DISSERTATION

EXPLORATION OF THE ARTHROPOD VIROME, ITS BIOLOGICAL IMPACTS ON HOST HEALTH, AND ITS POTENTIAL IMPLEMENTATION IN BIOCONTROL.

Submitted by

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ABSTRACT

EXPLORATION OF THE ARTHROPOD VIROME, ITS BIOLOGICAL IMPACTS ON HOST HEALTH, AND ITS POTENTIAL IMPLEMENTATION IN BIOCONTROL.

With the advent of next generation sequencing, viruses are being discovered at an unprecedented rate. The collection of these viruses, known as the virome, and their impact on the host is relatively understudied compared to the bacterial microbiome. The underlying goal of this thesis work was to better understand how the virome interacts with the host, and this has been accomplished in two ways. First, we biologically characterized predominant virus constituents in arthropod viromes, namely arthropod-infecting partitiviruses. In a subsequent study we measured how one of these partitiviruses, galbut virus, impacted the fitness of *Drosophila melanogaster*. Second, we searched for evidence that these viruses are active in their interactions with fellow microbial constituents within the host. Specifically, we addressed how the virome may change disease vectors' competence in harboring and transmitting pathogens with a focus on *Ixodes scapularis* ticks.

Partitiviruses are segmented, multipartite dsRNA viruses that until recently were only known to infect fungi, plants, and protozoans. Metagenomic surveys have revealed that partitivirus-like sequences are also commonly associated with arthropods. One arthropod-associated partitivirus, galbut virus, is common in wild populations of *D. melanogaster*. To begin to understand the processes that underlie this virus's high global prevalence, we established colonies of wild-caught infected flies. Infection remained at stably high levels over three years, with between 63-100% of individual flies infected. Galbut virus infects fly cells and replicates in tissues throughout infected adults, including reproductive tissues and the gut epithelium. We detected no evidence of horizontal transmission via ingestion but vertical transmission from either infected females or infected males was ~100% efficient. Vertical transmission of a related partitivirus, verdadero virus, that we discovered in a laboratory colony of *Aedes aegypti* mosquitoes was similarly efficient. This suggests that efficient biparental vertical transmission may be a feature of at least a subset

of insect-infecting partitiviruses. To study the impact of galbut virus infection free from the confounding effect of other viruses, we generated an inbred line of flies with galbut virus as the only detectable virus infection. We were able to transmit the infection experimentally via microinjection of homogenate from these galbut-only flies. This sets the stage for experiments to understand the biological impact and possible utility of partitiviruses infecting model organisms and disease vectors.

Using the galbut virus and *D. melanogaster* system, we set forth to answer: what are the biological effects, if any, of galbut virus infection on *D. melanogaster* fitness? Using multiple lines of flies from the *Drosophila* Genetic Reference Panel (DGRP) that differed only in their galbut virus infection status, a variety of fitness measurements were performed across both sexes. Galbut virus minimally impacted lifespan and had no effects on fecundity, but infection did significantly impact developmental speeds of flies. When challenged with various viral, bacterial, and fungal pathogens, some galbut virus infected flies had altered sensitivity to these pathogens. These susceptibility changes varied by both genetic background and sex. Galbut virus overall has minimal influences on host transcriptional responses, consistent with minimal phenotypic impacts of galbut virus infection. Major constituents of the microbiome were not perturbed by galbut virus infection. All fitness measurements alterations attributable to galbut virus were small, but they were dependent by strain and sex, highlighting the importance of these variables in phenotype outcomes. However, these altered measurements in galbut virus infected flies were dwarfed in comparison to those measurements attributable solely by fly strain and sex. These findings further support a trend of predominately cryptic phenotypes of partitivirus infections.

To understand how the virome interacts with other microbial constituents, we specifically searched for polymicrobial interactions within the field of vector-borne diseases. *I. scapularis* ticks harbor a variety of microorganisms, including eukaryotes, bacteria and viruses. Some of these can be transmitted to and cause disease in humans and other vertebrates. Others are not pathogenic but may impact the ability of the tick to harbor and transmit pathogens. A growing number of studies have examined the influence of bacteria on tick vector competence but the influence of the tick virome remains less clear, despite a surge in the discovery of tick-associated viruses. In this study, we performed shotgun RNA sequencing on 112

individual adult *I. scapularis* collected in Wisconsin, USA. We characterized the abundance, prevalence and co-infection rates of viruses, bacteria and eukaryotic microorganisms. We identified pairs of tick-infecting microorganisms whose observed co-infection rates were higher or lower than would be expected, or whose RNA levels were positively correlated in co-infected ticks. Many of these co-occurrence and correlation relationships involved two bunyaviruses, South Bay virus and blacklegged tick phlebovirus-1. These viruses were also the most prevalent microorganisms in the ticks we sampled and had the highest average RNA levels. Evidence of associations between microbes included a positive correlation between RNA levels of South Bay virus and *Borrelia burgdorferi*, the Lyme disease agent. These findings contribute to the rationale for experimental studies on the impact of viruses on tick biology and vector competence.

Follow-up analyses on a second population of *I. scapularis* ticks derived from New York, USA revealed that these potential functional relationships may be population-specific. When evaluating South Bay virus, blacklegged tick phlebovirus-1, and *B. burgdorferi* in these individual ticks, no correlative or cooccurrence associations were observed. The lack of concordance between populations suggests that interactions between microbial constituents may be fluid, and change based upon location and populations. To characterize the biology of tick-associated viruses, an attempt to isolate South Bay virus was performed. Despite using mammalian and tick cell lines, we were unsuccessful in isolating South Bay virus through *in vitro* cell culture. The lack of success accents the challenge for understanding the biology of these arthropod-specific viruses. Further additional attempts to acquire infectious South Bay virus, such as creating a reverse genetics system, are warranted for its biological characterization.

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CHAPTER 1: INTRODUCTION

1.1: Review of Virus Discovery Techniques and Metagenomic Sequencing

Since the discovery of tobacco mosaic virus (TMV) in 1892 by Dmitri Ivanovsky, biologists have become fascinated with viruses. Viruses have enlightened our world of understanding host biology and have shaped how we understand topics such as infectious diseases, genetics, and cell biology [1]. This introduction begins with a brief overview of conventional tools and next generation sequencing used for virus discovery.

1.1.1: Conventional Methods of Virus Discovery

Although there has been a plethora of technological advances since the days of early virus discovery, there have been staple methods in the identification of novel viruses. Virus discovery started with the identification of TMV in 1892. Ivanovsky showed that a filtered homogenate derived from affected plants could be inoculated onto unaffected plants, and those inoculated plants would begin to show the same, distinct mosaic leaves. This opened an entirely new world of microbiology, a world where there were infectious agents that were smaller than bacteria.

In the early days of microbial discovery, the microscope provided a useful tool in exploring the depths of the microbial world [2]. For viruses, this tool is severely limited as most viruses cannot even be viewed under a basic light microscope (giant viruses being the exception [3]). However, light microscopy can be the starting point of identifying a novel virus as identification of histopathological lesions associated with infectious disease can lead to viral pathogen detection. Since light diffraction proved to be the limiting factor in microscopy tools in virus discovery, scientists began creating microscopes (the first being the PhD project of Ernst Ruska [4]) that harnessed the power of shorter electromagnetic waves, such as X-rays and electrons. These advances opened the field of electron microscopy, which has been a key component of many virus discoveries, including norovirus, Ebola virus, severe acute respiratory syndrome virus (SARS), lymphocytic choriomeningitis virus, and Nipah virus [5–9]. A major strength of this tool is does not require

a priori knowledge of the virus, giving an unbiased approach and detection of both known and novel viruses [4]. Disadvantages however include lack of sensitivity, special handling and image interpretation that requires extensive training, and superficial identification based on morphological characteristics (often on the family level) [4]. Despite proving useful at times, microscopy other methods that provide more specific classification have been used.

Culture techniques have long been an essential tool in virology [10]. The filtering of fluids and homogenates, and subsequent infection of *in vivo* systems was key in identifying many of the first viruses, such as yellow fever virus [11], poliovirus [12], and influenza virus [13]. Filtering lysed bacterial cells, followed by inoculation on live bacterial cells led to the discovery of bacteriophages [14]. The rise of cell culture has proven fruitful for virus discovery as well [15]. Although culturing has proven invaluable for our knowledge of virology, there are still challenges. Culturing in this era is considered by some as time intensive and expensive. More importantly, many viruses are completely unculturable in cells, such as Kaposi sarcoma-associated herpesvirus [16]. As molecular techniques have advanced, culturing techniques for virus discovery have begun to diminish [16].

Serology and immunoassays (immunofluorescence assay, immunohistochemistry, western blots, enzyme-linked immunosorbent assays, and neutralization assays) detect the presence of a virus by either the presence of an antigen or antibodies resulting from the body's response to infection. These assays have been useful in the identification of the causative viruses responsible for outbreaks, such as the West Nile virus outbreak in New York [17] and the SARS coronavirus in China [18]. This technology is more suited for already known viruses, as cross-reacting antibodies are necessary to detect related viruses. However, recent advances in immunoassays allows for the detection of novel viruses through technologies like Luciferase Immunoprecipitation Systems (LIPS) [19]. Yet, these technologies are based on algorithms that build upon already known viral protein sequences; therefore, they are limited by the necessity of *a priori* knowledge.

A transformative technology emerged in 1983 when Kary Mullis developed polymerase chain reaction (PCR) [20]. This technology allows one to amplify a nucleic acid target of interest through design

of complementary DNA sequences known as primers. With proper primer design, this assay carries a high specificity for detection of known sequences (e.g. viral sequences). Multiple sequences can be identified through combination of panels of primers and probes in a process called multiplexing. Although these assays are key for detecting known viruses, they can be modified to use degenerate primers to reduce specificity of a specific target, but increase sensitivity for related viruses [21]. PCR techniques founded in this idea of multiplexing with degenerate primers have been useful for the detection of a handful of novel viruses [22–25] but is low throughput. Further strengths of PCR include the low cost, rapid and real-time data generation, and portability for use in the lab and the field. Several molecular techniques have arisen using the fundamentals of PCR.

Another technology making use of complementary nucleic acid sequences for detection are microarrays. Microarrays consist of numerous oligonucleotide probes (oligos) that are attached to a solid substrate base [26]. These oligos can then hybridize with complementary nucleic acid sequences. During sample preparation, the nucleic acid sequences of interest are modified to have fluorophores and thus can be used to identify which oligos bound to the sample. Because microarrays can contain thousands of oligonucleotide combinations, they have strength as a high-throughput method. Microarrays have been successful in identifying novel viruses from a diverse number of virus families [27–30]. Similar to serological methods for detection of novel viruses, these microarrays are rooted in *a priori* knowledge of viral sequences. This subjects them to continuously needing regular updates as new genomic information emerges. Viruses diverge easily from one another in their genomes [31]; therefore, this technology cannot identify highly divergent viruses. Additional flaws include background autofluorescence and inappropriate hybridization with non-complementary DNA [26].

Although serology/proteomics, PCR, and microarrays have all been useful in the identification of novel viruses, they all build upon previous knowledge of closely related viruses. This results in them failing in the identification of highly divergent or completely novel viruses. The continual need for unbiased methods for the detection of viruses is necessary. One such technology has arisen in recent years: next

generation sequencing. With increasing availability and affordability [32], next generation sequencing has become a foundational tool for virologists shaping a new era in the hunt for viruses.

1.1.2: History and Development of Sequencing Technologies

The history of sequencing technologies is relatively young. The beginning of the sequence era (often referred to as first-generation sequencing) began with "chemical cleavage" and "chain termination" techniques [33,34]. The latter is still a widely used technique, commonly known as dideoxy or Sanger sequencing. Sanger sequencing, developed by Fredrick Sanger in 1977, makes use of chain-terminating nucleotide analogs. In this technique, a nucleic acid sequence is elongated, but when one of these nucleotide analogs is incorporated, it terminates the synthesis. An additional characteristic of these chain-terminating nucleotides is that they are fluorescently labeled with colors specific to each nucleotide type (adenosine, guanine, cytosine, thymine), allowing the sequence to be derived by identification of specific termination sites and subsequent "stitching" of nucleotide sequences. This technique has continually developed over the years and can now, with high accuracy, sequence reads up to ~1000 bases in length. Though this sequencing technology dominated the field for decades and was the gold standard, it's restricted by low-throughput and high costs for the amount of data generated [35].

Building upon the foundation of Sanger sequencing, technologies entered the world of second-generation sequencing. This era is marked by high-throughput short read sequencing, known as next generation sequencing (NGS). A key characteristic of these platforms is the massive parallel sequencing. Although several technologies were developed in this era (e.g. Roche 454 and Ion Torrent sequencing [36,37]), today this sequencing technology is essentially synonymous with Illumina sequencing platforms and is colloquially referred to as such. This platform is based on a technique known as "sequencing by synthesis". Similar to Sanger sequencing, this technology makes use of fluorescently labeled nucleotides and tracks the fluorescence as DNA sequences are elongated. Because many sequences are copied in a massively parallel fashion, this technology allows an almost unfathomable amount of data and throughput (the newest machine can produce up to 20 billion sequences in a single run). Because of the large scalability,

sequencing costs are also relatively cheap [38]. This technology also outputs high precision sequences, referred to as reads (less than 0.1% error rate) [35,38]. However, these reads are relatively short (usually 75-300 bases in length), but given the large amount of data generated, sequences can achieve high depth (number of reads mapping to a sequence) and accuracy offsetting this weakness [35].

Although not discussed further in this dissertation, another era of next generation sequencing has been ushered in recently with a third-generation known as "long-read sequencing". These technologies are dominated by two groups: Pacific Biosciences and Oxford Nanopore Technology. As the era name infers, these sequencing technologies produce very long reads. The long reads can overcome shortcomings of short-read sequencing, such as accurately sequencing through tandem repeats or detecting epigenetic modifications directly [39]. Although getting progressively better, these long read sequencing technologies suffer from a high error rate and cannot output nearly as many reads as any Illumina platform [39]. One major strength of one of these platforms, Nanopore MinION sequencing, is the extreme portability and cheap cost of the sequencing machine (~$1000). The MinION sequencer can be plugged into a simple USB port and sequencing can be done essentially anywhere (we, for example, have sequenced samples in the back of a car traveling between universities). This portability in field use proves to be very fruitful in quick diagnoses and surveillance of pathogens [40,41].

1.2: The Arthropod Virome

1.2.1: An Incredible Expansion of the Arthropod Virome

Although many fields of science have rapidly advanced with NGS technologies, these platforms have drastically changed the field of virology. As scientists continue to sequence more and more samples, new viruses are continually being discovered. This has led to an extensive expansion of the viral genome databases and taxonomic groups (**Figure 1.1**). Viruses have been identified across wide ranges of hosts and environments, such as human pathogens [42–44], exotic animal pathogens [45,46], and even at the bottom of the ocean [47]. A review on all the expansions of viruses through metagenomic sequencing

could occupy many pages alone, will not be discussed here, but is reviewed nicely elsewhere [48–50]. Greninger put it nicely when he said, “Growth in virome and metagenomics papers is outpaced only by that of the viruses discovered.” [48]

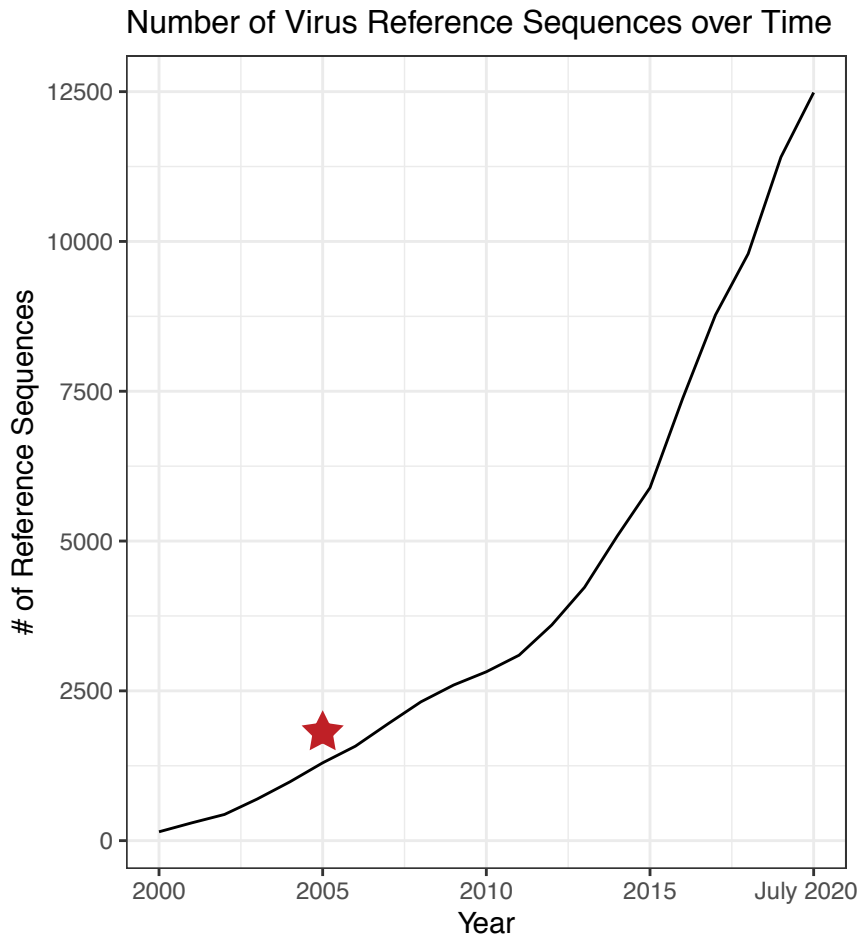

Figure 1.1. Expansion of virus reference sequences over the two decades. Graph depicting the number of virus reference sequences from 2000 to today (July 2020). Also depicted as a red star is the start of the next generation sequencing (NGS) revolution.

Before proceeding forward, it is essential to define the virome. The microbiome, generally speaking, is the collection of all microorganisms within a system, such as an animal host. The virome is a subset of the microbiome, which includes all the viruses within the given system. Although acute viruses, those that cause transient infections, can be considered virome constituents, most viruses within the

virome are persistent in nature. Persistent viruses are not cleared from the host and remain long-term for the remainder of the host's life. Sometimes persistent viruses are present the entire duration of the host's life if the persistent virus is acquired via vertical transmission [51]. In this dissertation I focus on exogenous viruses, but endogenized – that is, viruses whose sequences have integrated into the host genome – viruses can be grouped within the virome and are capable of providing benefits to the host [52]. Although the *bacterial* microbiome has gained considerable appreciated for its role and influence on host health and well-being [53,54], the virome is comparatively understudied.

Perhaps obvious, to study the virome, the viral constituents within different systems must be identified and discovered first. Though many areas of the virome are rapidly expanding, one field that has highly benefited from metagenomic sequencing endeavors is arthropod-associated viromes. Arthropod viromes have rapidly expanded as arthropods are fairly easy to collect and, in many cases, the entire organism can be homogenized and sequenced. Multiple individual arthropods can be to increase the yields of nucleic acid, resulting in high levels of ribonucleic acid (RNA). A caveat to this approach is when the entire organism is sequenced, it is not just comprised solely of arthropod tissues or cells. Instead, all collections of microorganisms associated with the host are sequenced (referred to as the holobiont), making it difficult at times to link a viral sequence to its host.

Though there have been large numbers of papers and groups focused on arthropod viromes, two studies in particular have had extensive influence. A group at the Chinese Center of Disease Control and Prevention, led by Yong-Zhen Zang, first published a paper describing the discovery of 112 novel viruses [55], including a new virus family (*Chuviridae*), and when they expanded their search to include more hosts (including non-arthropod invertebrates), they identified an additional 1445 novel RNA viruses [56]. This has revolutionized our understanding of the true depth and diversity of virus families that can be found in arthropod viromes.

These papers alone did not collectively and completely characterize the global arthropod virome. Studies rapidly moved forward in search of more novel viruses and did so very successfully. At the time of this writing, a search of the NCBI PubMed database with the key input of "arthropod AND virus discovery",

it returns over 600 publications. As NGS technologies continue to advance, costs decrease, and ease and knowledge of the associated analyses expands, we will likely see increases in the number of new viruses for years to come.

1.2.2: The Biological Significance of the Arthropod Virome

In insects, the first case of an RNA metagenomics sequencing approach was in screening of honey bees. The goal of this study was to identify viruses or other microbes that may be responsible for colony collapse disorder. Several candidate viruses were identified, with Israeli acute paralysis virus having the strongest correlation with colony collapse disorder [57]. This study gave support to the utility of metagenomic sequencing in identifying novel viruses and correlations to phenotypes of interest. Further metagenomic exploration in honey bees has expanded the range of pathogenic viruses that can infect honey bees and may be responsible for colony collapse disorder [58,59].

Exploration of arthropod viromes has led to an augmented view of how viruses have evolved [60,61]. A massive expansion of available RNA virus sequences has come from screening arthropod and invertebrate samples. This large expansion has helped enable sophisticated studies on the origin of RNA viruses. For example, Wolf et al. built a phylogenetic tree describing how various RNA virus families may have evolved and branched off from one another [62]. They concluded that double-stranded (ds) RNA viruses evolved from positive-sense RNA viruses, and that negative-sense ssRNA viruses evolved from dsRNA viruses.

Relevant to this thesis, metagenomic sequencing of mosquito viromes has led to a sharp increase of insect-specific viruses (those that infect insects but not mammalian cells), with a large expansion within the *Flaviviridae* family. These flaviviruses are suspected to be ancestral forms of current pathogenic (vector-borne) flaviviruses and represent a base from which scientists can begin to understand the evolution of this important group of viruses [63–65]. These flaviviruses may interfere with their pathogenic cousins; therefore they have potential utility in biocontrol measures [63,66]. Further studies of the disease vector viromes may prove useful for identification of novel biocontrol agents. A secondary goal of my thesis work

was to identify potential functional interactions between the virome and arthropod-borne pathogens, which I have done within *Ixodes scapularis* ticks (see Chapter 4 for a review and my research on this subject).

Through the continual sequencing of arthropod viromes, what has become increasing clear is that co-infection by multiple viruses is the norm, not an exception. I have participated in sequencing of arthropods with close connection to human health, including *D. melanogaster*, various mosquito species, and *Ixodes scapularis* ticks. When evaluating individual *Drosophila* collected from Fort Collins, CO, USA and Paulista, Brazil, we found (perhaps unsurprisingly) that individual flies were rarely infected with a single virus (**Figure 1.2**). Only 1 of the 56 flies sequenced was completely free of any detectable viral infection. What did surprise us was the large fraction of RNA derived from viruses in individual flies. These flies were sequenced starting from total RNA with no enrichment or depletion of any RNA type. In some individuals, viral RNA can account for up to ~25% of total RNA (**Figure 1.2**). In sequencing of individual *Ixodes scapularis* ticks, the stark abundance of viral reads was again noted with two viruses (South Bay virus and blacklegged tick phlebovirus 1) being the most prevalent and most abundant microbial constituents of ticks (**Figure 1.3**; see Chapter 4).

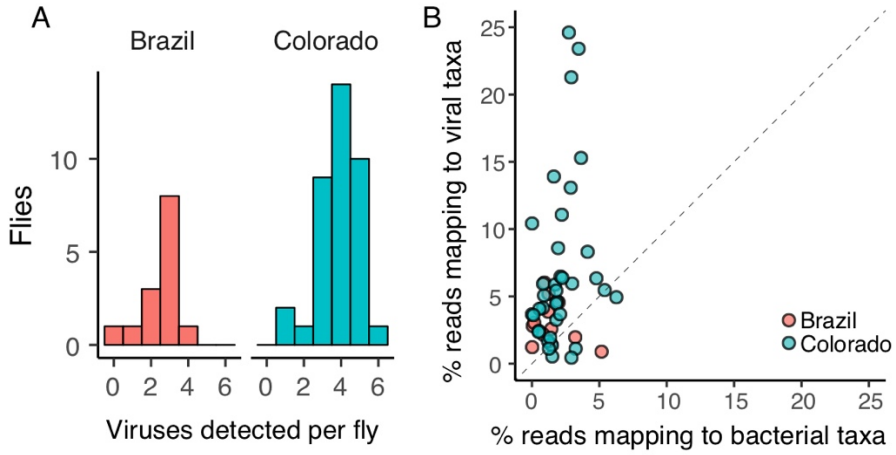

Figure 1.2. **Virus co-infections are common in *Drosophila melanogaster* and account for a large fraction of total RNA.** A) Histograms illustrating the number of viruses found in individual flies from Paulista, Brazil and Fort Collins, CO, USA B) The percent of virus- and bacteria-mapping reads in individual flies. Colorado flies are colored in teal while Brazil flies are colored in red.

