one could determine which glial cells mediate syncytia formation and consequently identify the cells and tissues affected by hypercapnia.

Such information would be useful in a genetic screen to identify genes facilitating hypercapnic tolerance. Because these genes are potentially necessary for flies to develop to the adult stage, a random mutagenesis screen would most likely be inappropriate. Instead, a more advantageous approach would be to use the pertinent Gal4 drivers to screen a RNAi library. Temporally controlling gene knockdown could help avoid missing candidate genes that are necessary for hypercapnic tolerance during the larval stage. After inducing gene knockdown, one could subject adult flies to CO_2 anesthesia. Since these flies do not express VSV G, they should be capable of recovering from anesthesia. However, if a gene involved in anesthesia recovery is knocked down, flies will have a delayed recovery rate or will not be able to recover at all. These flies would be of interest, and the genes knocked down would be the focus of further studies in order to understand how they mediate CO_2 tolerance. Such tolerance to CO_2 is relevant to human physiology as hypercapnia is known to contribute to neuronal and pulmonary damage (Butterworth, 1999).

Concluding remarks

Insects are a fundamental part of our ecosystem. Some species are integral to agriculture, pollinating crops. Others are disease vectors for myriad pathogens. Like us, insects can succumb to infections, offering an opportunity for comparative analysis to our own biological responses to pathogens. As described in this thesis, organisms have multiple strategies for responding to pathogens such as viruses. Disease resistance mechanisms utilized by the insect immune system help limit pathogen growth. Although antiviral mechanisms have been identified, greater detail is needed to fully understand how they function. Disease tolerance mechanisms do not necessarily limit pathogen load, but they help stabilize the host during these stressful onslaughts. When a host is no longer able to maintain homeostasis, the host dies. Many complex interactions occur between a pathogen and host at the molecular, cellular, tissue and organismal levels over the course of time. *Drosophila melanogaster* provide an opportunity to manipulate these interactions to understand insects and to understand ourselves. Although one might suppose such studies are basic scientific research, diverse practical applications can arise. Plenty more has yet to be learned and this dissertation provides a mandate for further study.

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