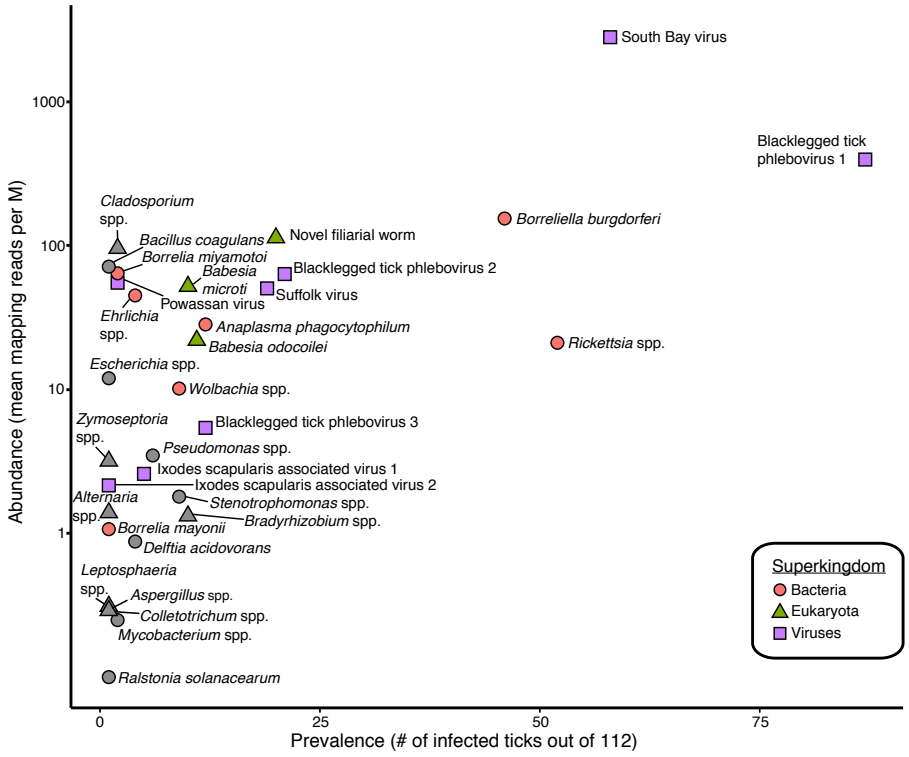

Figure 1.3. South Bay virus and Blacklegged tick phlebovirus 1 are the most abundant and prevalent microorganisms in sampled *I. scapularis*. The prevalence (number of infected ticks out of 112) and average RNA level (average mapping reads per million unique reads on a log scale) for the indicated taxa are plotted. The superkingdom of each taxa is indicated by shape and color as indicated. The 18 taxa that were selected for focused analysis in Chapter 4 are colored (other taxa in grey) [67].

Although the discovery of pathogenic viruses is important, surveys of organisms show that viral infections are ubiquitous and are not associated with any apparent disease [48,55,56,68,69]. In fact, there are increasing cases in which viruses benefit their hosts. Examples of mutualistic phenotypes attributed to viruses include: increased drought tolerance and deterrence of aphid pests in plants [70,71], toxin production in yeasts that kills competing virus-free strains of yeast (known as 'killer' viruses) [72], resistance to pathogenic bacteria in a mouse model [73], and faster development, increased fecundity, and pathogen protection in moths [74]. Though not all constituents of the virome may provide mutualistic benefits, these examples still provide glimpses into the importance of further investigating how viruses influence host health and biology.

A shortcoming of metagenomic sequencing is that the information acquired is only a viral sequence. Although helpful in providing evolutionary context of the new virus, it provides little information into the biology of the virus. For example, many arthropod viruses were discovered when sequencing the holobiont (host and all associated microorganisms), and as a result of this sequencing method, the true host of these viruses must be merely inferred. This is a particular challenge for novel viruses found in families that were not initially associated with arthropods until this massive expansion of virus discovery and diversity occurred. Additionally, the lack of follow-up studies on these viruses, in most cases, leaves open the question: what is the biological impact of these viruses on their hosts?

In previous metagenomic screening of *Drosophila* spp. [69] and *Anopheles gambiae* mosquitoes [75], many new viruses were discovered. Of particular interest were newly discovered partitiviruses. This was of initial interest as the discovery of partitiviruses in arthropods indicated a potential expansion of partitivirus host range. Until recently, partitiviruses had only been known to infect plants, fungi and protozoa [76]. Partitiviruses (family *Partitiviridae*) are non-enveloped viruses with segmented dsRNA genomes. These viruses are persistent in nature, and in many cases do not cause any apparent harm or phenotypic changes in general; as such, they are often referred to as cryptic viruses [76]. Besides the expansion of potential new hosts, we were interested in these viruses because the published sequences were only those encoding the RNA-dependent RNA polymerase. Given the segmented nature of partitiviruses, my rotation project was to find and link the additional segments of these arthropod-associated partitiviruses. I did this within *D. melanogaster* and *An. gambiae*. Through screening individual flies and mosquitoes by metagenomics and RT-PCR, we linked other previously described virus-like sequences [69,75] as the additional segments of these partitiviruses. Segments were identified through relative abundances of virus-like sequences that always cooccurred within individual samples.

When we continued to explore the range of partitiviruses, we found that partitiviruses were found in additional important mosquito vectors, namely *Aedes aegypti* and *Culex* spp. We also sequenced additional populations of *Drosophila* spp. and found that an associated partitivirus, known as galbut virus, was present in every population sequenced. Using our data, and mining through others' data, we found that

galbut virus is globally ubiquitous (**Figure 1.4**). Upon further investigation we also observed that galbut virus is highly prevalent within every population tested (~60% prevalence), comparable to the well-studied endosymbiont *Wolbachia*, and much higher than other well-studied *Drosophila*-infecting viruses (**Fig. 1.4B**).

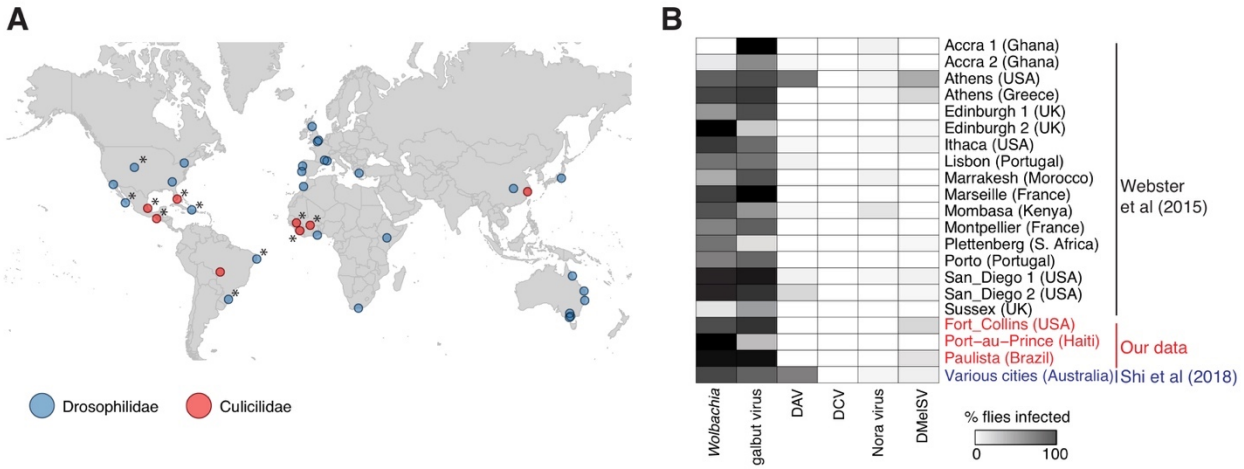

Figure 1.4. **Partitiviruses are globally present in mosquito and *Drosophila* populations.** A) World map illustrating distribution of *Drosophila* (*Drosophilidae*) and mosquito (*Culicidae*) populations that have tested positive for partitiviruses. * denotes these populations were screened by ourselves, other data collected from [56,69,77,78] B) The prevalence of galbut virus within various fly populations screened by ourselves, Webster et al. [69] and Shi et al. [77].

No previous literature describing arthropod-associated partitiviruses existed outside of those reporting novel viral sequences. We aimed to take these new partitivirus-like sequences beyond where most metagenomic sequencing studies end by characterizing their biology further. This research is subsequently a large portion of my thesis work (Chapters 2 and 3). We first verified that these arthropod-associated partitiviruses are actually infecting the arthropod itself and not a symbiont and evaluated how these viruses are transmitted (Chapter 2). A challenge to biologically characterizing novel viruses in arthropods is that most of the hosts are not model organisms. This impairs the ability to carefully dissect underlying biological impacts by the virus. In the case of galbut virus however, we have an invaluable benefit that it is capable

of infecting a premier model organism, *D. melanogaster*. Taking advantage of this model system, we further pursued to evaluate how galbut virus may alter *D. melanogaster* fitness (Chapter 3).

To pursue the studying of *Drosophila*-infecting partitiviruses (and the virome of flies in general), essential laboratory techniques needed to be established. The Stenglein lab had expertise in performing metagenomic sequencing, including sequencing of arthropod samples, but there was no infrastructure built for performing wet-lab work with *Drosophila*. During the course of my thesis, I have taken the lead in establishing the fundamentals and turning the Stenglein lab into a fully functioning *Drosophila* lab. What began as small collection of wild flies being reared on single individual grapes during my rotation, the Stenglein lab now houses dozens of *Drosophila* lines and has the capability to perform fairly sophisticated techniques as described throughout Chapters 2 and 3.

Given that a majority of this dissertation work has been performed using *D. melanogaster*, the remainder of this introduction will be a review on how *D. melanogaster* has proven fruitful in elucidating host-microbe interactions.

1.3: *Drosophila melanogaster* as a Model for Host-Microbe Interactions

1.3.1: History of *Drosophila melanogaster* as a model organism

D. melanogaster, or commonly referred to as vinegar or fruit flies, has been a powerful tool in deciphering many key aspects of basic biology. Fruit flies are particularly good model organisms because of their low cost, ease of rearing, quick developmental time, and extensive genetic tools available. This organism gained its foothold as a model organism and grew famous as Thomas Hunt Morgan used it to confirm the chromosome theory of inheritance in 1910 [79]. Fruit flies continued, and still continue, to be useful in unraveling key aspects of biology. Evidence of this are the several Nobel prizes awarded to groups working with *D. melanogaster* through the 20th century [80–83]. Fields that have benefited from this model organism include genetics and evolutionary biology, neurology and behavior, developmental biology, drug discovery, and bioengineering [84–88].

Another major feat that was accomplished using *D. melanogaster* was showing the utility of the "shotgun approach" for genome sequencing, providing impetus to use this strategy to sequence the human genome [89]. With both human and *Drosophila* genomes available, scientists found that 75% of genes associated with human diseases have orthologues in the fruit fly genome, validating its previous and continual use as a model system for human biology [90]. Other genetic strengths of the fruit fly have since arisen such as the development of the *Drosophila* Genetic Reference Panel (DGRP). This panel consists of >200 inbred strains of flies that can be used to precisely map genetic information responsible for a phenotype of interest with high statistical power [91].

The fields of microbiology and immunology have significantly benefited using *D. melanogaster* due to its capability of being infected with a large variety of microbes through various inoculation routes (oral, pricking, and microinjection) [92]. Key host-microbe interactions, host responses to infection, and immunological pathways have been elucidated using the fruit fly. The remainder of this section will focus on how the fruit fly is used across various host-microbe systems.

1.3.2: Bacteria

Bacteria can be generally classified into two large groups based on their cell wall composition. These groups are Gram-negative and Gram-positive bacteria [93]. *Drosophila* have distinct immune effectors produced for microbial infections, the best described being the antimicrobial peptides (AMPs) [94]. These include diptericin, drosocin, and attacin for Gram-negative bacteria, and defensin for Gram-positive bacteria. Signaling pathways that recognize bacterial peptides and result in subsequent AMP production are the *immune deficiency* (*imd*) pathway for Gram-negative bacteria [95] and the Toll pathway for Gram-positive bacteria [96]. The mechanisms of immune responses regulating bacterial infections that are shared in part with higher order organisms, were first elucidated in fruit flies. For example, relatives of the Toll protein receptors are conserved as a regulatory pathway in humans (Toll-like receptors) [97]. Also importantly, orthologues of these innate immune responses are conserved across various insect systems helping identify interactions in other medically important arthropods, such as disease vectors [98,99].

D. melanogaster has been a useful model for not only elucidating the anti-bacterial signaling pathways but have been useful as models for understanding bacterial pathogenesis. For example, fruit flies have now become models for the human pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* [100,101]. Though the fruit fly system cannot entirely replicate the process that occurs in higher order organisms, like humans, it provides insights to understand the fundamentals of pathogenesis [102,103].

Not only have we better understood pathogenic bacteria interactions using the fruit fly, but our understanding of bacterial-host interactions within the microbiome has grown immensely. The bacterial microbiome of fruit flies is comparatively simple compared to higher order organisms [104,105]. An additional strength of *D. melanogaster* as a model for microbiome studies is the relative ease of creating gnotobiotic lines of flies [106]. The combination of gnotobiotic flies and the low-diversity microbiome has been useful in teasing apart meaningful interactions between these commensal bacteria and their host. Interactions that have been identified include growth across developmental stages, fecundity and lifespan [53,107,108], behavior [109,110], and immune system functions [111–113]. A particular immune system function of the fly microbiome to highlight is the role of commensal organisms in priming the fruit fly immune system against viral infections [114].

1.3.3: Fungi

Similar to bacterial infections, fruit flies produce AMPs in response to fungal infections. The AMPs produced against fungal infections include drosomycin and metchnikowin [94]. Two fungal species have been described extensively using *D. melanogaster* as a model. The first is an entomopathogenic fungus, *Beauveria bassiana*, and the second is a human pathogen *Candida albicans*. Studies of these fungi have shown the Toll pathway to be essential for immune response to fungal infections [115,116]. In addition to antifungal immune responses, fruit flies have been studied in-depth via ecological studies as they play a role in the dissemination of phytopathogenic fungi [117–119].

1.3.4: Parasites

The predominant parasite-host relationship studied in *D. melanogaster* is the interaction between the flies and parasitic wasps. These wasps are members of a group of insects known as parasitoids. This parasitoid name is derived as they lay their eggs in a host after which the larvae develop while feeding on the host tissues, ultimately leading to the host's death [120]. The response of *Drosophila* to these invasions is cellular encapsulation. This host-parasite system is more often studied to understand how the parasitoids evade host immune responses [120]. *Drosophila* have also been useful models for understanding protozoan parasites where fruit flies do not act as the natural host, such as the *Plasmodium* parasite which is the causative agent for malaria and is vectored by *Anopheles* mosquitoes [121].

1.3.5: Viruses

Although the antibacterial and antifungal pathways were initially the predominant immune pathways studied in fruit flies, the antiviral immune responses have quickly gained appreciation and a depth of knowledge has been acquired. The best studied antiviral pathway in fruit flies is the RNA interference (RNAi) pathway which creates antiviral small RNAs. Although there are 3 classes of small RNAs (micro (mi) RNA, small interfering (si) RNA, and piwi (pi) RNA), the siRNAs are responsible for the predominant antiviral effects in fruit flies [122–124]. The formation of siRNAs occurs when host cells recognize double stranded (ds) RNA, and subsequently cleave it into 21 nucleotide fragments with the protein dicer-2 (Dcr-2). These fragments are then loaded onto a complex of proteins known as the RNA-induced silencing complex (RISC) complex. This complex is comprised of Argonaute 2 (AGO2), R2D2, and Dcr-2. With this complex, the siRNA fragments act as guide RNAs, allowing recognition and cleavage of target RNA (i.e. viral RNA). The RNAi pathway is highly effective against RNA viruses, because all RNA viruses must go through a dsRNA intermediate during their replication cycle (though do so at different detectable levels) [125,126]. This pathway broadly acts against RNA viruses [125,127–129] and recent studies show it can also target DNA viruses [130–132]. The highly characteristic and predictable nature of these siRNAs provides a useful source to screen for and identify novel viruses [69,133].

Not unexpectedly, viruses have developed mechanisms to suppress the RNAi pathway [128,134–136]. Although RNAi is the predominant antiviral mechanism, other intrinsic antiviral restriction factors exist. These include ref(2)P, CHKov1, Ge-1, and Pastrel [137–140]. These antiviral suppressors and intrinsic factors have allowed *D. melanogaster* to act as a model to understanding the evolutionary molecular arms race engaged in by host and virus. The JAK/STAT pathway and NF-kB transcription factors have also been associated with antiviral immunity [141–143].

The RNAi pathway has also been heavily used as a genetic tool to reduce expression of interest. This has been useful for high-throughput screenings of gene function both *in vitro* and *in vivo* [144]. Large-scale resources exist now for RNAi in *Drosophila* including fly stocks that can express RNAi target sequences either broadly or in specific tissues [145,146]. Although RNAi is still a very useful tool, CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 technologies are continually becoming more common which allows for complete knockout (loss-of-function) or addition of genes (gain-of-function) [147,148]. However, some speculate that CRISPR-Cas9 and RNAi systems will continue to both exist simultaneously to complement each other in studying genes of interest [144].

The RNAi pathway is a conserved pathway across invertebrates, and therefore has had implications in understanding vector-arthropod-borne (arbo) virus interactions [99,149–151]. Although not an arbovirus vector, *D. melanogaster* can be infected with various arboviruses and therefore can act as a model system for understanding innate antiviral pathways against arbovirus infections [131,152–156]. Fruit flies have also been used as models for some human pathogenic viral infections as well [157].

Drosophila viromes are some of the best characterized insect viromes. In two metagenomic studies screening over 2000 wild flies, >45 new putative RNA viruses and a single DNA virus were identified in *Drosophila* spp. [69,158]. Now to date, there are more than 85 *Drosophila*-associated viruses across ~15 virus families, with the vast majority from virus families with RNA genome structures [69,158,159]. A new group of segmented RNA viruses (Quenyaviruses) were recently identified in *Drosophila* spp. [160], supporting the idea that there are still virome constituents yet to be described. An interesting phenomenon with the *D. melanogaster* virome is the lack of concordance between wild and colonized (laboratory)

populations, with only a few viruses conserved between the two [69]. In subsequent PCR screening of wild flies, only 3 viruses were observed at a prevalence above 50%, galbut virus being one of these constituents [69]. This suggests that although there are diverse viruses associated with the *Drosophila* virome, there are only a subset of common viruses across populations.

Despite being a core constituent of the wild *D. melanogaster* virome, no further information was available regarding galbut virus. To address this gap in understanding, we proceeded forward looking to characterize the biology of galbut virus and its impact on host fitness. Given that partitiviruses appear to be core constituents of mosquito viromes as well [75,78,161,162], we searched for new mosquito-associated partitiviruses and compared similarities between *Drosophila*-associated and mosquito-associated partitiviruses. The following two chapters encompass these research findings.

CHAPTER 2: PARTITIVIRUSES INFECTING *DROSOPHILA MELANOGASTER* AND *AEDES AEGYPTI* EXHIBIT EFFICIENT BIPARENTAL VERTICAL TRANSMISSION [163]

2.1: Introduction

Metagenomic surveys of wild organisms have revealed a breathtaking abundance and diversity of viruses [48,55,56,161,164–169]. Recent studies describing hundreds or thousands of new virus or virus-like sequences have contributed substantially to our understanding of virus evolution and genome structure [55,56,165,169,170]. The explosion of virus discovery from metagenomics has been an important advance in virology, but questions remain about the biological impact of these viruses. Virus sequences can often not even be assigned to a particular host, since many metagenomic datasets derive from intact organisms (holobionts) or from pools of organisms. A virus sequence could represent infection of microbiota of the targeted organism. For example, Webster *et al.* described a variety of novel virus-like sequences associated with wild *Drosophila* fruit flies [69]. A subsequent study on one of these viruses, Twyford virus/Entomophthovirus, revealed that it was in fact a virus of a fungal parasite of flies [171].

Like many groups of RNA viruses, the partitiviruses (family *Partitiviridae*) have undergone a recent expansion via metagenomics [55,56,69,75,78,162,172]. Partitiviruses were previously only known to infect plants, fungi, and protozoa, and most of what is known about these viruses is from studies in these hosts [76,173–177]. Partitivirus genomes are composed of two or more segments of double stranded (ds) RNA. Partitivirus particles are non-enveloped and multipartite: individual particles contain a single segment. Although infections are persistent and generally not associated with phenotypic differences, partitivirus infection can alter hosts in measurable ways. Partitivirus RNA levels were correlated with increased fecundity of *Cryptosporidium parvum* parasites [178]. Some partitiviruses of fungal pathogens confer hypovirulence to their hosts [179–182]. A partitivirus of jalapeño pepper plants made infected plants less attractive to aphids [71].

There is emerging evidence that partitiviruses also commonly infect insects. We had observed partitivirus-like sequences in wild-caught *Anopheles gambiae* mosquitoes from Liberia, Senegal, and Burkina Faso [75]. Partitivirus-like sequences have also been identified in a variety of other types of

mosquitoes, and spodopteran moth-infecting partitiviruses have recently been characterized [75,78,161,162,183,184]. At the time we identified the partitivirus-like sequences in *Anopheles* mosquitoes, among the most closely related sequences were those of galbut virus, a partitivirus-like sequence that had been identified in *Drosophila melanogaster* [69]. Galbut virus was ubiquitous in sampled populations of *D. melanogaster* and present in related species in the melanogaster group [69,77,158,172]. Although small RNA profiles suggested that galbut virus legitimately infected fly cells, it was not clear whether this was indeed the case. Recognizing this uncertainty, Webster et al named galbut after the Lithuanian word galbūt, meaning maybe [69].

We therefore undertook studies to better understand the biology of insect-infecting partitiviruses. We established colonies of wild-caught galbut virus infected *D. melanogaster*. We discovered that a laboratory colony of *Aedes aegypti* mosquitoes harbored a pre-existing infection of a related partitivirus, which we named verdadero virus. We confirmed infection of *Drosophila* cells, quantified efficiency of horizontal and vertical transmission, isolated galbut virus through inbreeding, and established a system for experimental infection by microinjection. These results contribute to a broader understanding of partitivirus biology and potential impacts on arthropod hosts.

2.2: Materials and Methods

2.2.1: *Drosophila* collections

Wild *Drosophila* were collected in Fort Collins, Colorado, USA, from a backyard compost bin. Subsets of flies were moved to bottles for colony establishment or stored at -80˚C. Laboratory reared *D. melanogaster* strain w^{1118} was provided by Dr. Susan Tsunoda. Additional stocks of flies from the *Drosophila* Genetic Reference Panel (DGRP; strains 360, 399, and 517) were obtained via the Bloomington Drosophila Stock Center [91].

2.2.2: Arthropod maintenance and rearing

Both wild-derived and laboratory-derived *Drosophila* were reared at 25°C on the Bloomington Drosophila Stock Center cornmeal *Drosophila* medium (https://bdsc.indiana.edu/information/recipes/bloomfood.html). *Drosophila* stocks were moved to new bottles every 2 weeks.

Aedes aegypti strain Poza Rica (collected in Poza Rica, Mexico and colonized at CSU in 2012; verdadero virus infected) [185] and strains New Orleans (collected from New Orleans, USA with colony donated and established at CSU in 2005; partitivirus uninfected) and Vergel (collected from Vergel, Mexico and colonized at CSU in 2011; partitivirus uninfected) [186] were used for vertical transmission experiments. Mosquitoes were reared as previously described [187].

2.2.3: Vertical and horizontal transmission experiments

To quantify vertical transmission of galbut virus, virgin male and female flies were crossed. Infected wild-caught colonized flies (colony FoCo-17) and uninfected flies (strain w^{1118}) were used for experiments. Individual mating pairs were maintained in the same bottle with an apple agar egg plate and yeast paste (1:1 yeast and water) to promote egg laying. Egg plates were removed daily and eggs collected. Eggs were placed in a 50% bleach solution for 2 min to remove the outer chorion layer, after which they were immediately transferred to clean ddH_2O to remove residual bleach. Bleached eggs were then placed either in vials containing media either together (experiment 1) or as individuals (experiment 2), allowing offspring to rear to adulthood. FoCo-17 parents from which the eggs were derived were tested via quantitative reverse transcription polymerase chain reaction (qRT-PCR) for galbut virus, and if positive, 2-5 day old offspring were collected and also tested via qRT-PCR. Primer sequences were: galbut virus: 5′-CCGTGAAGCAAGGAATCAAT-3′, 5′-TGCCGATTTTCTGCTCTTTT-3′; RpL32: 5′-TGCTAAGCTGTCGCACAAATGG-3′, 5′-TGCGCTTGTTCGATCCGTAAC. Crosses consisted of either a FoCo-17 female and w^{1118} male (maternal transmission) or w^{1118} female and FoCo-17 male (paternal transmission). Exact binomial 95% confidence intervals were calculated with the binom R package.

For vertical transmission of verdadero virus, virgin male and female *Aedes aegypti* were crossed. Verdadero-infected mosquitoes from the Poza Rica colony were crossed with the uninfected New Orleans and Vergel strains. Previous qRT-PCR screening showed that 100% of the males and females of the Poza Rica colony were infected by verdadero virus, while verdadero infection was undetectable in the other colonies. Primer sequences were: verdadero virus: 5′-ATATGGGTCGTGTCGAAAGC-3′, 5′-CACCCCGAAATTTTCTTCAA-3′. Groups of 30 male and 30 female mosquitoes were placed together for 2 days to allow mating. After this period, female mosquitoes were blood fed with defibrinated calf blood (Colorado Serum Company, Denver, USA) for egg production, and eggs were collected 3 days post blood meal. Eggs were then reared to adulthood and adults collected 0-2 days post eclosion. These offspring were then tested for virus presence via qRT-PCR. Groups consisted of either infected females and uninfected males (maternal transmission) or infected males and uninfected females (paternal transmission). Exact (Clopper-Pearson) 95% binomial confidence intervals were calculated with the binom R package [188].

Horizontal transmission of galbut virus was measured by allowing uninfected (DGRP 399 and 517) flies to ingest homogenate from FoCo-17 line #30 galbut virus infected flies. This homogenate was infectious by microinjection (see DGRP experiments below). Homogenate was created by grinding 200 *Drosophila* with a dounce homogenizer in 150 μL 1X Phosphate-buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). To remove cellular debris, homogenate was spun down at 12,000xg for 1 min, and supernatant was transferred to a new vial. This was repeated for a total of three spins. The 150 μL of homogenate was mixed with 850 μL of 5% sucrose and 5% yeast extract in ddH_2O. As a negative control, a second food solution was prepared with sterile 1X PBS instead of homogenate. A single drop of blue food coloring was added to the solution to allow visualization of ingestion. Capillary tubes were filled with 7 μL of food solution. Capillary feeder systems were set up following the protocol by Zer et al. [189]. Two exceptions to this setup included the lack of piercing holes in the vial and wetting the cap. 3-5 day old virgin female and male flies were starved for 9 hours, after which they were placed in the capillary feeding vials containing either virus inoculated food or control inoculated food. Flies were allowed to feed for 17 hours. Flies that had a blue abdomen were moved to vials containing standard fly

medium. Flies were moved to fresh media as needed until 21 days post inoculation, at which point they were frozen at -80°C. Flies were tested for virus presence via qRT-PCR as above.

2.2.4: RNA extractions

We developed and validated a high throughput, magnetic bead-based method to extract RNA from flies and mosquitoes. Individual flies or mosquitoes were added to a 96-well round bottom plate (Corning catalog #3958) with 1 metal BB ball and 100 µL lysis buffer (5M guanidine thiocyanate, 0.1M Tris-HCl, pH 7.5, 0.01M Na_2EDTA, pH 8.0, and 6.25mL 2-mercaptoethanol (βME)) and homogenized at 30Hz for 3 min in a TissueLyzer II (Qiagen). 60 µL of 100% isopropanol was added to each tube and incubated for 1 min. To remove cellular debris, samples were spun down in a centrifuge at 1,200xg for 1 min. Supernatant was removed and added and mixed well by pipetting to 96-well plates containing 90 µL RNA magnetic beads (1 mM trisodium citrate, 2.5 M NaCl, 20% PEG 8000, 0.05% Tween 20, pH 6.4, and 1 mL Sera-Mag SpeedBeads (Thermo Scientific) in a total volume of 50 mL) and 10 µL enhancer (Proteinase K 200ug/mL, 20% glycerol, and 0.5% SDS). Samples were incubated in beads for 5-10 minutes. Using magnetic racks, beads were separated from the supernatant by incubation for 2-3 min, and the supernatant was removed. Tubes were removed from the rack, and the beads were washed with 150 µL wash buffer 1 (20% ethanol, 900 mM guanidine thiocyanate, and 10mM Tris-HCl, pH 7.5) for 2 min. Beads were pelleted again using the magnetic rack and supernatant removed. Beads were then washed with 150 µL wash buffer 2 (WB2; 1X Tris-EDTA buffer (10 mM Tris HCl, 1.0 mM EDTA) pH 8.0, and 80% ethanol) for 2 min. Supernatant was removed. Following this step, beads were resuspended in 30 µL of a DNase I mixture, consisting of 3 µL DNase I (NEB), 3 µL 10xDNase I Buffer (NEB), and 24 µL WB2. Beads were incubated in this mixture for 30 minutes at room temperature. Following incubation, tubes were removed from the magnetic rack and resuspended by pipetting with 100 µL binding buffer (5M Gu-HCl, and 30% isopropanol), and incubated for 5 min. Beads were added to magnetic rack and supernatant removed as previously described. Beads were then washed twice with WB2 as previously described. Following the second wash, tubes were left on the magnetic rack and beads air dried for 3 min. To elute RNA from the beads, tubes were removed from

the magnetic rack, the beads were resuspended by pipetting with 25 µL of nuclease-free H_2O, and the suspension was incubated for 5 min at room temperature. Tubes were placed back on the magnetic rack, and 23 µL of the supernatant, containing the extracted RNA, removed. RNA was quantified fluorometrically using a Qubit instrument (Thermo Fisher) and stored at -80°C.

2.2.5: cDNA synthesis and virus screening

Drosophila were screened for various viruses, including galbut virus, via qRT-PCR. First, RNA was subjected to cDNA synthesis: 5.5 µL of RNA was added to 200 pmol of random 15-mer oligonucleotides and incubated for 5 min at 65 °C, then set on ice for 1 min. A RT mixture containing the following was added (10 µL reaction volume): 1× SuperScript III (SSIII) FS reaction buffer (Thermo Fisher), 5 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleotide triphosphates (dNTPs; NEB), and 100 U SSIII reverse transcriptase enzyme (Thermo Fisher), then incubated at 42 °C for 30 min, 50 °C for 30 min, then at 70 °C for 15 min. 90 µL of nuclease-free H_2O was added to dilute the cDNA to a final volume of 100 µL.

Following cDNA synthesis, qPCR reactions were set up using Luna Universal qPCR Master Mix (NEB) following the manufacturer's protocol. The qPCR reaction was performed on LightCycler 480 (Roche) with the following protocol: 95°C for 3 min, 40 cycles of 95°C for 10s, then 60°C for 45s, and then followed by a melting curve analysis of 95°C for 5s, 65°C for 1 min, and an acquisition starting from 97°C with a ramp rate of 0.11 °C/s and 5 acquisitions per degree. Some qPCR products were validated by running on a 2% agarose gel and Sanger sequencing. Gels were stained with ethidium bromide and imaged on a Gel Doc (Bio-Rad). Primer sequences were: vera virus: 5′-CGTCGGGTGTTTAGAGGTAA-3′, 5′-TAACGATGGTGTTCCAAGGT-3′; La Jolla virus: 5′-ACCGTATGGCGTCGTACTTC-3′, 5′-AAAGTATCAGCAGCGCGAAT-3′; Thika virus: 5′-CAGCAGGTCCCTTGCTAAAG-3′, 5′-TGGTCAGCATATGACCGAAA-3′; Nora virus: 5′-GCACCTGGTCGATTGAATCC-3′, 5′-CGTTCAGGGCATAGTCAAGC-3′.

2.2.6: Shotgun metagenomic library preparation

Library preparation for the *Drosophila* and mosquito samples utilized a KAPA HyperPrep kit following the manufacturer's protocol. All libraries were sequenced on an Illumina NextSeq 500 instrument and NextSeq 500 Mid Output v2 Kits (Illumina). Samples were sequenced using single-end 1x150bp reads.

2.2.7: Sequence and data analysis

Metagenomic sequencing datasets were processed to taxonomically assign viral reads as previously described [67]. Species were assigned to individual flies or pools of flies using a competitive mapping approach. A collection of cytochrome C oxidase subunit 1 (COX1) sequences were collected and curated. Sequences were retrieved from the NCBI nucleotide database by BLASTN searching using as a query the *D. melanogaster* COX1 sequence (NC_024511.2:1474-3009) with an e-value cutoff of 10^{-12} [190]. Sequences longer than 1400 bp were retained and collapsed into a set of representative COX1 sequences using cd-hit-est and a similarity threshold of 97% (-c 0.97) [191]. These operations produced a set of 233 representative COX1 sequences. Quality and adapter trimmed reads from sequencing datasets were aligned to these sequences using bowtie2 with parameters --local and --score-min C,120,1, and the number of reads mapping to the various COX1 sequences were tabulated [192]. Code and the set of representative sequences available at https://github.com/scross92/partitivirus_transmission_paper. All additional data analysis scripts can be found at this location as well. All sequencing datasets have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA645199. Assembled genome sequences are deposited in Genbank under accessions MT742160-MT742176.

2.2.8: Phylogenetic analysis

Sequences similar to the predicted galbut virus RNA dependent RNA polymerase were retrieved from the NCBI protein database using blastp with an E-value cutoff of 10^{-30}. Sequences longer than 400 amino acids were retained and collapsed into a set of representative sequences using cd-hit and a similarity threshold of 95% (-c 0.95). Sequences were aligned using the MAFFT aligner using the --auto setting [193].

Multiple sequence alignments were trimmed with the trimAL tool using setting -automated1 to remove uninformative columns [194]. The highest scoring model for tree inference (LG+I+G4) was selected using the modeltest-ng software [195]. Maximum likelihood trees were inferred using raxml-ng and bootstraps were allowed to run until convergence [196]. Trees were visualized using the ggtree R package [197]. Code and alignments are available at the above mentioned github repository.

2.2.9: Antibody creation

Polyclonal rabbit sera targeting the putative galbut virus capsid protein (encoded on RNA 2) was generated by Pacific Immunology (Ramona, California) using the synthetic peptide Cys-QPRRMIRDKPSLREEAHES.

2.2.10: Western blotting

Whole flies were homogenized in a cold protein extraction buffer [198] containing 20 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM DTT and Complete protease inhibitor cocktail (Sigma). Samples were spun for 5 minutes at 12,000 x g and the supernatant was retained. The protein samples were quantified using the BCA Protein Assay Kit (Thermo Fisher) and diluted 1:10. 15 µg of protein were suspended in NuPAGE LDS Sample Buffer (4X) (Thermo Fisher), heated at 70°C for 10 minutes, then loaded onto a NuPAGE 4-12% Bis-Tris, 1.0mm x 12 well gel (Thermo Fisher). Protein samples were separated via SDS-PAGE and transferred onto a 0.45 µm nitrocellulose membrane (BioRad). The membrane was incubated with anti galbut virus capsid rabbit serum at a dilution of 1:100 and pre-immune rabbit serum at a dilution of 1:100. Primary antibody was detected using a fluorescently-labeled goat anti-rabbit secondary antibody (Li-Cor). Blots were imaged using an Odyssey scanner (Li-Cor).

2.2.11: Immunofluorescence assay

Slides were prepared according to Stenglein et al. [45]. Whole adult *D. melanogaster* tissue sections were prepared for immunofluorescence assays. Adult *Drosophila* were collected, knocked down at 4°C,

and legs and wings were removed. Flies were placed in 4% paraformaldehyde in 1X PBS and fixed for 24 hours at 4°C. Following fixation, *Drosophila* were removed from paraformaldehyde and stored in 1X PBS. Fixed *Drosophila* were sent to Colorado State University Veterinary Diagnostic Laboratories' histology lab for paraffin embedding and sectioning. Sections were deparaffinized in xylene, rehydrated in graded ethanol, placed in 1 mM EDTA at 99°C for 20 min, and rinsed with water. Sections were washed 2 x 5 min in 1X Tris-buffered saline (TBS; 50 mM Tris-Cl pH 7.5, 150 mM NaCl) + 0.025% Tween-20, permeabilized in 1X PBS + 0.1% Triton X-100 for 10 min, and washed 3 x 5 min in TBS + 0.05% Tween-20 (TBS-T). Slides were then blocked with 1X TBS + 1% BSA for 20 min, incubated for 30 min with anti-galbut rabbit serum at a dilution of 1:500 and pre-immune rabbit serum at a dilution of 1:500, washed in TBS-T, incubated for 30 min with Alexa-Fluor 488-conjugated goat anti-rabbit secondary antibody, and washed again. The second wash included 0.5 µg/ml Hoechst 33342. Sections were mounted in Vectashield, coverslipped and sealed with clear nail polish, then imaged with an Olympus IX81 Inverted Confocal Laser Scanning Microscope.

2.2.12: Intrathoracic microinjections

100 adult flies from the galbut-only line (FoCo-17 line #30) were placed in 200 µL 1X PBS and homogenized by hand with a 1.5 mL homogenizer. Homogenate was spun down at 12,000xg in a microcentrifuge for 1 min to remove cellular debris. This was performed three times to remove all cellular debris. Homogenate was filtered at 0.22 µM filter to remove any cellular material. 50 nL of the homogenate was injected into flies intrathoracically using a Drummond Nanoject II.

2.3: Results

2.3.1: Galbut virus in local wild *Drosophila melanogaster* populations and establishment of colonies of galbut virus infected flies

Previous work described galbut virus as a ubiquitous infection of wild *D. melanogaster* [69]. Motivated to better understand the biological impact of galbut virus on infected flies and the dynamics that

produced this high global prevalence, we sampled local fly populations and established colonies of wild-caught infected flies. We collected flies from Fort Collins, Colorado and used qRT-PCR to detect galbut virus RNA in individual flies, which were – as expected – infected with galbut virus (**Figure 2.1**). We collected flies from the same backyard compost bin over 3 years and found 94%, 84%, and 67% of individual flies infected in 2017, 2018, and 2019 (**Figure 2.1A**). We used subsets of collected flies to establish colonies, which we called FoCo-17, -18, and -19.

We first determined whether colonized flies would retain galbut virus infection, as a previous analysis of laboratory-reared *Drosophila* transcriptomic datasets showed a general absence of galbut virus [69]. We tested galbut virus stability in the FoCo-17 population over four generations and then again after 2 years in the laboratory. We found that the parental generation had a prevalence of 93% while F1-F4 offspring maintained a prevalence between 63% and 92% (**Figure 2.1B**). After two years, 66 out of 66 sampled FoCo-17 flies were infected (**Figure 2.1B**). We used metagenomic sequencing to quantify levels of all of the galbut virus genome segments in 20 randomly selected adult flies from the FoCo-17 and FoCo-18 populations (**Figure 2.1C**). FoCo-17 flies were sampled 2 years post colony establishment and FoCo-18 flies after 1 year. 19 of 20 FoCo-17 flies were galbut virus positive by metagenomic sequencing (>10 reads mapping to galbut virus RNA 1 per million reads). All 20 sampled FoCo-18 flies were galbut virus positive (**Figure 2.1C**). "Chaq virus" sequences, which represent either an optional galbut virus segment or a satellite virus of galbut virus, were detected in most but not all galbut virus infected flies (**Figure 2.1C**) [69,77].

2.3.2: Identification of additional partitiviruses in flies and mosquitoes

We identified the sequence of a second partitivirus in our metagenomic datasets from wild flies and from flies in our FoCo-17 and FoCo-18 colonies (**Figure 2.1C**). We named this virus vera virus (vera means true in Esperanto). The vera virus genome consisted of an RNA encoding an RdRp (RNA #1), an RNA encoding a putative capsid protein (RNA #2), and a chaq virus-like RNA segment (21% identical at an amino acid level to the predicted galbut virus-associated chaq protein) (**Figure 2.2**). We did not identify

any contigs with detectable sequence similarity to the predicted protein encoded on galbut virus RNA 3, nor contigs with co-occurrence patterns and coverage levels that were similar to vera virus RNAs 1 and 2. Like galbut virus, vera virus was detectable in wild-caught flies, and remained as a stable persistent infection in colonized populations over two years (**Figure 2.1C**). In addition to these two partitiviruses, the FoCo-17 and FoCo-18 populations harbored infections by Nora virus, La Jolla virus, and Thika virus [69,199]. Craigies Hill and Motts Mill virus sequences were additionally detected in FoCo-18 flies [69].

We also serendipitously identified using metagenomic sequencing another partitivirus as a persistent infection of a colony of *Aedes aegypti* mosquitoes originally derived from Poza Rica, Mexico. We named this virus verdadero virus (verdadero meaning true in Spanish, **Figure 2.2**). The verdadero virus genome consisted of an RNA encoding an RdRp (RNA #1), an RNA encoding a putative capsid protein (RNA #2), and a chaq virus-like RNA segment. As was the case for vera virus, we could not identify a segment with similarity to RNA #3 of galbut virus. We tested 48 (24 male, 24 female) adult mosquitoes in the colony and 100% were positive for verdadero virus RNA by qRT-PCR (confidence interval 92.6-100%). Other *Ae. aegypti* colonies housed at Colorado State University (strains New Orleans and Vergel) were negative for verdadero virus by qRT-PCR.

We created maximum likelihood phylogenies using these new partitivirus sequences and previously described similar sequences (**Figure 2.3**). This tree includes a number of sequences from metagenomic surveys of invertebrates and some fungi-derived sequences. The invertebrate-derived sequences did not form a well supported monophyletic cluster. In some cases, sequences from related hosts clustered together. For instance, Atrato Partiti-like virus 2 and Partitivirus-like 1 from *An. darlingi* and *An. gambiae* formed a well-supported group. But overall there was a notable lack of concordance by host. Mosquito-derived sequences were spread throughout the tree. Galbut virus and vera virus, both from the same populations of *Drosophila melanogaster*, were distantly placed on the tree.

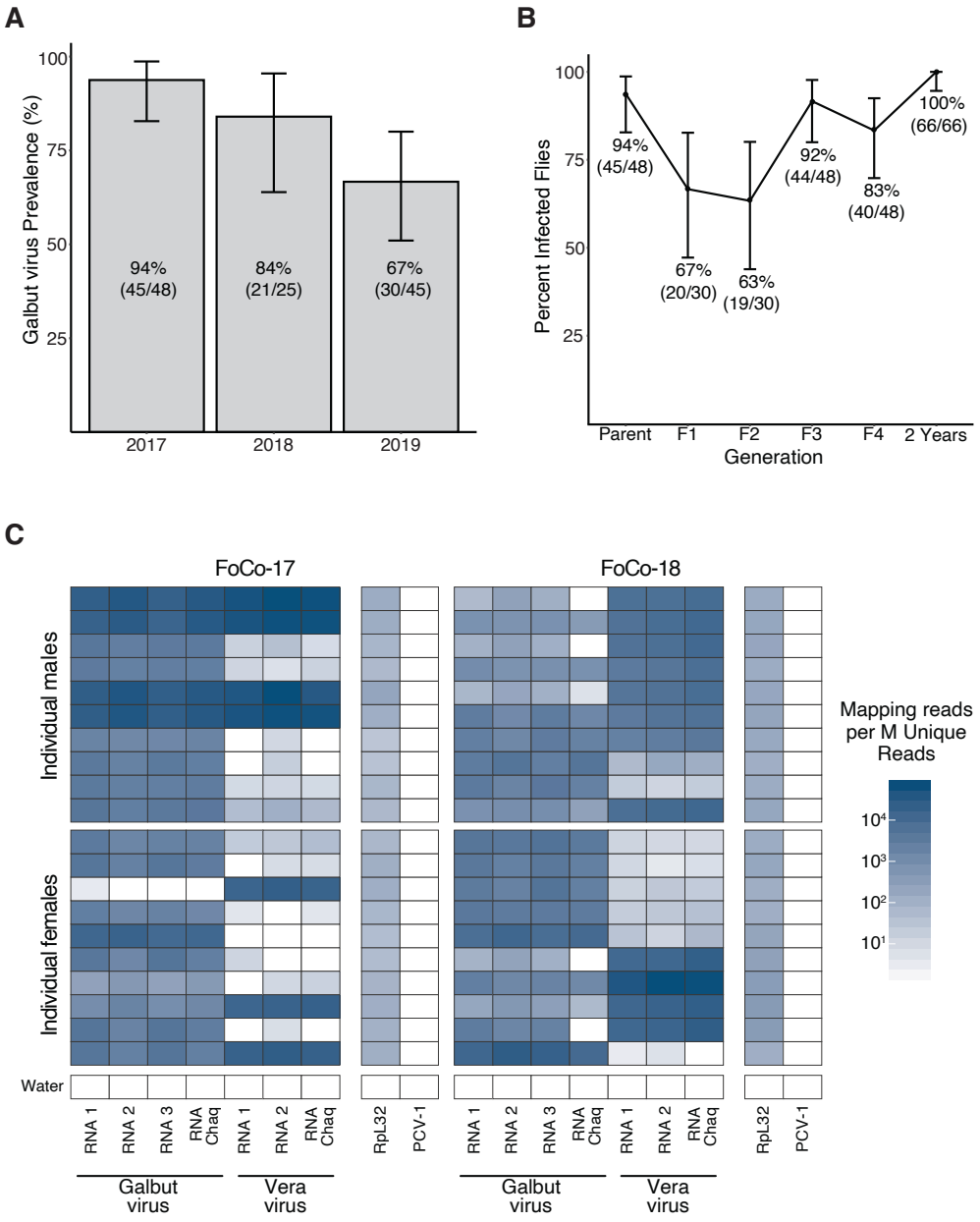

Figure 2.1. **Galbut virus is found in wild populations consistently and can be maintained in colonized populations of *Drosophila melanogaster* over multiple years.** A) Prevalence of galbut virus in wild-caught flies from a backyard compost bin in Fort Collins, Colorado over 3 years. Numbers of flies positive by qRT-PCR and number of flies sampled are indicated. Error bars indicate 95% confidence intervals. B) Galbut virus was maintained for at least two years in colonized populations of wild *D. melanogaster* collected in Fort Collins, CO, USA in 2017 (FoCo-17). C) Prevalence and relative loads of all segments of galbut virus and vera virus in individual FoCo-17 and FoCo-18 adult flies. Each row represents an individual fly and values indicate mapping reads per million unique reads in each metagenomic sequencing dataset. Mapping to host ribosomal protein L32 (RpL32) served as a positive control proportional to dataset sizes, and pepper cryptic virus-1 (PCV-1) served as a mapping negative control. A library prepared from water served as an additional negative control.

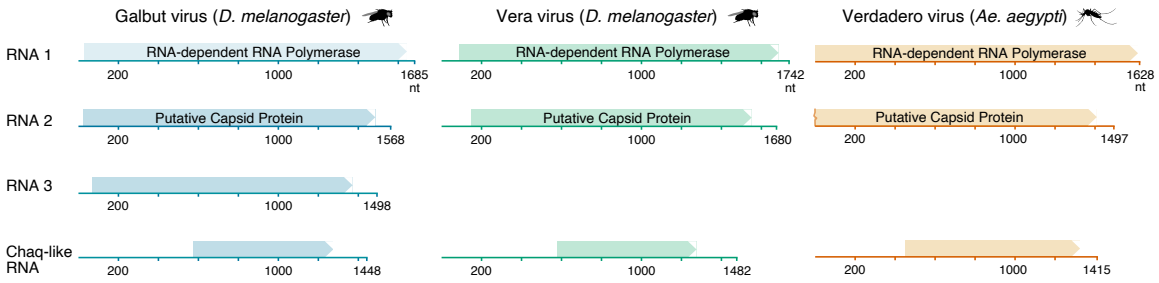

Figure 2.2. **Genome structure of galbut virus, vera virus, and verdadero virus.** Depictions of the genome structures of these viruses with predicted open reading frames indicated. RNA 1 of these viruses is predicted to encode RNA dependent RNA polymerases and RNA 2 a putative capsid protein. RNA 3 and the chaq virus-like segments are predicted to encode proteins of unknown function. The open reading frame on verdadero virus RNA 2 extends beyond the partial sequence that we recovered.

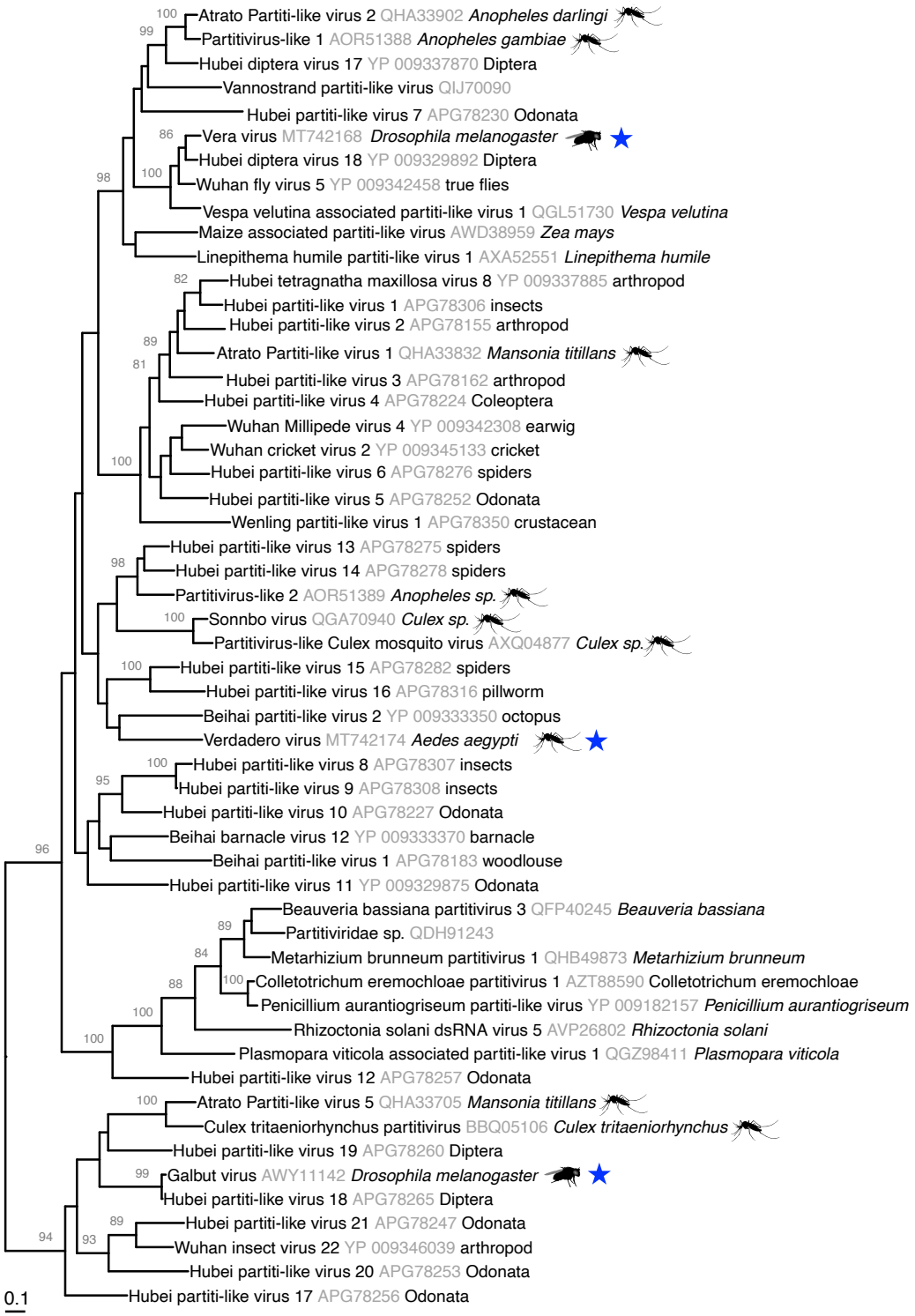

Figure 2.3. **Phylogenetic tree of partitivirus-like sequences.** Unrooted maximum likelihood phylogenetic tree of partitivirus-like RdRp sequences related to those described here (galbut virus, vera virus, and verdadero virus; marked with blue star). Sequences from mosquitoes and *Drosophila* are indicated with images. The host from each sequence's metadata in NCBI is noted after the accession number. In many cases, exact hosts are uncertain because sequences derive from pools of different organisms.

2.3.3: Galbut virus replicates in *Drosophila* cells

Two lines of evidence suggested that galbut virus actually infects flies. First, galbut virus derived small RNA profiles matched those expected for a virus that replicated in fly cells [69]. Second, galbut virus clusters phylogenetically with a large group of partitivirus-like sequences from arthropod samples (**Figure 2.3**). Nevertheless, direct evidence of galbut virus replication in fly cells had not been published, and it remained possible that galbut virus sequences actually derived from another microbial symbiont or were dietary in origin.

We therefore raised an antibody against the galbut virus putative capsid protein encoded on RNA segment 2. Laboratory strain w^{1118} flies tested negative for galbut virus RNA both by qRT-PCR and subsequent metagenomic sequencing. Extracts from FoCo-17 and w^{1118} flies were subjected to western blotting to confirm the specificity of the antibody. A band of the expected size, ~63 KDa, was present in galbut-infected FoCo-17 flies but not in uninfected w^{1118} flies (**Figure 2.4A**). We used immunofluorescence microscopy using this antibody to locate virus protein in sections of formalin fixed paraffin-embedded adult flies. We observed foci of staining within cells and tissues throughout adult male and female flies. Locations of infection included gut tissues (foregut, midgut, hindgut) and in egg chambers. These fluorescent foci were not evident in galbut virus uninfected w^{1118} flies (**Figure 2.4B**).

We also evaluated galbut virus RNA levels in dissected tissues from adult FoCo-17 flies. Midguts, ovaries, and testes were all positive for galbut virus RNA by qRT-PCR (**Figure 2.4C**). In general, galbut virus RNA levels were higher in midguts and in reproductive tissues than in whole bodies (**Figure 2.4C).** Galbut virus RNA levels were higher in male bodies than in female bodies (2.6x higher median RNA level; **Figure 2.4D**). In all samples, galbut virus RNA levels were higher than those of ribosomal protein L32 (RpL32) mRNA, which is categorized as having "extremely high expression" in the modEncode database [200]. The highest level of galbut virus RNA was in testes, where median levels were 57x higher than those of RpL32 mRNA.

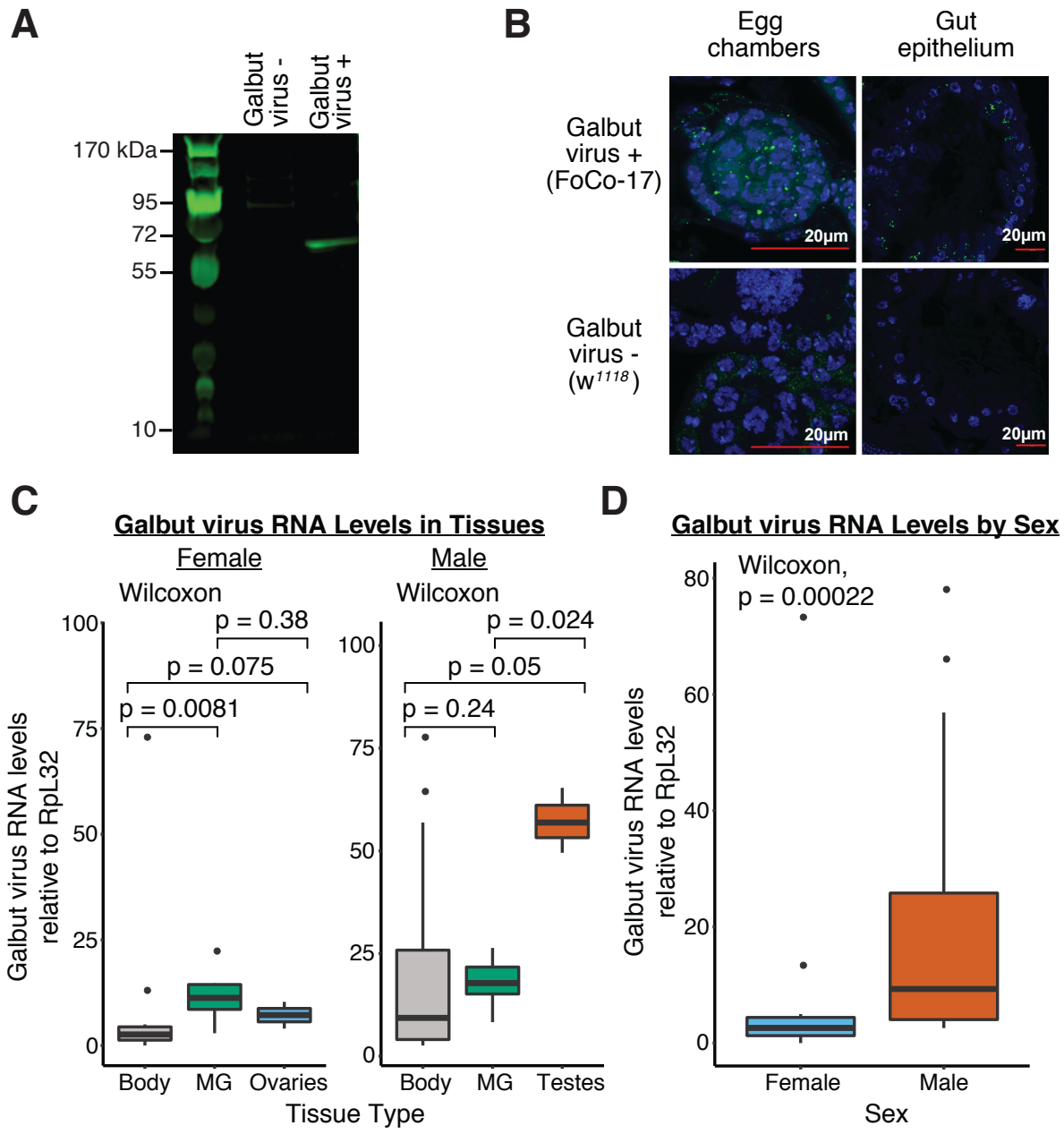

Figure 2.4. **Galbut virus protein and RNA detection in *Drosophila* tissues.** A) The specificity of a polyclonal antibody raised against the predicted galbut virus capsid protein was confirmed by detection of a single band of the expected size of ~63kDa in infected FoCo-17 flies that was absent in uninfected w^{1118} flies. B) Detection of galbut virus predicted capsid protein in adult FoCo-17 flies showed foci of staining in cells in various tissues including the egg chambers and gut epithelium. Green: anti-galbut capsid antibody; blue: Hoescht 33342 (DNA). Scale bars represent 20 µm. C) Boxplots showing galbut virus RNA levels relative to the housekeeping gene RpL32 ($2^{-\Delta Ct}$ method) in dissected midguts (MG), ovaries, and testes. RNA levels were quantified in 20 whole bodies of each sex, from 6 pools of 5 midguts per sex, or from 3 pools of 10 ovaries or testes. Bonferroni adjusted p-values from Wilcoxon test are indicated. D) Galbut virus RNA levels in male and female adult bodies as in panel C.

2.3.4: Galbut virus exhibits efficient biparental vertical transmission but inefficient horizontal transmission by ingestion

We set out to determine what modes of transmission could contribute to galbut virus's exceptional high global prevalence. Having visualized viral protein in the egg chambers (**Figure 2.4B**) and because dissected testes and ovaries tested positive for galbut virus by qRT-PCR (**Figure 2.4C)**, we first evaluated maternal and paternal vertical transmission. We performed several experiments to quantify transmission efficiency. For all experiments, vertical transmission efficiency was quantified by testing for galbut virus RNA in adult offspring collected 2-5 days post eclosion.

In the first experiment, we set up individual crosses between FoCo-17 infected and w^{1118} uninfected adults, collected and bleached eggs, and placed eggs together in one vial per cross. Of the 6 independent maternal crosses (infected female, uninfected male), 34 of the 34 offspring were positive (100% transmission efficiency, confidence interval 89.7-100%; **Table 2.1**). Of the 5 independent paternal crosses (uninfected female, infected male), 27 of the 28 offspring tested positive (~96% transmission efficiency, confidence interval 81.7-99.9%; **Table 2.1**).

In the second experiment we separated bleached eggs into individual vials to avoid possible horizontal transmission between siblings during development. Of the 4 independent maternal crosses, 44 of 44 offspring tested positive (100% transmission efficiency, confidence interval 92.0-100%; **Table 2.1**). Of the 2 independent paternal crosses, 18 of 18 offspring tested positive (100% transmission efficiency, confidence interval 81.5-100%; **Table 2.1**).

In the third experiment, to verify that this high efficiency was not an artifact associated with laboratory-reared flies, we trapped wild female *D. melanogaster*, separated them into individual bottles, allowed them to lay eggs, and then tested mothers and offspring for galbut virus RNA. Offspring from 7 independent galbut virus positive females were tested, and we found a vertical transmission efficiency of 99% (119/120 offspring; confidence interval 95.4-100%; **Table 2.1**), indicating that efficient vertical

transmission was not only associated with laboratory environments. We could not discern the infection status of the unknown fathers.

To quantify galbut virus horizontal transmission (experiment 4), adult flies were exposed by ingestion of filtered homogenate from galbut virus infected flies. This homogenate was infectious by microinjection (see below: Experimental infection of DGRP flies with galbut virus). Homogenate was mixed with a dyed food solution and placed in capillary feeding tubes. Ingestion was confirmed by visual inspection of fly abdomens. After feeding, flies were returned to normal food for 21 days then screened for galbut virus RNA. Galbut virus RNA levels in experimental flies were compared to levels in flies that were immediately flash frozen after ingestion of homogenate to establish an upper limit for residual RNA levels in the absence of viral replication. Although 3 of the 54 exposed flies did test positive for galbut virus RNA after 21 days, their C_t values were on average 4.9 higher (~30 fold less RNA), than C_t values from immediately-frozen injected flies. We interpreted these low level signals as deriving from residual ingested RNA. Therefore, we detected no evidence of active replication in any of the 54 flies tested (0% horizontal transmission; confidence interval 0-13.2%). This indicated that horizontal transmission, at least by ingestion, is unlikely to contribute substantially to maintenance of galbut virus infection in fly populations.

2.3.5. A mosquito partitivirus also exhibits efficient biparental vertical transmission

We performed similar experiments to quantify vertical transmission efficiency of verdadero virus in *Aedes aegypti* by crossing infected female or male mosquitoes from the Poza Rica colony with uninfected counterparts from the New Orleans and Vergel colonies (experiment 5). Maternally, verdadero virus was transmitted at 100% efficiency (48/48 offspring, confidence interval 92.6-100%, **Table 2.1**) and paternally it was transmitted at 97% efficiency (38/39 offspring, confidence interval 86.5-99.9%, **Table 2.1**). This indicated that high vertical transmission efficiency is a characteristic of multiple dipteran infecting partitiviruses.

Table 2.1: *Drosophila melanogaster* and *Aedes aegypti* partitiviruses exhibit efficient biparental vertical transmission. Transmission efficiencies of vertical (maternal, paternal, and wild maternal) and horizontal (ingestion) routes. Numbers in brackets represent exact binomial confidence intervals.

Experiment	Transmission	Species	Type	Group	# Crosses	# Screened*	Total Positive	Transmission Efficiency
1	Vertical	*D. melanogaster*	Maternal	Female+/Male-	6	34	34	100% [89.7, 100%]
		D. melanogaster	Paternal	Female-/Male+	5	28	27	100% [81.7, 99.9%]
2	Vertical	*D. melanogaster*	Maternal	Female+/Male-	4	44	44	100% [92.0, 100%]
		D. melanogaster	Paternal	Female-/Male+	2	18	18	100% [81.5, 100%]
3	Vertical	*D. melanogaster*	Maternal	Wild Female+	7	120	119	99% [95.4, 100%]
4	Horizontal	*D. melanogaster*	Ingestion	Female	NA	28	0	0% [0, 12.3%]
		D. melanogaster	Ingestion	Male	NA	26	0	0% [0, 13.2%]
5	Vertical	*Ae. aegypti*	Maternal	Female+/Male-	4	48	48	100% [92.6, 100%]
		Ae. aegypti	Paternal	Female-/Male+	4	39	38	97% [86.5, 99.9%]

**Offspring screened in vertical transmission, exposed adults screened in horizontal transmission*

2.3.6. Partitivirus RNA levels in *Drosophila* and mosquito offspring

Although both maternal and paternal transmission were ~100% efficient (**Table 2.1**), we were curious whether the route of vertical transmission impacted viral loads in offspring. For sigma virus, another biparentally transmitted virus in *Drosophila*, lower viral levels following paternal transmission lead to decreased paternal transmission in subsequent generations [201,202]. We quantified galbut virus RNA levels in offspring of crosses between FoCo-17 and w^{1118} parents (**Table 2.1; Figure 2.5**). Offspring were collected 2-5 days post eclosion. Offspring from paternal transmission (n=18) had significantly higher galbut virus RNA levels (5-fold median difference) compared to those infected by maternal transmission (n=44) (**Figure 2.5A**, p = 0.0016). However, in *Aedes aegypti*, we saw the opposite trend: offspring from maternal transmission had significantly higher verdadero virus levels (107-fold median difference) than offspring infected via paternal transmission (**Figure 2.5B**, $p = 5.2 \times 10^{-6}$). In both flies and mosquitoes, individual offspring exhibited broad distributions of viral loads that overlapped between sexes. Nevertheless, the route of vertical transmission impacted average partitivirus loads in offspring, and the direction of this effect was opposite for these two viruses.

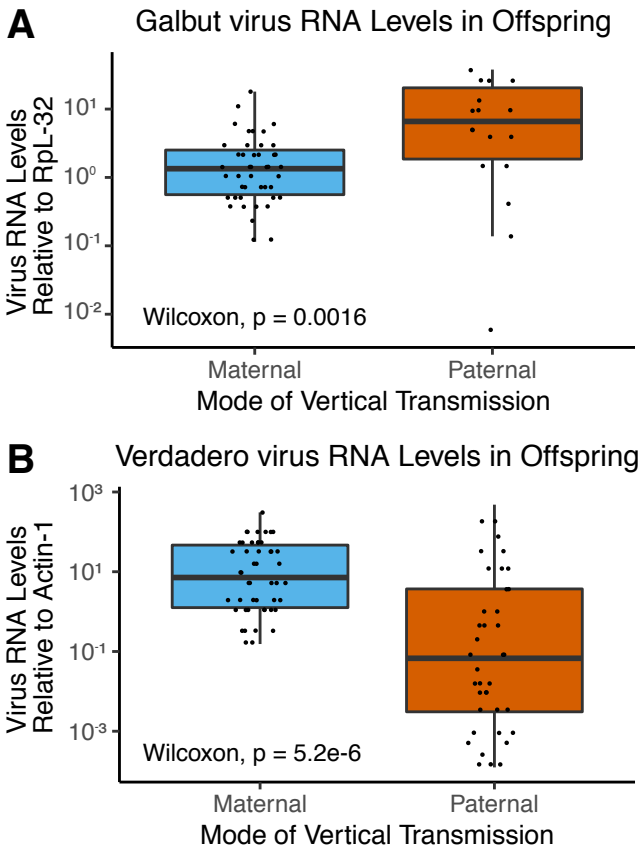

Figure 2.5. **Galbut virus and verdadero virus RNA levels in offspring infected via maternal or paternal transmission.** A) Galbut virus RNA levels in offspring (maternal n=44; paternal n=18) relative to the housekeeping gene RpL-32 (Wilcoxon test, $p = 1.6 \times 10^{-3}$). B) Verdadero virus RNA levels in offspring (maternal n=48; paternal n=38) relative to the housekeeping gene Actin-1 (Wilcoxon test, $p = 5.2 \times 10^{-6}$).

2.3.7. Isolation of galbut virus through breeding

We designed a breeding scheme to create inbred fly lines that were only infected with galbut virus that took advantage of galbut virus's efficient vertical transmission. We crossed pairs of virgins from the FoCo-17 population that harbored 5 viruses: galbut virus, vera virus, La Jolla virus, Nora virus, and Thika virus [69,199]. Eggs from 33 independent crosses were collected, bleached and placed in individual vials. This created bottlenecks that removed horizontally transmitted viruses and those with less efficient vertical transmission than galbut virus [203]. Parents were retrospectively tested by qRT-PCR for all 5 viruses, and if both parents had fewer viruses than the previous generation, sibling offspring were randomly crossed, eggs collected and screening repeated. We were able to generate a line with galbut virus as the only

detectable viral infection after 4 generations, which we named FoCo-17 line #30 (**Figure 2.6**). Also observed in this breeding scheme was the eventual purge of vera virus, the other partitivirus we identified in *Drosophila* (**Figure 2.1, Figure 2.2**). This suggested that although galbut virus and verdadero virus were transmitted efficiently at ~100% over at least one generation (**Table 2.1**), this efficiency may not be a universal feature of all arthropod-infecting partitiviruses.

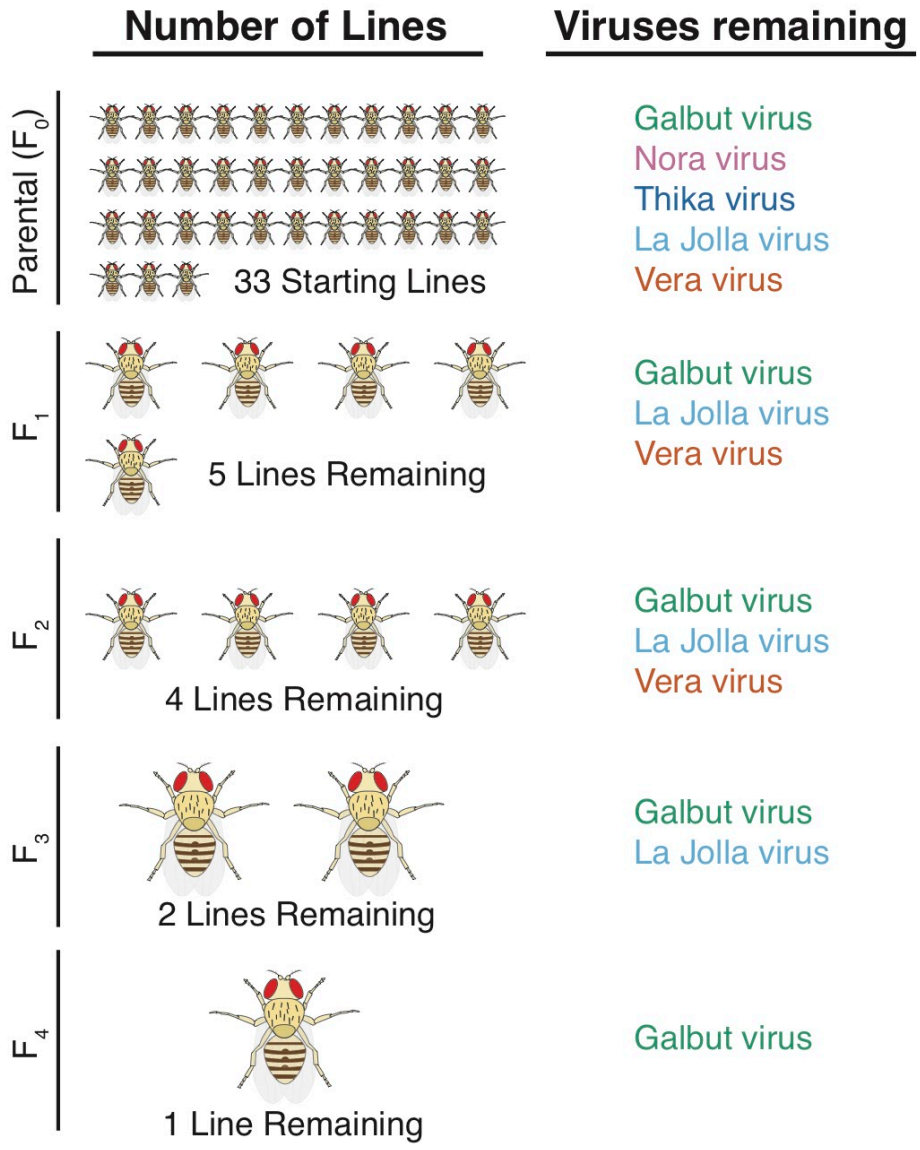

Figure 2.6. **Creation of a line of *D. melanogaster* singly infected with galbut virus.** Iterative inbreeding of *D. melanogaster* coupled to purging of lines that did not contain fewer viruses, lead to the establishment of a *D. melanogaster* line with galbut virus as the only detectable virus after four generations. Virus names on the right of the figure indicate the viruses present in any of the lines remaining at the indicated generation.

2.3.8. Experimental infection of galbut virus via microinjection

To establish a system for experimental infection we performed intrathoracic (IT) microinjection of virgin uninfected flies with a filtered homogenate from FoCo-17 line #30. We injected between 61 and 120 flies from each of three *Drosophila* Genetic Reference Panel (DGRP) strains (~50% of each sex) [91]. 9 days post injection, we tested 5 male and 5 female injected flies per strain using qRT-PCR and in all cases 10/10 injected flies tested positive (**Table 2.2**). To verify that these flies were truly infected and that we were not detecting residual injected viral RNA, the remaining flies were crossed and we tested offspring from IT injected parents at 9, 15, and 19 days post injection. Offspring whose eggs were laid 9 or 15 days post injection were not uniformly infected (range 0-100%; **Table 2.2**). But, by 19 days post injection, all offspring tested were positive by qRT-PCR (**Table 2.2**). This confirmed that injected parents were legitimately infected and that infection had disseminated to tissues involved in vertical transmission in all DGRP strains by 15 days post injection. These infected offspring were used to establish colonies that differed from the original inbred DGRP lines only in their galbut virus infection status. Testing these colonies 50 days later (~4-5 generations), we found that DGRP strains 399 and 517 maintained 100% prevalence (24/24 flies for each positive by qRT-PCR, **Table 2.2**). However, DGRP 360 only had a galbut virus prevalence of ~21% (5/24 flies, **Table 2.2**). This suggested that multigenerational vertical transmission efficiency may be a function of host genetic background.

Table 2.2: Experimental infection of galbut virus by microinjection and subsequent transmission to offspring. Adult flies from three DGRP strains were injected with a homogenate from galbut virus infected line #30. Ten injected flies per strain were tested for galbut virus RNA by qRT-PCR 9 days post injection; the percent positive of each set of 10 is indicated. Additional injected flies were allowed to mate, with subsequent testing of offspring from the first and 5th generation to monitor vertical transmission from injected parents and stability of transmission across multiple generations. First generation offspring whose eggs were laid 9, 15, or 19 days post injection were tested; the percent positive of each set of 12 flies are indicated. Infected colonies were established using offspring from days 15 and 19, and prevalence was tested 50 days after establishment (~4-5 generations). Numbers in brackets represent the lower and upper 95% confidence intervals. DGRP: *Drosophila* Genetic Reference Panel.

Strain	Adults Positive (n=10)	Days Post Injection	Prevalence in F_1 Offspring (n=12)	Prevalence in F_5 Offspring (n=24)
DGRP-360	100% [69.2, 100%]	9	0% [0, 26.5%]	21% [7.1, 42.2.%]
		15	92% [61.5, 99.8%]	
		19	100% [73.5, 100%]	
DGRP-399	100% [69.2, 100%]	9	25% [5.5, 57.2%]	100% [85.7, 100%]
		15	100% [73.5, 100%]	
		19	100% [73.5, 100%]	
DGRP-517	100% [69.2, 100%]	9	100% [73.5, 100%]	100% [85.7, 100%]
		15	100% [73.5, 100%]	
		19	100% [73.5, 100%]	

2.4: Discussion

In this study, we began to characterize the biology of a recently discovered virus – galbut virus – that is unusually common in an important model organism. We confirmed that this "maybe" virus is indeed a virus of its putative fruit fly host and found that it can be transmitted efficiently from infected fathers or mothers to their offspring. This property is shared by at least one more insect-infecting partitivirus, verdadero virus, which we identified as a persistent infection in a colony of *Aedes aegypti* mosquitoes. This expands the known host range of the partitiviruses and suggests that the large number of partitivirus sequences that have been identified in a broad range of arthropods are likely legitimate viruses of those hosts. Efficient vertical transmission may be a mechanism that generally supports the success of these viruses.

An apparent paradox associated with this efficient biparental vertical transmission is that galbut virus has only been detected in ~60% of individual wild flies tested (**Figure 2.1**, [69,77]). Modeling indicates that highly efficient biparental transmission should eventually produce 100% of susceptible individuals to be infected, unless infection exacts a high fitness cost [204]. Several hypotheses could account for this apparent discrepancy. First, it is possible that galbut virus is increasing or decreasing in

frequency. In our sampling, prevalence decreased slightly over three years, though it is unclear whether this was just a stochastic effect nor whether these flies and viruses represented a single population lineage (**Figure 2.1A**). Previous work suggested that the common ancestor of galbut virus in *D. melanogaster and D. simulans* populations existed ~200 years ago [69], and perhaps galbut virus has not settled to an equilibrium frequency. Additional longitudinal sampling will shine light on this.

A related hypothesis is that galbut virus might exact enough of a fitness cost that it is driving an increase in host resistance alleles [140,205–207]. This phenomenon has been observed for *D. melanogaster* sigma virus: although sigma virus exhibits biparental vertical transmission, it negatively impacts host fitness resulting in prevalences of 0-30% worldwide [69,208]. In our laboratory colonies, galbut virus infection frequency rose to ~100% in 2 populations (**Figure 2.1C**). It may be that fitness costs are negligible in a laboratory environment. Our experiments using different DGRP strains provided evidence that host genetics might modulate galbut virus transmission efficiency (**Table 2.2**). However, we only tested only 3 of the >200 DGRP lines. It is possible that other traits that vary between DGRP lines, such as differential microbiomes, could impact galbut virus replication or transmission efficiencies [114,209]. We noted that DGRP 399 and 517 were *Wolbachia* negative, whereas DGRP 360 was *Wolbachia* positive (our metagenomic sequencing data and [210]). *Wolbachia* are bacterial endosymbionts with antiviral properties [211], and *Wolbachia* infection status may underlie the differences in galbut virus vertical transmission efficiency that we observed (**Table 2.2**). However, Shi et al. reported similar galbut virus infection rates in wild *Drosophila* with and without *Wolbachia* [77].

The efficient vertical transmission observed for these insect-infecting partitiviruses is reminiscent of what has been observed for their plant and fungus-infecting counterparts. Plant-infecting partitiviruses are completely dependent on vertical transmission, and horizontal transmission does not occur even when infected plants are grafted onto uninfected counterparts [173,175,177]. Fungus-infecting partitiviruses also exhibit efficient vertical transmission [76,176,177], but the efficiency varies [212–218]. Unlike the plant-infecting partitiviruses, fungus-infecting partitiviruses are able to transmit horizontally through processes such as hyphal anastomosis [76,217,219,220]. It appears that galbut virus and verdadero virus depend on

vertical transmission like their plant-infecting counterparts (**Table 2.1**). Vera virus on the other hand, did not appear to have similarly efficient vertical transmission, as we were able to purge it relatively easily during the creation of *Drosophila* singly infected with galbut virus (**Figure 2.6**). Similarly, partitiviruses infecting armyworm moths exhibited 100% maternal vertical transmission transmission, but no paternal vertical transmission was detected [184].

Although we did not observe any evidence for horizontal transmission of galbut virus, there is evidence that galbut virus and other partitiviruses can transmit across species boundaries, so horizontal transmission *does* happen. First, there was a general lack of host-virus phylogenetic concordance, consistent with past cross-species transmission (**Figure 2.3**). Second, galbut virus has been detected in a number of *Drosophila* species in the melanogaster group, including *D. simulans* and *D. suzukii* [69,77,158,172]. Moth-infecting partitiviruses could be transmitted across species boundaries experimentally [184]. And, cross-species transmission of fungus-infecting partitiviruses has been documented [217,221–223]. Several possible mechanisms could allow cross-species transmission: The lack of horizontal transmission that we observed for galbut virus may not be representative of related viruses, and we only tested a single mode of horizontal infection. Other modes of horizontal transmission including sexual transmission, possibly during interspecific mating attempts, could contribute. Mites or other parasites could serve as vectors for partitiviruses, as has been proposed for sigma viruses [224].

Some instances of cross species partitivirus transmission may involve jumps to distantly related organisms in other eukaryotic kingdoms. There is a well-supported clade of fungi-associated partitiviruses nested within the arthropod-associated partitiviruses (**Figure 2.3)**. Similar phylogenetic interspersion of plant and fungus infecting partitiviruses has been noted [76,225]. Additional phylogenetic and experimental studies will be needed to address the intriguing possibility that partitiviruses are capable of long-range host switches.

Chaq virus was originally described as a virus-like sequence whose presence was correlated with galbut virus. It was postulated that chaq virus might be a satellite virus of galbut virus (chaq also means maybe, in Klingon) [69,77]. Consistent with previous reports, we found that chaq virus usually but not

always co-occurred with galbut virus segments (**Figure 2.1C**). We also identified chaq virus-like segments associated with vera and verdadero viruses, which are only distantly related to galbut virus (they share ~25-30% pairwise amino acid identity in their RdRp sequences). This indicates that if chaq-like segments are satellites of partitiviruses, this association has existed over long evolutionary time frames. An alternative interpretation is that chaq-like segments represent "optional" partitivirus segments not strictly required for replication, as has been described for multipartite mosquito-infecting Jingmenviruses [226]. A reverse genetics system for insect infecting partitiviruses would allow characterization of the function of individual viral proteins.

The highly efficient biparental vertical transmission that we documented for galbut and verdadero viruses is unusual for insect infecting viruses. Although many insect viruses are maintained both vertically and horizontally [227–230], there are several cases where insect viruses, like galbut virus, seem to be dependent on vertical transmission [208,231,232]. In most documented cases however, maternal transmission is more efficient than paternal [208,227,232–234]. An exception is rice stripe virus, where paternal transmission is more efficient in its leafhopper vector [235]. The best precedent is probably *Drosophila*-infecting sigma viruses, which can be transmitted biparentally, allowing for sweeps through fly populations [202,208]. Parental transmission of *D. melanogaster* sigma virus is less efficient than maternal (~100% vs 65%), and paternally infected flies transmit infection less efficiently to the subsequent generation [201]. We did observe different viral loads in offspring following paternal or maternal transmission, although for galbut virus, paternally infected flies had higher viral loads (**Figure 2.5**). Whether a similar multigenerational phenomenon occurs for partitiviruses remains to be determined.

A particular challenge associated with understanding the biology of novel viruses identified via metagenomics is the inability to isolate them through classical methods such as cell culture. Here, we circumvent that obstacle through an iterative breeding scheme to generate flies with galbut virus as the only detectable infection (**Figure 2.6**). We propose that this isolation method could be applied to other newly identified unculturable viruses. The isolation of galbut virus and the initial characterization described here

establishes essential groundwork for further understanding the biological impacts and possible utility of arthropod-infecting partitiviruses.

CHAPTER 3: GALBUT VIRUS MINIMALLY INFLUENCES *DROSOPHILA MELANOGASTER* FITNESS IN A STRAIN AND SEX-DEPENDENT MANNER

3.1: Introduction

Galbut virus is a partitivirus identified in *Drosophila* spp. that until recently was unknown whether it was a legitimate virus or not (galbūt meaning "maybe" in Lithuanian). Furthermore, whether it was legitimately infecting the arthropod or a symbiotic microorganism within them was unclear (partitiviruses historically were known to infect plants, fungi, and protozoa [76]). We recently showed that galbut virus is a *bona fide* arthropod-infecting virus using the model organism *D. melanogaster* ([163] and see Chapter 2). Additional partitivirus-like sequences have recently been identified in other arthropod species [56,69,75,78,161,162,172,183,184]. Given this relatively young timeline of arthropod-infecting partitiviruses, very little is known about the biological impact of these viruses on their hosts. Given that *D. melanogaster* has been an extremely useful tool for teasing apart host-microbe interactions [236–239], we continued our exploration of the galbut virus-*D. melanogaster* system asking: what is the biological impact of galbut virus on *D. melanogaster*?

An interesting characteristic we noted in our initial biological characterization of dipertan-infecting partitiviruses was highly efficient biparental vertical transmission (~100% both maternally and paternally) ([163] and see Chapter 2). Although galbut virus is transmitted at ~100% efficiency through vertical transmission, the global prevalence of galbut virus is maintained at only ~60% ([69,163] and see Chapter 2). We hypothesized several possibilities for why this phenomenon could occur. One such explanation is that galbut virus exacts a fitness cost on flies. Modeling demonstrated that efficient biparental transmission should eventually result in infection 100% of susceptible individuals in a population unless a high fitness cost is observed [204]. Previous studies of fungal, plant, and protozoan partitiviruses have shown that infection is generally not associated with any particular phenotype, therefore were originally called cryptic viruses. Yet, there is a growing body of literature showing they may not be as cryptic as previously thought [76]. Studies have shown cases where partitiviruses have clear hypovirulence [179,180,182], hypervirulence [178], or even mutualistic [71] effects on the host. Though little is known about arthropod-

infecting partitiviruses, an initial exploration of moths co-infected with partitiviruses showed a reduction of fecundity and developmental speed, but at the same time these partitiviruses provided protection against a viral pathogen [184].

Host genetics play a large role in the outcome of infection [132,240–244]. In some cases, host genetics can determine whether a given microbial infection may actually be beneficial [245,246]. We previously observed that although multiple colonies can reach a 100% fixation of galbut virus infection, host genetics may have an impact on virus infection and subsequent transmission ([163] and see Chapter 2). How genetics may have an impact on galbut virus infection phenotypes is unclear. In host-microbe interactions another key player dictating infection-associated phenotypes is sexual dimorphism [132,247–251]. Galbut virus is efficiently transmitted by both female and male flies, but other features of infection phenotypes dictated by sex remain unexplored.

We continued to characterize the biology of galbut virus by measuring its biological impacts on *D. melanogaster* across different genetic backgrounds and sexes. Using male and female flies from multiple *Drosophila* Genetic Reference Panel (DGRP) strains that only differed in their galbut virus infection status, we tested a broad range of phenotypes. We measured standard factors of fitness including lifespan, fecundity, and development speed. To observe how galbut virus may be interact with other microbes, we challenged galbut virus infected flies with viral, bacterial, and fungal pathogens. We also explored how galbut virus influences host transcriptional responses and core microbiome constituents. Overall, fitness impacts appeared to be minimal, with the DGRP strain and sex having much larger impacts on the observed phenotypes than galbut virus infection. Effects associated with galbut virus did vary as a function of sex and DGRP strain.

3.2: Materials and Methods

3.2.1: *Drosophila* rearing and maintenance

All flies were reared on the Bloomington Drosophila Stock Center (BDSC) standard cornmeal diet (https://bdsc.indiana.edu/information/recipes/bloomfood.html). Stocks were reared at 25°C and changed every 14 days. All experiments were performed with *Drosophila* Genetic Reference Panel (DGRP) stocks 399 and 517 [91]. Original stocks were acquired from BDSC. Generation of galbut virus infected stocks was performed previously ([163] and see Chapter 2).

3.2.2: Lifespan and fecundity fitness assays

Lifespan and fecundity fitness assays of galbut-infected and uninfected flies were performed as previously described [53]. Flies were reared in 5 replicate groups of 10 adults (5 female, 5 male). Flies were checked daily for survival of adults, and living adults were moved to fresh media every 3 days. Longevity of adults was measured using the R survival package [252]. After adults were moved, original vials were kept for an additional 14 day period, after which offspring were counted and sexed. This offspring count was used as a measurement of fecundity.

As an additional measurement of fecundity, eggs were collected and counted. Flies were housed together in individual bottles (10 males, 10 females per bottle) with an apple agar egg plate and yeast paste (1:1 yeast and water), to promote egg laying. Egg plates were replaced every 24 hours, and the used plates containing eggs were frozen at -20°C until the eggs were quantified. Plates were collected for a total of 3 days, and were performed in 3 biological replicates and 2 technical replicates. Images of egg plates were captured and eggs were counted individually by hand using the ImageJ cell counter program [253]. All fecundity measurements were analyzed with custom R scripts that can be found at:https://github.com/scross92/galbut_fitness_analysis.

3.2.3: Developmental speed assays

Developmental assays were performed as previously described [254]. Eggs were collected using standard apple agar plates with a mixture of 1:1 yeast and water applied. Every hour for one day, agar plates were discarded and replaced to encourage egg synchronization. Agar plates were replaced a final time and

incubated for several hours. The plates were removed and rinsed with sterile water through a nylon, mesh sieve (120 µm pore size), and sieve was rinsed into a clean petri dish for egg collection. 20 eggs were collected and moved to non-nutritive agar plates containing 5% sucrose/2% agar with no antimicrobials added (e.g. tegosept). Agar plate was placed inside a larger petri dish with a damp paper towel on the bottom and moved to a 25°C incubator with a 12 hour light/dark cycle. Every 2 days, yeast paste was added as a nutrition source for developing flies. Yeast were killed prior to use in the paste by microwaving for 45 seconds on high to prevent overgrowth of yeast on the agar plates. Vials were checked daily for pupae to determine speed of pupation. Once pupation began, vials were checked approximately every 5 hours (morning, midday, evening). Continual monitoring occurred from pupation to emergence of adults in the same ~5 hour increments hours for measuring total time it took for flies to reach the adult stage. This was performed in 6 replicates per group (strain and galbut virus infection status).

3.2.4: *Drosophila* experimental groups and analysis code.

For downstream methods (pathogen challenges, transcriptomic analysis, galbut virus RNA levels and microbiome analysis), a core set of experimental groups were used. Experimental groups consisted of DGRP 399 females, DGRP 399 males, DGRP 517 females, and DGRP 517 males. Experimental comparisons each consisted of 2 groups: one galbut virus infected, one galbut virus uninfected. This results in a total of 8 experimental groups. All flies were 3-5 day old virgins and reared in a 12 hour light/dark cycle at 25°C, unless otherwise stated. All code and data were deposited in the GitHub repository: https://github.com/scross92/galbut_fitness_analysis.

3.2.5: *Pseudomonas aeruginosa* oral challenge.

The first pathogen challenge performed was an ingestion assay as adapted from Lutter et al [255]. A glycerol stock of *Pseudomonas aeruginosa* (Strain PA01) was provided by the Borlee lab at Colorado State University. A culture from this initial stock was obtained by inoculating 200mL of brain, heart infusion (BHI) broth and stirred at 220 rpm overnight at 37°C. The following day, the culture was spun

down in a centrifuge at 4200 g for 5 minutes until a loose pellet was formed. Excess supernatant was decanted and culture was resuspended to an OD_{600nm} of ~7 using a sterile 5% sucrose solution. Autoclaved filter disks were inoculated with 290µL of the *P. aeruginosa* culture. Disks were placed on 5% sucrose agar vials. Control disks were inoculated with the 5% sucrose solution in place of bacterial culture. Twelve flies that were previously starved for 5 hours were put into the bacteria-containing vials for each group replicate. Flies were checked at the end of this first day for death, which was likely due to starvation stress rather than the bacterial pathogen and were censored from statistical analyses. Flies were observed daily for dead flies for a total of 12 days. Survival analysis of adults was measured using the R survival package [252]. A total of 3 technical replicates were performed.

3.2.6: Intrathoracic microbial pathogen challenges

All the following pathogen challenges were performed through intrathoracic microinjection. All experimental injections were performed in 3 biological replicates (12 flies per replicate) per technical replicate, and a total of two technical replicates were performed. An exception is the *Staphylococcus aureus* pathogen challenge which was performed in 3 technical replicates. Control injections (flies injected with 1X Phosphate Buffer Solution (PBS)) were performed alongside these experimental injections. Flies were checked at 10-12 hours post-injection, and any flies that were dead at this point were assumed to have died from microinjection stress rather than by the specified pathogen. Survival analysis of adults was measured using the R survival package [252]. Injected volumes, inoculum dose, and subsequent intervals for checking fly survival are stated below for the respective pathogen.

<u>*Pseudomonas aeruginosa*</u>: Flies were microinjected with *P. aeruginosa* (strain PA01). A culture was obtained by inoculating 150mL of brain, heart infusion (BHI) broth and stirred at 220 rpm overnight at 37°C. The following day, the culture was spun down in a centrifuge at 4200 g for 5 minutes until a loose pellet was formed. Excess supernatant was decanted and the culture was resuspended to an OD_{600nm} of 0.03 using 1X PBS. Flies were injected with 9.2nL of this diluted *P. aeruginosa* culture, which corresponds to ~100 CFUs [256]. Flies were incubated overnight and checked at 24 hours post injection, 28 hours post

injection, and every 2 hours after 28 hours post injection. Flies were checked until 52 hours post injection, at which point any living flies were censored from downstream statistical analyses.

Staphylococcus aureus: Flies were microinjected with *S. aureus* (strain XEN36), which was provided by the Borlee lab at Colorado State University. A culture was obtained by inoculating 150mL of brain, heart infusion (BHI) broth and stirred at 220 rpm overnight at 37°C. The following day, the culture was spun down in a centrifuge at 4200 g for 5 minutes until a loose pellet was formed. Excess supernatant was decanted and the culture was resuspended to an OD_{600nm} of 0.1 using 1X PBS. Flies were injected with 23nL of this diluted *S. aureus* culture, which corresponds to ~100 CFUs [250]. Flies were checked daily until 8 days post injection, at which point any living flies were censored from downstream statistical analyses.

Drosophila C virus: *Drosophila* C virus (DCV) stocks were provided by the Andino lab at the University of California San Francisco. DCV stocks were amplified and titrated on *Drosophila* S2 cells. DCV infections of flies were performed as previously described [203]. Flies were microinjected with DCV at a titre of 100 50% Tissue Culture Infective Dose units ($TCID_{50}$) in a total volume of 50nL. Flies were checked daily until 14 days post injection, at which point any living flies were censored from downstream statistical analyses.

Candida albicans: *Candida albicans* pathogen challenge was performed as previously described [257]. Original *C. albicans* (strain SC5314) stock was obtained from ATCC. A yeast extract peptone dextrose (YPD) agar plate was streaked with the glycerol stock and incubated at 30°C for 18 hours. 150mL of YPD broth was inoculated with a single yeast colony from the YPD plate and stirred at 220 rpm overnight at 30°C until the culture was at an OD_{600nm} of ~1. The culture was spun down in a centrifuge at 4200 g for 5 minutes until a loose pellet was formed. Excess supernatant was decanted and the pellet was resuspended using 1X PBS. Yeast cells were counted and culture was diluted to 10^7 cells/mL. Flies were microinjected with 50nL of this dilution, resulting in ~500 yeast cells injected into flies. Flies were incubated at 30°C and were checked daily until 6 days post injection, at which point any living flies were censored from downstream statistical analyses.

3.2.7: RNAseq library preparations.

Pools of 10, 5-day old, virgin flies were collected, flash frozen in liquid nitrogen and stored at -80°C. RNA from pooled flies was extracted using the NEB Magnetic mRNA Isolation Kit according to the manufacturer's protocol. Sequencing libraries were created using Kapa HyperPrep RNA Library Prep Kit (Roche) according to the manufacturer's protocol. Final library molecules had an average size of 348 base pairs and were sequenced on an Illumina NextSeq using the NextSeq 500/550 High Output Kit v2.5, generating single-end reads (1x75).

3.2.8: Transcriptomic computational analysis.

RNAseq datasets were first processed to remove low quality filtering and adapter sequences through using cutadapt tool [258] version 1.13 with the following settings: -a AGATCGGAAGAGC -A AGATCGGAAGAGC -g GCTCTTCCGATCT -G GCTCTTCCGATCT -a AGATGTGTATAAGAGACAG -A AGATGTGTATAAGAGACAG -g CTGTCTCTTATACACATCT -G CTGTCTCTTATACACATCT, -q 30,30, --minimum-length 40, and -u 1. Remaining reads were mapped to the *Drosophila melanogaster* genome assembly BDGP6.28 (downloaded from Ensembl) using HISAT2 version 2.2.0 [259]. Reads were counted using featureCounts version 2.0.0 [260] to the BDGP6.28 gtf file with the following settings: -s 2 -t exon -g gene_id. The resulting read count table was used as input for differential gene expression analysis using DESeq2 version 1.26.0 [261] in R version 3.6.3 [262]. Differential gene expression analyses on the condition of galbut virus infection status and was performed for each experimental group. Gene set enrichment and gene ontology (GO) term analyses were performed using the clusterProfiler R package version 3.14.3 [263] using a pre-ranked gene list ordered by the log2 fold changes and the gse function.

3.2.9: Validation of galbut virus infection status and microbiome constituents via qPCR.

To measure galbut virus RNA levels, RNA was extracted from 5 day old, virgin flies using a bead-based protocol as previously described [163] (and see Chapter 2). cDNA was synthesized by adding 5.5 µl of RNA to 200 pmol of a random 15-mer oligonucleotide and incubated for 5 min at 65°C, then set on ice for 1 min. A RT mixture containing the following was added (10 µL reaction volume): 1× SuperScript III (SSIII) FS reaction buffer (Invitrogen), 5 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleotide triphosphates (dNTPs) (NEB), and 100 U SSIII reverse transcriptase enzyme (Invitrogen), then incubated at 42°C for 30 min, 50°C for 30 min, then at 70°C for 15 min. 90 µL of nuclease-free H_2O was added to dilute the cDNA to a final volume of 100 µL.

For microbiome screening, total genomic DNA was extracted from 4-5 day old virgin flies. 10 flies per pool (total of 3 pools per group) were surface sterilized by vortexing in 70% ethanol for 2 minutes, followed by 2 rinses with autoclaved ddH_2O and vortexing for 1 minute each. Flies were then stored at -80°C until DNA was extracted. DNA was extracted using the DNeasy Tissue and Blood extraction kit (Qiagen) following the manufacturer's protocol for insect tissues with three modifications. First, samples were added to 180 µL ATL buffer (provided in kit) along with a single BB bead and homogenized using a Qiagen TissueLyzer for 3 minutes at 30Hz rather than homogenizing by hand. Second, samples were incubated in proteinase K for a duration of 4 hours. Last, following incubation with proteinase K, samples were treated with 20 µL of RNase A (2mg/mL; Sigma Aldrich) for 30 min at 37°C. After RNase treatment, samples were processed as stated in the protocol.

Following cDNA synthesis or DNA extraction, qPCR reactions were set-up using Luna qPCR Master Mix (NEB) following the manufacturer's protocol. The qPCR reaction was performed on LightCycler 480 (Roche) under the following protocol: 95°C for 3 min, 40 cycles of 95°C for 10s, then 60°C for 45s, and then followed by a melting curve analysis of 95°C for 5s, 65°C for 1 min, and an acquisition starting from 97°C with a ramp rate of 0.11 °C/s and 5 acquisitions per degree. Microbiome analysis primer sequences were predominately acquired from Early et al. [264]. Primer sequences can be found in **Supplemental Table 3.1**.

3.3: Results

3.3.1: Confirmation of galbut virus infection status and galbut virus RNA levels in individual flies.

We first verified that our stocks of galbut virus-infected flies established previously [163] (and see Chapter 2) were still persistently infected. We screened 20, 3-5 day old flies from each DGRP line (10 male, 10 female; total of 40 flies per line) for galbut virus. Some DGRP 517 individuals had relatively high galbut virus RNA levels (relative to the housekeeping gene RpL32). However, DGRP 399 flies, overall, had median galbut virus RNA levels that are 2.3X higher than DGRP 517 flies (Wilcoxon, p=0.0016; **Figure 3.1**).

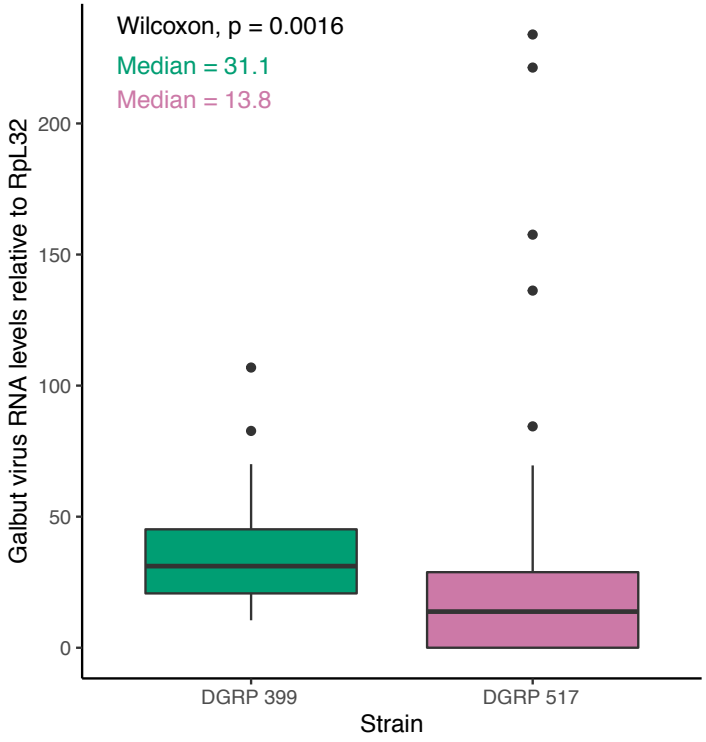

Figure 3.1. **Relative galbut virus RNA levels in DGRP strains.** Boxplots depicting galbut virus RNA levels relative to the housekeeping gene RpL32 ($2^{-\Delta Ct}$ method) in adult flies from infected DGRP strains used in this study (n = 20 per strain). DGRP 399 infected flies had significantly higher median galbut virus RNA levels than DGRP 517 infected flies (Wilcoxon, p = 0.0016). DGRP: *Drosophila* Genetics Reference Panel

3.3.2: Galbut virus has varied impacts on *Drosophila* lifespan, fecundity, and development.

Given that fitness is a function of multiple factors, including lifespan, fecundity, and development (time to adulthood) [53,265], we first measured whether there was a difference in these measurements between galbut virus infected and uninfected flies. Vials (n=5 per experimental group) of newly eclosed adults were housed together in groups of 10 flies (5 males, 5 females). Flies were checked daily for survival. Galbut virus infected flies in the DGRP 399 genetic background had a slight reduction in lifespan with infected flies having a mean lifespan 8.1 days shorter than uninfected flies (R survival package, p=0.02, **Figure 3.2**), while infection status had no impact on the average lifespan of DGRP 517 flies (p=0.27, **Figure 3.2**). The small change in lifespan attributable to galbut virus infection status was smaller than that attributable to DGRP strain, where DGRP 517 flies lived on average 14.9 fewer days than DGRP 399 flies ($p=2.1x10^{-4}$, **Supplemental Figure 3.1**).

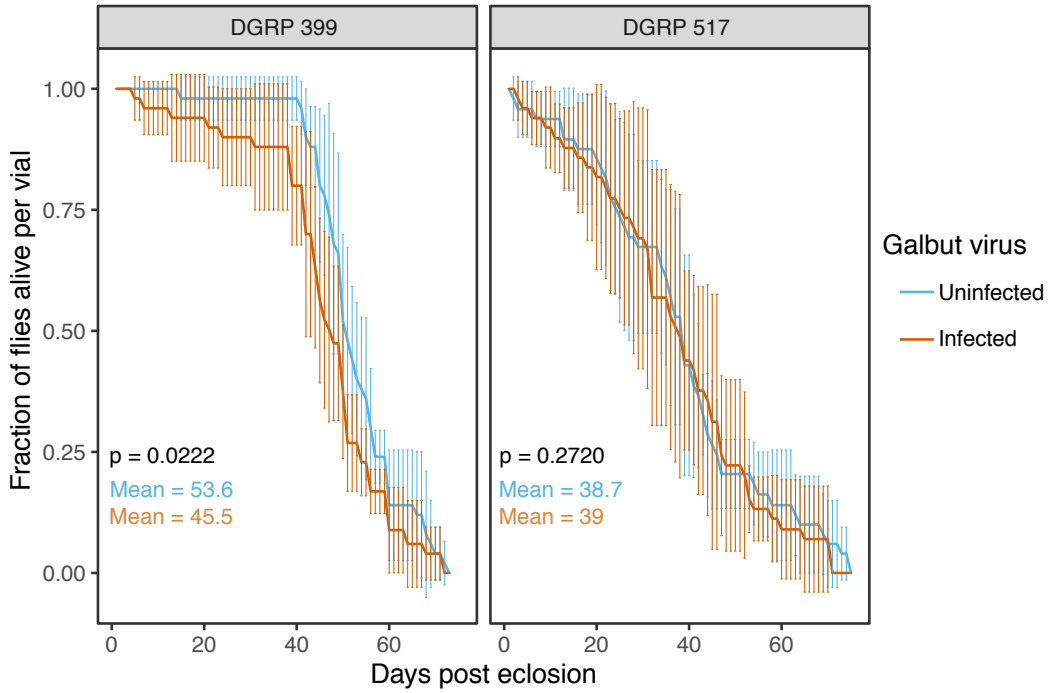

Figure 3.2. Lifespan of galbut virus infected and uninfected flies. Galbut virus infected and uninfected groups were housed in batches of 10 flies and monitored daily for survival. Galbut virus infected DGRP 399 flies exhibited significantly decreased survival (p=0.0222) while there was no significant difference in DGRP 517 flies.

We compared fecundity of infected flies and uninfected flies by counting total adult offspring. Galbut virus infected DGRP 399 flies produced fewer offspring than their uninfected counterparts, but this was not significantly different (t-test; female offspring p=0.77, male offspring p=0.83; **Figure 3.3A**). Galbut virus infected DGRP 517 flies also produced fewer offspring, but the decrease was only significant for male offspring numbers (t-test; female offspring p=0.1588, male offspring p=0.0270; **Figure 3.3A**). Galbut virus infection did not significantly change offspring sex ratios (t-test; DGRP 399: p=0.6346, DGRP 517: 0.7462, **Supplemental Figure 3.2**). Similar to lifespan, differences in offspring number between the different DGRP strains dwarfed differences attributable to galbut virus infection status ($p=2.812x10^{-6}$, **Supplemental Figure 3.3**).

As an additional measure of fecundity, we recorded the cumulative number of eggs laid when one or both parents were infected with galbut virus by counting the total number of eggs laid over 3 days. There were no significant differences in the number of eggs laid when either or both parents were infected with galbut virus (ANOVA, DGRP 399: p=0.85, DGRP 517: p=0.72; **Figure 3.3B**).

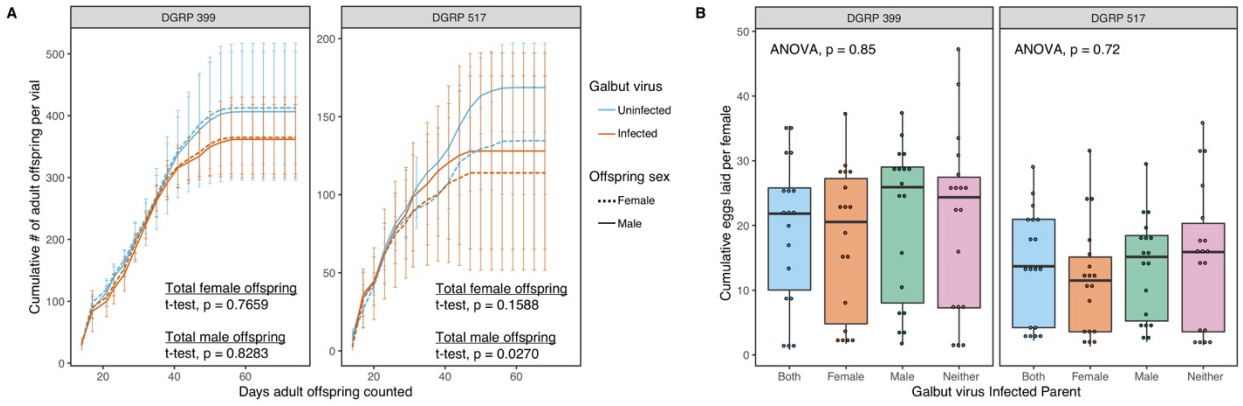

Figure 3.3. Impacts of galbut virus infection on *Drosophila* fecundity. (A) Galbut virus infected and uninfected groups were housed in batches of 10 flies (5 males and 5 females per vial) and cumulative number of female and male offspring were counted. In all groups, galbut virus infected flies produced fewer offspring, but this difference was only statistically significant for DGRP 517 flies, which produced significantly fewer male offspring (p=0.0270). (B) 10 male and 10 female flies were crossed with different combinations of galbut virus infection status, and eggs were collected daily for a total of 3 days. Cumulative eggs laid per female were counted.

The final general fitness parameter measured was developmental speed. Eggs from galbut virus infected and uninfected flies from both DGRP stains were collected, and the times from oviposition to pupation and oviposition to adulthood were measured. There were no statistically significant differences in the time it took galbut virus infected and uninfected DRGP 399 flies to pupate (~5 days to pupation, **Figure 3.4A**). No differences in the total time to reach adulthood was noted between infected and uninfected DGRP 399 females (~9 days to adulthood; **Figure 3.4B**), but galbut virus infected DGRP 399 males reached adulthood significantly slower (9 hour average difference; Wilcoxon, p=0.0097, **Figure 3.4B**). In DGRP 517 flies, those infected with galbut virus pupated on average 6 hours faster than uninfected flies (Wilcoxon, $p=6.6x10^{-10}$; **Figure 3.4A**). DGRP 517 infected females significantly reached adulthood faster than uninfected females by an average 5 hour difference (Wilcoxon, p=0.0053; **Figure 3.4B**). DGRP 517 galbut virus infected males also reached adulthood significantly faster than their uninfected counterparts by an average 10 hour difference (Wilcoxon, $p=2.1x10^{-7}$; **Figure 3.4B**). When removing galbut virus as a variable, DGRP 399 flies pupated significantly faster (average 8 hour difference; Wilcoxon, $p=1.13x10^{-13}$, **Supplemental Figure 3.4**). Additionally, DGRP 399 males, but not females, reached adulthood significantly faster than DGRP 517 males (average 10 hour difference; Wilcoxon, $p=2.8x10^{-8}$, **Supplemental Figure 3.4**).

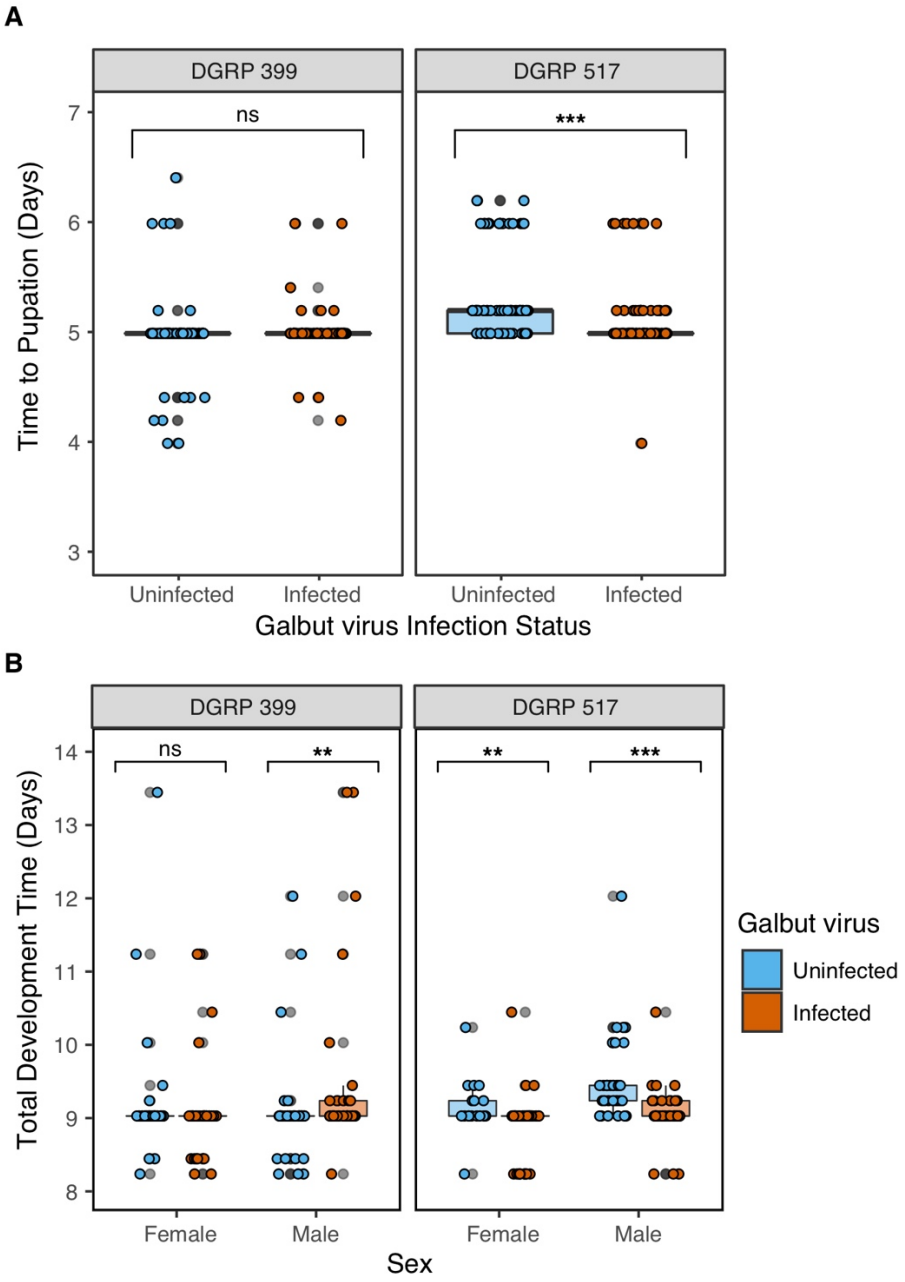

Figure 3.4. Galbut virus infected flies have altered developmental speed dependent on strain and sex. (A) Galbut virus infected and uninfected eggs were collected, reared, and checked daily for until pupation began. Once pupation began, flies were checked approximately every 5 hours (morning, midday, evening). DGRP 399 flies had no significant differences by galbut virus infection status, whereas DGRP 517 infected larvae reached pupation significantly faster (Wilcoxon, $p=6.6x10^{-10}$). (B) Galbut virus infected and uninfected flies were continually monitored until flies eclosed, and this time was recorded as the total developmental time. Once flies began to eclose, flies were checked approximately every 5 hours (morning, midday, evening). Sex of adult flies was recorded and total development time between infected and uninfected flies was compared by sex. DGRP 399 infected male flies reached adulthood significantly slower (Wilcoxon, p=0.0097), while there were no differences in female flies. In DGRP 517 infected flies, both sexes reached adulthood faster than their uninfected counterparts (females, Wilcoxon, p=0.0053; males, Wilcoxon, $p=2.1x10^{-7}$). ns: not significant; **: $p < 0.01$, ***: $p < 0.001$

3.3.3: Galbut virus alters the susceptibility of flies to viral, bacterial, and fungal pathogens.

Inter-microbial interactions are becoming increasingly appreciated in the outcomes of host-microbe interactions [114,266–269]. We hypothesized that galbut virus infection might alter the ability of flies to resist or tolerate infection by pathogenic microbes. To test this hypothesis, galbut virus infected and uninfected flies were challenged with a variety of pathogens. The first pathogen was a viral pathogen, *Drosophila* C virus (DCV) [205]. Flies were challenged with 100 $TCID_{50}$ units through intrathoracic microinjection and checked daily for survival. In most cases, there was no significant difference between galbut virus infected and uninfected flies in susceptibility to DCV. However, one group, DGRP 517 female, galbut virus-infected flies, was significantly less susceptible to DCV, though this effect was minor (**Figure 3.5**, p=0.028). It should be noted that these DGRP strains are *Wolbachia* negative, thus changes in susceptibility in either direction (increased or decreased) may be more attributable to galbut virus rather than the known protective effects of *Wolbachia* against DCV [211,270–273].

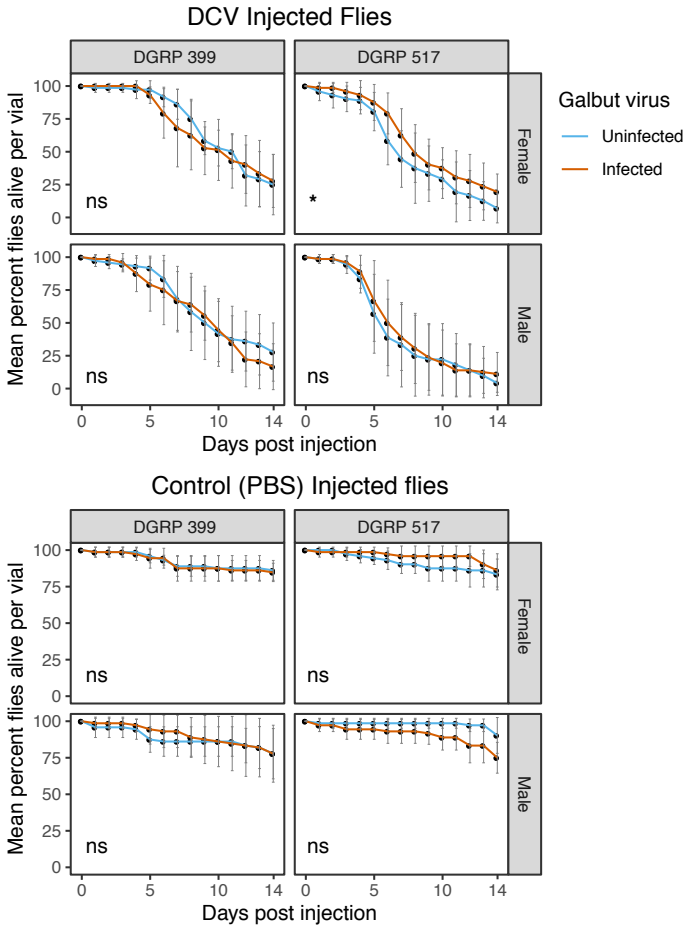

Figure 3.5. Galbut virus changes susceptibility of some flies to a viral pathogen. Galbut virus infected and uninfected flies were challenged with 100 $TCID_{50}$ units of *Drosophila* C virus (DCV) through intrathoracic microinjection. Flies were monitored daily for survival for 14 days. Top panels show survival curves of flies challenged with DCV and bottom panels show control flies (injected with PBS). DGRP 517 female, galbut virus infected flies have a significantly increased survival (p=0.0279) while other groups remained insignificant. ns: not significant; *: $p < 0.05$.

The next challenge performed was with bacterial pathogens. Flies were first challenged orally with *Pseudomonas aeruginosa* and checked daily for survival. Galbut virus infected DGRP 399 female flies were significantly more susceptible to *P. aeruginosa* bacterial challenge (**Figure 3.6**; $p=4.5x10^{-6}$). All other groups of flies were not significantly different between infection status. When removing galbut virus infection and sex as variables, DGRP 399 flies were significantly more susceptible to *P. aeruginosa* than DGRP 517 flies ($p=6.639x10^{-9}$).

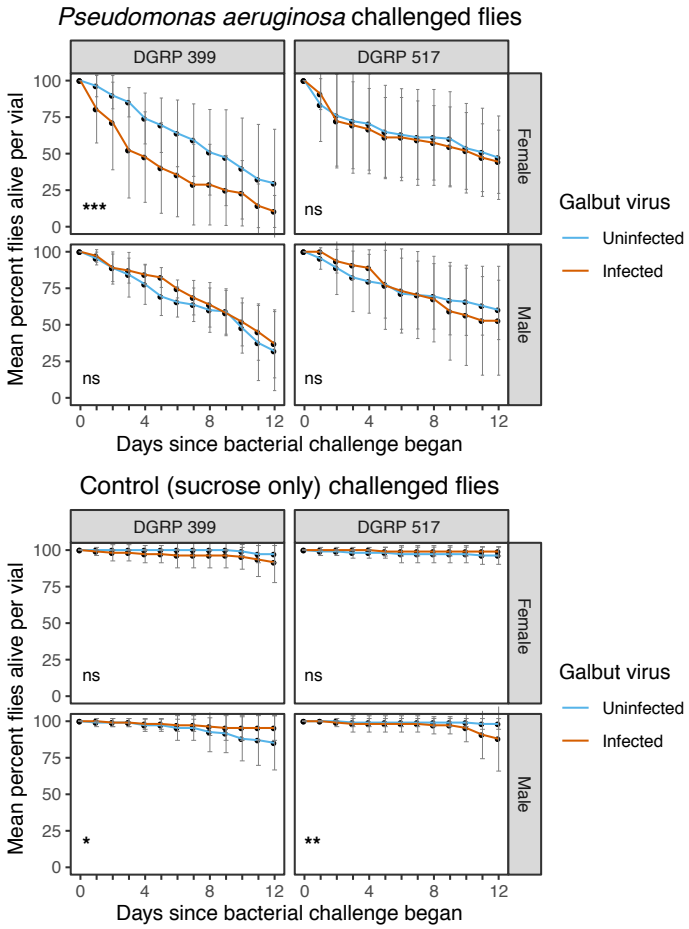

Figure 3.6. Galbut virus alters susceptibility of DGRP 399 female flies when challenged orally with *Pseudomonas aeruginosa*. Galbut virus infected and uninfected flies were challenged with *Pseudomonas aeruginosa* through ingestion. Flies were then monitored daily for survival. Top panels show survival curves of flies challenged with *P. aeruginosa* and bottom panels show control flies (fed only with sucrose). DGRP 399 female, galbut virus infected flies have a significantly reduced survival ($p=4.5x10^{-6}$) while other groups remained insignificant. ns: not significant; *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

Although it may be considered more of a natural route of infection, oral inoculation has little control over total CFUs ingested by the flies and leads to only fair reproducibility [256]. To circumvent this, we shifted to a controlled bacterial pathogen challenge through intrathoracic microinjection. When challenging flies with ~100 CFUs of *P. aeurginosa*, galbut virus infected DGRP 399 female no longer were significantly more susceptible (**Figure 3.7A**; p=0.1362). All other groups maintained no statistically significant differences. This suggests that interactions occurring between galbut virus and *P. aeruginosa* might be occurring at the gut epithelial interface, and not systemically.

Since the *Drosophila* innate immune system responds differently to Gram negative and Gram positive bacteria [274], we continued our bacteria pathogen challenges by microinjecting flies with *Staphylococcus aureus*. When flies were microinjected with ~100 CFUs, certain groups of infected flies were significantly less susceptible (**Figure 3.7B**), suggesting that galbut virus may be conferring protection. though these differences were not profound in any case. Galbut virus infected DGRP 399 male flies were less susceptible (p=0.026) as well as DGRP 517 female, galbut virus infected flies (p=0.0026).

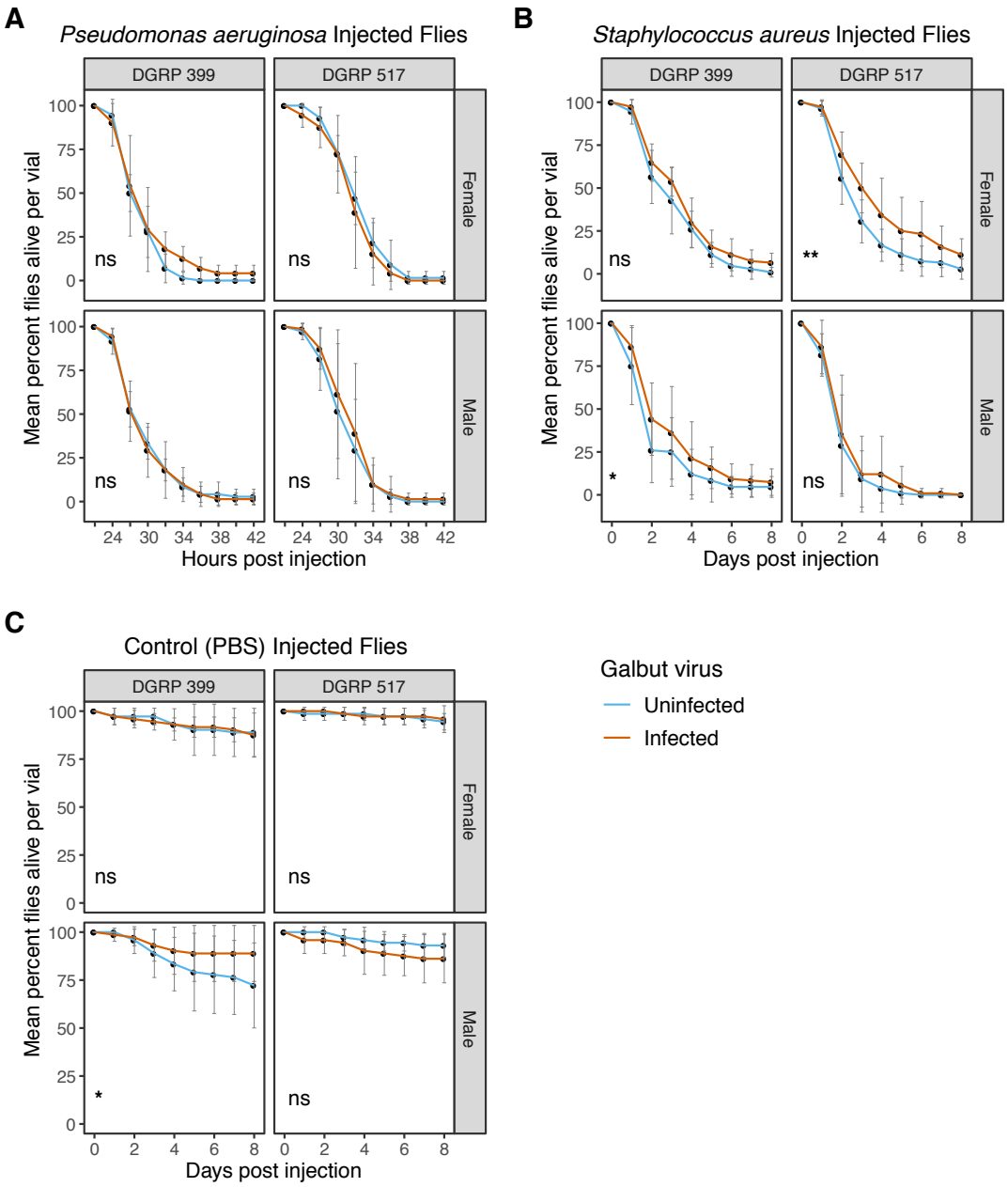

Figure 3.7. Galbut virus alters susceptibility of some flies when challenged via intrathoracic microinjection with *Staphylococcus aureus*, but not *Pseudomonas aeruginosa*. (A) Galbut virus infected and uninfected flies were challenged with ~100 CFUs of *Pseudomonas aeruginosa* through intrathoracic microinjection. Flies were checked at 12 hour and 24 hour time points, then monitored every 2-4 hours for survival (see *Materials and Methods*) and survival curves are depicted. No group was statistically significant based on galbut virus infection status. (B) Galbut virus infected and uninfected flies were challenged with ~100 CFUs of *Staphylococcus aureus* through intrathoracic microinjection. Flies were then monitored daily for survival and survival curves are depicted. DGRP 399 male, galbut virus infected flies and DGRP 517 female, galbut virus infected flies have a significantly increased survival (DGRP 399 male p=0.02577; DGRP 517 female p=0.00262) while other groups remained insignificant. (C) Galbut virus infected and uninfected flies were challenged with PBS through intrathoracic microinjection as a control. Flies were then monitored daily for survival and survival curves are depicted. ns: not significant; *: $p < 0.05$; **: $p < 0.01$.

The final pathogen challenge was a fungal pathogen, *Candida albicans*. Flies were injected with ~500 cells and monitored daily for survival for a period of 6 days. Regardless of sex, DGRP 399 galbut virus infected flies were significantly more susceptible to *C. albicans* challenge (**Figure 3.8**; DGRP female $p=6.5108x10^{-6}$ and DGRP male $p=3.4735x10^{-5}$). No significant differences were observed in DGRP 517 flies based upon galbut virus infection status in either sex (**Figure 3.8**).

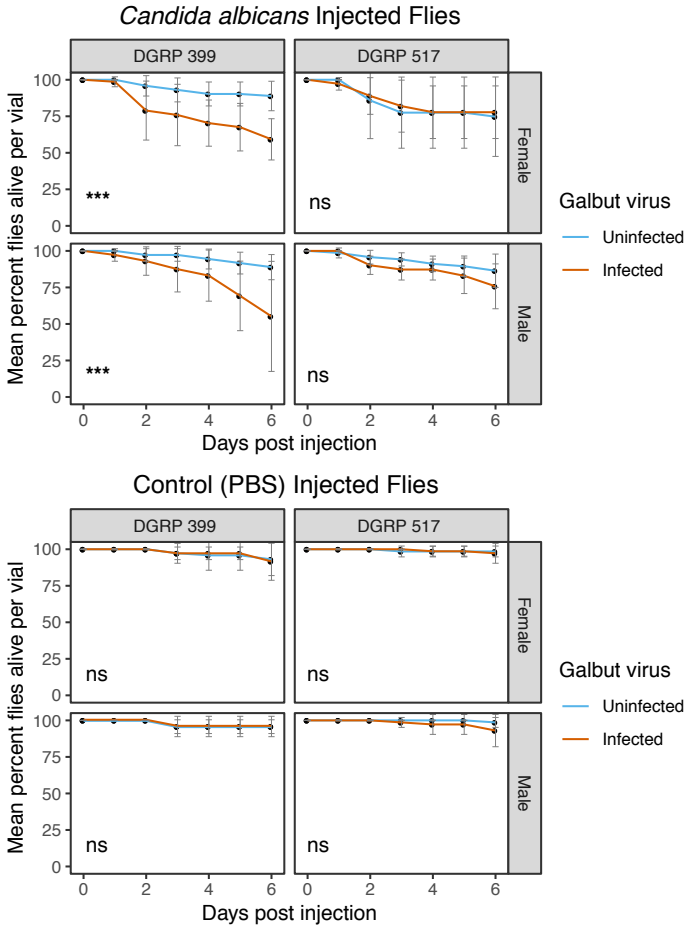

Figure 3.8. Galbut virus sensitizes DGRP 399 flies to a fungal pathogen (*Candida albicans*). Galbut virus infected and uninfected flies were injected with ~500 *Candida albicans* cells. Flies were then monitored daily for survival. Top panels show survival curves of flies challenged with *C. albicans* and bottom panels show control flies (injected with PBS). Both female and male, galbut virus infected DGRP 399 flies have a significantly reduced survival ($p=6.5108x10^{-6}$ and $p=3.4735x10^{-5}$) while other there was no significant difference in survival of DGRP 517 flies. ns: not significant; ***: $p < 0.001$.

3.3.4: Galbut virus induces strain and sex specific changes in the transcriptomes of flies.

To explore possible transcriptional changes responsible for the observed phenotypes, we performed an RNAseq experiment to understand how galbut virus infection may impact the transcriptome of *D. melanogaster*. When transcriptional responses were clustered hierarchically, they were first separated by sex, followed by strain and galbut virus infection (**Figure 3.9**). Few genes were significant in their differential expression in all flies when compared by galbut virus infection status alone. To tease apart any meaningful interactions between flies and galbut virus, transcriptional responses were examined within each DGRP strain and sex (as previously divided for the pathogen challenges). Within each of these subsets, the response to galbut virus infection was varied by both the number of differentially expressed genes and those that passed a significant threshold (**Figure 3.10**). Given a lack of consistent fitness phenotypes across any one sex or strain in most cases, this may not be unsurprising.

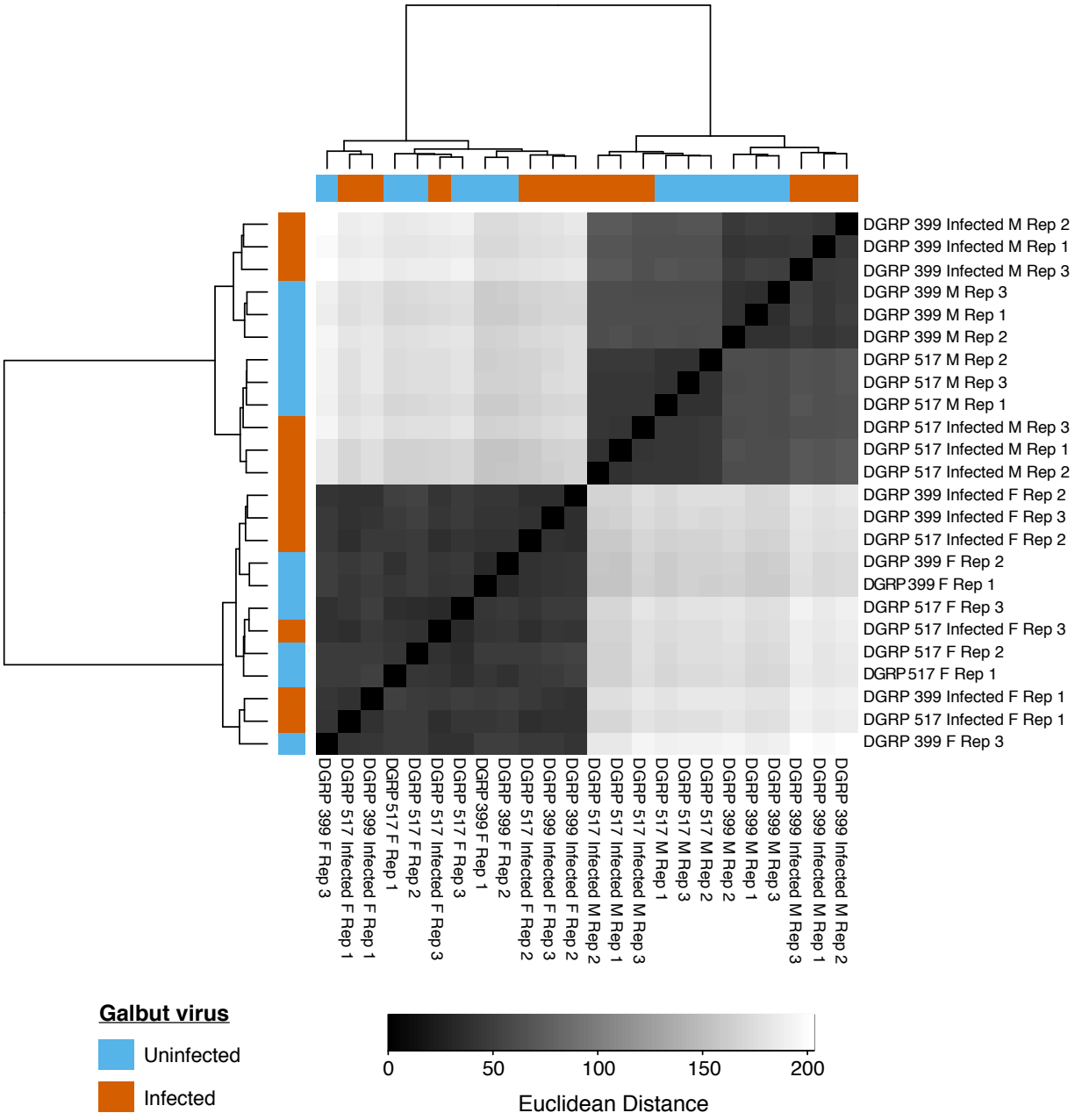

Figure 3.9. Galbut virus appears to have minimal influence on overall distinct transcriptional responses in flies. A sample distance matrix (Euclidean distances) evaluating the transcriptome similarities between all galbut virus infected and uninfected flies. Similarity between flies were first separated by sex, followed by strain and galbut virus.

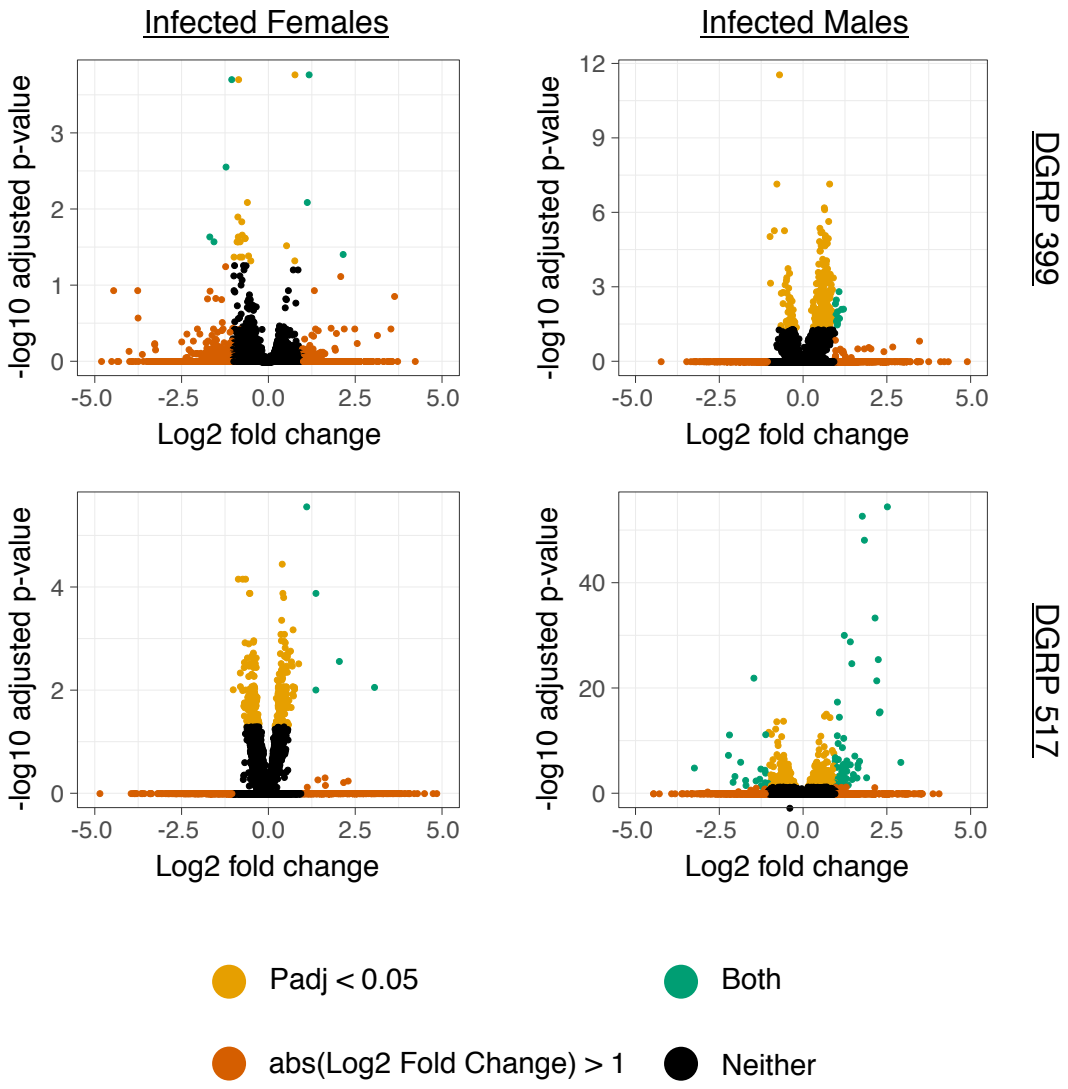

Figure 3.10. Volcano plots of differential gene regulations in galbut virus infected flies. Plots depict the relative fold change of individual genes in galbut virus infected flies relative to uninfected flies (positive fold-change values indicate higher expression levels in galbut virus infected flies) and associated multiple testing corrected p-values. Individual genes that have a log2 fold change greater than 1 (orange), an adjusted p-value < 0.05 (gold), or both (green) are colored.

Of the top 15 up and downregulated genes (padj < 0.05), a large portion of genes in any subset were of unknown function (**Figure 3.11**). In DGRP 399 infected females, there were not 15 upregulated genes that passed the significance threshold, consistent with minimal phenotypic impacts of galbut virus infection. Little conservation of up or downregulation of genes existed across any two groups. However, genes that

were found multiple times included Kruppel homolog 1 (Kr-h1), which was significantly downregulated in both female groups (**Figure 3.11**). This gene is a transcriptional regulator that has links to development [275–277]. The downregulation of this gene did not have influence on development time in DGRP 399 flies as there were no statistically significant differences by infection status (**Figure 3.4B**). However, whether the downregulation of this gene leads to decreased developmental time in infected DGRP 517 females is of interest (**Figure 3.4B**). An additional gene that was conserved was Formin homology 2 domain containing (Fhos) which has both developmental implications (remodeling of muscle cytoskeleton) and immune response (directs macrophage movement) [278–280]. This gene was downregulated in both DGRP 399 infected females and males. We did see a significant increase of total development time in infected DGRP 399 male, but not female, flies (**Figure 3.4B**). The downregulation of this gene may be a contributing factor for DGRP 399 infected flies being more susceptible to *C. albicans* (**Figure 3.8**). For DGRP 399 infected females, this also may have implications in the increased susceptibility when orally challenged with *P. aeruginosa* (**Figure 3.6**).

Two genes with minimal information were also found in both sexes within each strain. For DGRP 399 infected flies, Glycogen binding subunit 76A (Gbs-76A) was downregulated. This gene is inferred to play a role in the glycogen biosynthesis pathway [281]. For DGRP 517 infected flies, there is a downregulation of CG14186 which is affiliated with the biological process of cilium assembly, but its exact molecular function is unknown. The cilium is an organelle that assists with cell motility, movement of particles, and response to stimuli. Cilia have been associated with growth, development, and behavior in flies [282,283]. The downregulation of this ortholog of this gene is associated with several diseases in humans, including a brain developmental disorder called Joubert syndrome [284].

Two genes that were conserved as top differentially regulated genes across groups, but in opposite directions, were CG17560 and Heat shock protein 70Bb (Hsp70Bb). CG17560 is predicted to have implications in metabolic processes [281]. In DGRP 399 infected females, this gene was upregulated, while in DGRP 517 infected females, it was downregulated (**Figure 3.11**). Hsp70Bb was downregulated in DGRP 399 infected females but was upregulated in DGRP 517 infected males (**Figure 3.11**). Heat shock proteins

were a large fraction of the upregulated genes in DGRP 517 infected males. Heat shock proteins, as implied by their name, are upregulated under heat and chemical stress, but these proteins have additional antiviral functions [285].

Of particular interest was the significant downregulation of several antimicrobial peptide genes in infected DGRP 517 males (attacin-C, AttC; diptericin A, DptA; metchnikowin, Mtk; **Figure 3.11**). Although these were downregulated, we observed no increased susceptibility of infected DGRP 517 males to any pathogen. These flies do however have an increased expression of prophenoloxidase genes (PPO; **Figure 3.11**) which are involved in the melanization process [286], and this increased expression may offset the downregulation of other antimicrobial peptides.

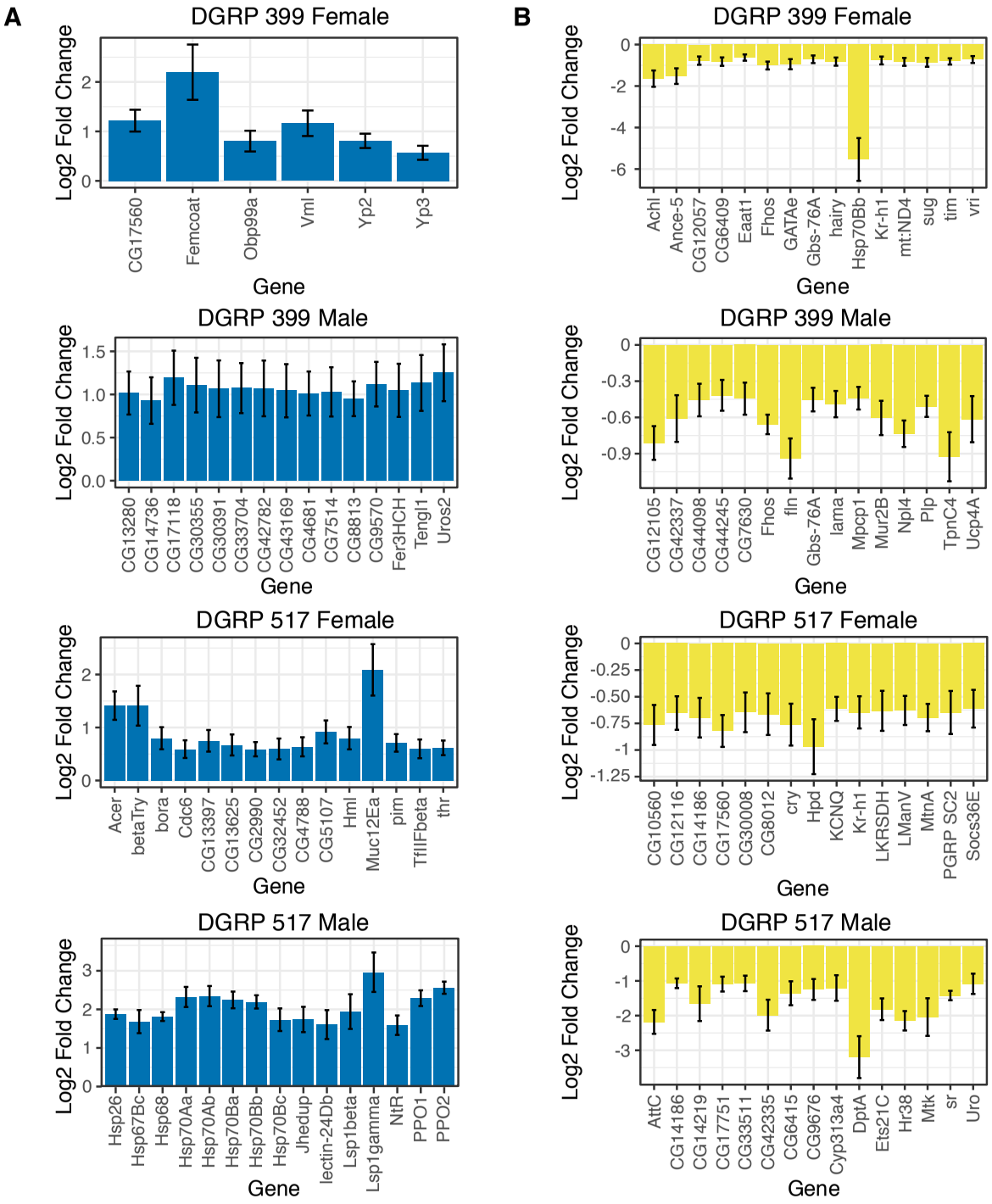

Figure 3.11. Top differentially expressed genes in infected flies relative to uninfected flies. (A) Top 15 most significantly upregulated genes (ranked by padj, with padj < 0.05) in each experimental group relative to uninfected flies (B) Top 15 most significantly downregulated genes (ranked by padj, with padj < 0.05) in each experimental group relative to uninfected flies.

We performed a gene set enrichment analysis (GSEA) with the genes pre-ranked by log2 fold change using the clusterProfiler package in R [263]. When looking at the top regulated gene ontology (GO) pathways, GO pathways associated with development, morphogenesis, and metabolic pathways were upregulated in infected DGRP 517 flies (**Supplemental Figures 3.7 and 3.8**). This is concordant with our developmental results (**Figure 3.4B**). Another interesting group of GO pathways that were regulated were ones associated with neuron development and differentiation and response to stimuli (**Supplemental Figures 3.5-3.8**). The influence of these upregulated pathways on host behavior is worth investigating.

3.3.5: Galbut virus infection does not have significant impacts on predominant microbiome constituents.

Previous studies have shown the microbiome can alter host fitness [53], that viral infections can potentially manipulate the microbiome [287], and commensal bacterial levels can vary by DGRP background when reared under the same conditions [264]. We were curious to observe whether galbut virus infection had detectable changes on the host microbiome. Previous shotgun metagenomic RNA sequencing on DGRP 399 and 517 uninfected flies revealed that major constituents of these flies' microbiomes in our lab include *Acetobacter persici, Lactobacillus brevis*, *L. planatarum*, *Corynebacterium* spp., and *Saccharomyces cerevisiae*. To measure how the major constituents of the microbiome may change upon galbut virus infection, microbial loads were acquired via qPCR using primers targeting these specific constituents. These loads were normalized using the single copy host gene *deformed* (*dfd*; as previously described [264]) and were statistically compared. No significant difference was seen in any of these major constituents in either strain or sex when comparing by galbut virus infection status (**Supplemental Figure 3.9**). This suggests that other factors associated with the DGRP strains or sex, and not perturbations to the gut microbiome, are responsible for any fitness impacts associated with galbut virus infection.

3.4: Discussion

A major goal of this study was to understand why galbut virus, despite a highly efficient vertical transmission rate (~100% from both parents), is maintained at a worldwide prevalence of only ~60% [69,163]. We hypothesized that galbut virus may exact a severe fitness cost reducing the prevalence, as suggested by previous modeling of vertically transmitted viruses [204]. To address this hypothesis, several aspects of fitness across two genetic backgrounds of flies (*Drosophila* Genetic Reference Panel; DGRP) that differed only in their galbut virus infection status were observed. We tested whether fitness was changed in a sex-dependent manner by evaluating both female and male flies in these strains. Galbut virus infection does modulate various fitness parameters, but these alterations are dwarfed in comparison to the differences observed when comparing by DGRP strain and sex (**Supplemental Figures 3.1, 3.3, 3.4, Figure 3.9**). The lack of any extreme phenotype is concordant with other partitiviruses. Partitiviruses were originally called cryptic viruses because of their minimal modulation of host biology and fitness [76]. Differences were not widely conserved but varied by strain and sex; a phenomenon not necessarily unusual for partitiviruses. A study showed that partitivirus infection of *Heterobasidion* fungi differed in the phenotypic outcomes based on the genetics of the host and environmental conditions [288]. Not all galbut virus-associated phenotypes observed in this study were harmful, suggesting that other factors may play a larger role in reduction in galbut virus prevalence, such as host resistance alleles within populations [140,205–207].

Fitness is a function of many variables including survival to reproductive age and fecundity [265]. Galbut virus infection minimally impacted both of these factors (**Figure 3.2, Figure 3.3**), with total lifespan and offspring more influenced by DGRP strain (**Supplemental Figures 3.1, 3.3**). Galbut virus infected DGRP 399 flies had a slight reduction in total survival time (on average 8 days shorter lifespan), while there was no difference by infection status in DGRP 517 flies (**Figure 3.2**). No major phenotypic impact of galbut virus on total fecundity was observed (**Figure 3.3**). DGRP 517 infected flies had a slight reduction in the total number of male offspring, but the point at which infected flies and uninfected flies began to diverge in this total number of male offspring was at ~20 days (**Figure 3.3A**). Furthermore, regardless of a parent's infection status, there was no impact on the number of eggs laid (**Figure 3.3B**). *D. melanogaster*

are thought to live for less than 20 days in the wild (lifespan estimates range from 1.3 to 6.2 days) [289]. This may suggest that this reduction in total male offspring is negligible, but that the impact on lifespan may be more influential. Partitiviruses have been known to alter the fecundity of their host, both positively and negatively. Examples include a partitivirus enhancing fecundity in *Cryptosporidium parvum* [178] and a reduction of spores in a partitivirus-infected fungus [290].

An additional life trait measured was developmental time. In DGRP 399 flies, no significant difference in time to pupate was noted by infection status, but galbut virus infected males did take longer to reach adulthood (**Figure 3.4**). Since total reproductive output of a population is a function of the number of females, slower development times in infected males may not necessarily contribute to overall fitness. However, in DGRP 517 flies, both the pupation speed and total developmental time was significantly shorter in galbut virus-infected flies than uninfected flies (**Figure 3.4**). This phenotype was supported by the transcriptional differences in these flies when compared by galbut virus infection. Gene ontology (GO) pathways associated with development and metabolic processes were upregulated in infected DGRP 517 flies (**Supplemental Figures 3.7, 3.8**). An initial assumption would be that a faster developmental time, in combination with the naturally short life cycle of flies in the wild [289], would be a mutualistic benefit. However, a study showed that when flies were selected for a faster developmental speed, it came with other fitness trade-offs, such as a reduction in body weight and size, a reduction in starvation and desiccation resistance, and an overall lower egg output [291]. Although we did not observe any significant differences in total egg output (**Figure 3.3B**), additional assays to measure how this development speed may impact other life history traits is necessary. Previous impacts of partitiviruses on host growth has been noted, particularly within fungi, where growth rate was stunted [179,292] or unchanged [293]. Current literature on plant partitiviruses show that they do not appear to have any obvious effects on domesticated plant development, but to date many of the impacts on wild plants remain largely unknown [294].

We further explored other aspects of fitness that may be modulated by galbut virus infection, namely pathogen susceptibility (**Figures 3.5-3.8**). Galbut virus provided protections but they were not conserved across any one strain or sex, and none of these protections were profound in any case. Female

DGRP 517 infected flies were slightly protected against a viral pathogen, *Drosophila* C virus (DCV; **Figure 3.5**). Slight protection against a Gram-positive bacterium, *Staphylococcus aureus*, was observed in infected DGRP 517 female and DGRP 399 male flies (**Figure 3.7B**). Pathogen protection by viruses has been described previously. Examples include a densovirus providing protection against a baculovirus pathogen in *Helicoverpa* larvae and a herpesvirus in mice providing protection against bacterial pathogens [73,74]. Insect-specific viruses also alter susceptibility of mosquitoes to arthropod-borne viruses (arboviruses) [295–299], and although not often considered as pathogens to the mosquito host, arboviruses can negatively impact mosquito fitness [300–304].

What was markedly different in galbut virus infected flies was the increase in pathogen susceptibility. When orally challenged with *Pseudomonas aeruginosa*, galbut virus infected DGRP 399 female flies were more susceptible (**Figure 3.6**). This susceptibility however was lost when the bacterium was inoculated via microinjection (**Figure 3.7A**). This may not be surprising as the gut epithelial immune response has key differences compared to a systemic infection [305]. An examination of transcriptome responses in the gut compared to the hemolymph may reveal transcriptional markers responsible for this difference in inoculation route susceptibility. DGRP 399 flies were also more susceptibile to a fungal pathogen, *Candida albicans* (**Figure 3.8**). This was also the sole occurrence where changes in susceptibility occurred in both sexes. The downregulation of Formin homology 2 domain containing (Fhos) in DGRP 399 flies (**Figure 3.11**) may be a key factor in the increased susceptibility to *C. albicans*. This gene helps direct macrophage movement [280]. Increased susceptibility to secondary fungal infections following viral infections have been noted in the literature, but these studies are predominately in higher order mammals [306–309]. Microbial interactions altering secondary fungal susceptibility has been noted within *Drosophila*, but was within the interactions of a commensal bacterium species and a fungal pathogen [310]. In this study, *Lactobacillus planatarum* was able to decrease the mortality rate of a fungal pathogen (*Diaporthe* sp.) by mitigating fungal toxicity and altering fly behavior to reduce infection risk. No significant changes in the relative loads of *L. planatarum* or other major microbiome constituents was observed in galbut virus infected flies (**Supplemental Figure 3.9**). Further investigation of additional

DGRP lines is warranted as galbut virus-infected flies may serve as a useful model for understanding changes in fungal susceptibility during persistent viral infections.

To date, the biological impacts of arthropod-infecting partitiviruses have only been described in partitiviruses infecting armyworm moths [184]. There are both similarities and differences in the biological impacts of galbut virus and these moth-infecting partitiviruses. In galbut virus infected flies, there were no major impacts on fecundity (**Figure 3.3**) while in moths, fecundity was severely reduced. Flies infected with galbut virus developed across a range of speeds (decreased, unaltered, or increased) depending on strain and sex (**Figure 3.4**); moths infected with partitiviruses were negatively impacted in developmental speed. Total adult lifespan however was minimally impacted in both hosts suggesting that this may be a general trait of arthropod-infecting partitiviruses. Pathogen protective phenotypes, noted in galbut virus infected flies (**Figure 3.5, 7B**), are also observed in the moth-infecting partitiviruses within the natural host (*Spodoptera exempta*). Only a single viral pathogen challenge (baculovirus) was performed in moths; how other pathogen sensitivities (bacterial and fungal) change in these infected moths is unknown. The authors suggested that these moth-infecting partitiviruses cause large transcriptome shifts, while galbut virus has minimal transcriptome disruptions in *D. melanogaster* (**Figure 3.9-10**). This study however used moths co-infected with multiple partitiviruses, therefore it is of interest to study how partitivirus co-infection may further alter these life history traits. The lack of complete concordance across biological fitness parameters between these arthropod-infecting partitiviruses warrants the need to elucidate how partitiviruses biologically impact other hosts, such as those partitiviruses found in diverse mosquito species [75,78,161–163,183].

CHAPTER 4: THE UTILITY OF THE VIROME FOR POSSIBLE BIOCONTROL MEASURES

4.1: Biocontrol Measures of Disease Vectors and Arthropod-borne Pathogens

Arthropod-borne diseases are an unrelenting threat to human health. These diseases account for more than 17% of all total infectious diseases and are responsible for more than 700,000 deaths worldwide [311]. A key feature, as the name implies, is that these disease-causing pathogens are harbored and spread by arthropods (known as vectors). Examples of vectors include mosquitoes, ticks, kissing bugs, sandflies, and tsetse flies. In an effort to reduce the burden of these diseases on the human population, scientists have developed strategies, referred as biocontrol, to reduce the number of viable or competent vectors available to spread these pathogens. Strategies include either targeting the vector itself resulting in subsequent death or by altering the biology of the vector to be unfit for harboring and transmitting these pathogens.

By far, the most-widely used strategy for the control of vectors is through insecticides. These chemical compounds are divided into 4 main categories: organochlorines, organophosphates, carbamates, and pyrethroids [312]. These chemical compounds target neuron-based pathways resulting in either hyperactivated or repressed neuronal activity leading to vector death [313]. Insecticides are usually applied through spraying, though infusion of insecticides into bed nets has also become commonplace, especially for the control of the malarial vector, *Anopheles* mosquitoes [312,314]. The major challenge of insecticide use is the rise of insecticide resistance. Vectors have evolved defense mechanisms, rendering these chemicals ineffective [315,316]. Alternative compounds have arisen such as the use of endectocides (e.g. ivermectin) for targeting *Anopheles* mosquitoes [317], use of bacterial insecticides [318], and insect growth regulators [319].

Other biological control techniques focus on the genetic modifications of the vector itself. An early example of this technique was the sterile insect technique (SIT). In SIT, males are sterilized via radiation and released into the wild populations where they successfully mate with females, but no viable offspring are produced [320]. This approach has been successful at the elimination of screwworm flies in certain areas [320]. A disadvantage to this approach is the necessity to continually mass produce and release sterile

males into the population which is costly. Alternative gene drive approaches, which allow self-propagation of deleterious genes through a population, using CRISPR-Cas9 systems are under investigation [321,322]. Resistance to gene drives can occur and reduce frequencies [323]. By targeting crucial genes needed, such as those associated with reproduction, mutation accumulation and subsequent resistance are highly unlikely to occur [322]. The ethics and ecological effects of these possible gene drive utilities remain a concern [324,325]. Alternative genetic approaches are aimed at not reducing the vector population, but rather at altering the vector to not be susceptible to harboring the pathogen, which has been done with *Aedes aegypti* mosquitoes [326–328].

An interesting approach to biocontrol is through vaccination. Two types of vaccines can be used. Those that prevent the vector from acquiring a pathogen (transmission blocking vaccines), and those that kill the vector (anti-vector vaccines). Transmission blocking vaccines function by challenging a vertebrate host with critical pathogen antigens which leads to the production of antibodies. When the vector takes its bloodmeal from a vaccinated host, and this host may also be infected with the pathogen, it acquires these antibodies preventing the vector from becoming infected by the pathogen. This has been studied in depth for malarial parasites [329–332] and *Borrelia burgdorferi* [333,334], the causative agent of Lyme disease. Anti-vector vaccines result in the production of antibodies against vector-based antigens. The vector then acquires antibodies against itself in the bloodmeal resulting in death [335,336]. Some vaccination strategies can even be transmission-blocking and detrimental to the vector [337].

A field that is gaining traction, and applicable to my thesis research, is the use of microbial symbionts for the control of pathogens within vectors. Symbionts from all areas of the microbiome, in a broad sense, are being explored. Bacterial symbionts are well studied features of the microbiome and research groups have shown their utility in different ways. Genetic modifications of symbionts and introduction of these genetically altered microbes into vectors is known as paratransgenesis. This method has proven fruitful in several studies. An example includes the modification of the endosymbiont *Rhodococcus rhodnii* in Triatomines (‘kissing bugs’). Kissing bugs vector *Trypanosoma cruzi*, the causative agent of Chagas disease. The genetically modified *R. rhodnii* produce an antiparasitic peptide,

thus when introduced into the kissing bug host, it reduces the capacity of the kissing bugs to harbor *T. cruzi* [338]. Another example is the genetic modification of a *Serratia* bacterium which subsequently makes *Anopheles* mosquitoes refractory to malarial parasites [339].

Without modifying the symbiont itself, one of the most successful implementations using microbes for biocontrol is the story of *Wolbachia*. *Wolbachia* is a bacterial endosymbiont that is widely found across arthropod species [340,341]. This endosymbiont is of interest as it has inherent properties that make the host oftentimes more resistant to viral infections [211]. When *Wolbachia* derived from *D. melanogaster* (stain *w*Mel) were introduced into *Aedes aegypti* mosquitoes, these mosquitoes became refractory to many arbovirus infections [342–346]. Another key feature of *Wolbachia* is that it induces a phenomenon called cytoplasmic incompatibility (CI). In this phenomenon, *Wolbachia*-uninfected females are unable to produce viable offspring when mating with *Wolbachia*-infected males [347]. The outcome of CI is the successful push and quick sweep of *Wolbachia* into arthropod populations [348]. *Wolbachia*-infected *Aedes aegypti* have been successfully deployed in communities, resulting in decreased disease burdens of dengue virus [349].

What has hopefully been a clear pattern throughout this dissertation is the role of understanding how viruses may modulate host biology. Viruses, too, have been studied for their potential utility as biocontrol agents. Insect-specific viruses (ISVs; those that replicate in arthropod cells but not mammalian cells) have grown in number through surveillance and metagenomic screenings of arthropod vectors. These have been identified in a wide variety of virus families [64]. Importantly, there are numerous studies that have shown that these ISVs are able to modulate arbovirus replication *in vitro* and *in vivo* [295–299,350–359], with the vast majority focused on ISVs in the *Flaviviridae* family. Most work examining interactions between ISVs and pathogens has been done in mosquito-infecting ISVs, but they can be found in other important insect vectors [360–363]. Although this group is commonly referred to as *insect*-specific, a broader term (i.e. arthropod-specific) may be necessary as similar viruses are found in important tick vectors, which are not classified as insects [55,56,364–369].

A key feature of ISVs that makes them promising for their use in biocontrol is how they are transmitted. Vertical transmission is the suspected dominate mode of transmission for maintenance of ISVs within mosquito populations, with maternal transmission rates up to 100% within naturally infected hosts [297,298,370–376]. ISVs can also be maintained transstadially (across the developmental life stages) [376] and in some cases through venereal (sexual) transmission [297]. By being vertically transmitted at a high efficiency, these viruses could reach fixation in a given population as long as no severe fitness costs are observed [204]. Verdadero virus, the partitivirus identified in *Ae. aegypti* (see Chapter 2), also has this high vertical transmission efficiency and should be investigated further as a potential biocontrol agent.

An important area of interest for ISVs is understanding how they interact with other microbial constituents of the host. It is clear that microbial symbionts do not act in an isolated manner but have regular interactions with other symbionts [377–381]. One key symbiont is *Wolbachia*, which as stated earlier has known antiviral effects [211]. Studies using *in vitro* analysis showed *Wolbachia* inhibited or did not alter virus levels [382–384], while an *in vivo* study showed *Wolbachia* enhanced virus replication [385]. Further investigation of how *Wolbachia* alters ISVs in their respective hosts is needed to understand how ISVs may be used in synergy with the current *Wolbachia*-based biocontrol measures. Though not a vector, in *D. melanogaster*, *Wolbachia* did not have an apparent impact on the natural virome of wild flies [77]. Additional future studies remain to understand how these viruses may be genetically altered. Currently an insect-specific alphavirus, Eilat virus, has been genetically modified as a potential vaccine platform for pathogenic alphaviruses, like Chikungunya virus [386].

To continue finding potential microorganisms (bacterial or viral) that may have importance in blocking the transmission of arthropod-borne pathogens, additional screening of vector populations must occur. I have contributed to this exploration by evaluating how constituents of the virome interact with fellow microbial symbionts within *Ixodes scapularis* ticks. More specifically, I identified interactions between the virome and known tick-borne pathogens to elucidate how the virome may influence the vector competence of these ticks.

4.2: Co-infection Patterns in Individual *Ixodes scapularis* Ticks Reveal Associations Between Viral, Eukaryotic, and Bacterial Microorganisms [67]

4.2.1: Introduction

Ixodes scapularis, the blacklegged or deer tick, is the main North American vector for *Borrelia burgdorferi*, the causative agent of Lyme disease. In the USA, there are an estimated 300,000 cases of Lyme disease per year, and the incidence of tick-borne diseases is increasing [387–389]. In addition to *B. burgdorferi*, *I. scapularis* ticks harbor other pathogens, including eukaryotic (*Babesia microti*), bacterial (*Anaplasma phagocytophilum*, *B. mayonii*, *B. miyamotoi* and *Ehrlichia muris eauclarensis*), and viral (Powassan virus) agents [390–394]. It is possible that individual ticks can be co-infected by more than one of these pathogens, and co-infection of a vertebrate can impact clinical outcome [395–397].

Ticks also harbor non-pathogenic microbes, and it has been recognized for some time that these have the potential to influence tick physiology and vector competence, the ability of the tick to acquire, harbor and transmit a pathogen [398–408]. For example, *Amblyomma americanum* ticks dysbiosed by antibiotic injection exhibited a marked decrease in reproductive success [407]. In *I. scapularis* larvae with decreased bacterial loads, *B. burgdorferi* colonization of the midgut was less efficient [408]. Also, *Anaplasma marginale* levels were lower in *Amblyomma americanum* ticks with altered microbiomes [406]. As in all organisms, it is clear that tick-associated microbiota can exert a significant effect on their host.

Metagenomic studies have also recently identified a number of new tick-associated viruses in the northeastern USA, in several European countries, and in China [55,56,364–369]. Two groups of bunyaviruses seem to be particularly common in *Ixodes* ticks: a lineage that includes South Bay virus in *I. scapularis* in the USA and Grotenhout virus in *I. ricinus* in Europe, and a lineage that includes the blacklegged tick phleboviruses in American *I. scapularis* and Norway phlebovirus in European *I. ricinus* [364,367,369]. Yet the biological impact of these viruses remains largely unknown, and previous studies have for the most part characterized the bacterial and viral microbiomes of ticks separately [364,366,369,400–402,409–411].

Therefore, to understand the possible influence of nonpathogenic viral components of the microbiota of *I. scapularis*, we simultaneously measured RNA levels of eukaryotic, bacterial and viral microbes in or on 112 individual adult ticks collected from Wisconsin, USA. This is an area of high Lyme disease risk, and the microbiome of *I. scapularis* from this region has not been evaluated in this manner [412]. We identified known microorganisms, including pathogens, as well as new virus-like sequences and a previously undescribed filarial worm. We characterized the prevalence, abundance and co-infection rates of microorganisms, and identified statistically significant co-occurrence and correlation patterns between microbiome constituents. We found that, as in other *I. scapularis* populations, South Bay virus and blacklegged tick phleboviruses were particularly common in these ticks [364,366,369]. These viruses were also involved in the majority of statistically significant associations with other microbes, including with *B. burgdorferi*.

4.2.2: Materials and Methods

4.2.2.1: Sample Collection

Adult *I. scapularis* were collected near Spooner Wisconsin by dragging in October 2015. Adult ticks were transported to the laboratory, identified to species and stored in individual cryovials in mosquito diluent (20% FBS, 1× PBS, 1× Penicillin/Streptomycin) at -80 °C until further processing. Ticks were not surface cleaned, so we sampled microorganisms present on the surface of ticks as well as those contained within ticks. Remaining tick halves were stored in this preservation medium for future possible virus isolation; 61 female and 51 male ticks were analyzed.

4.2.2.2: RNA Extraction

Ticks were sliced down the sagittal plane using a sterilized blade. One half of the tick was added to a 2 mL centrifuge tube along with a single sterile ball bearing, and 1 mL TRIzol (Ambion Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA); the other half was added to fresh mosquito

diluent and stored at -80 °C to be used for future analysis. The tick half in TRIzol was homogenized in a TissueLyzer II (Qiagen, Hilden, Germany) at 30 Hz for 4 min. 200 µL of chloroform (SigmaAldrich, St. Louis, MO, USA) was added, shaken by hand for 15 s, and incubated at room temperature (RT) for 2 min. RNA was further purified using RNA Clean and Concentrator-5 spin columns (Zymo, Irvine, CA, USA) as described [413]. RNA was quantified fluorometrically and stored at -80 °C.

4.2.2.3: Shotgun Metagenomic Library Preparation

Shotgun metagenomic libraries were prepared from total tick RNA as follows. 5 µL of RNA was added to 200 pmol of a random pentadecamer oligonucleotide and incubated for 5 min at 65 °C. Following incubation, the mixture was set on ice for 1 min. A reverse transcription mixture containing the following was added (12 µL reaction volume): 1× SuperScript III (SSIII) FS reaction buffer (Invitrogen, Carlsbad, CA, USA), 5 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleotide triphosphates (dNTPs) (NEB), and 100 U SSIII reverse transcriptase enzyme (Invitrogen). The RNA-oligomer with the reverse transcription mixture was incubated at 42 °C for 30 min, then at 50 °C for 30 min, then at 70 °C for 15 min. Total HeLa cell RNA and water were processed in parallel as controls. RNA templates were removed by adding a mixture 1 U RNase H (NEB) diluted in 160 pmol random pentadecamer and 5 µL 1× SSIII FS reaction buffer. Samples were incubated at 65 °C for 20 min followed by 94 °C for 2 min. This single-stranded cDNA was converted to double-stranded DNA by adding 2 mM each dNTPs, 1× SSIII FS reaction buffer, and 2.5 U Klenow DNA polymerase (3′ to 5′ exo-, NEB) and incubated at 37 °C for 15 min. The DNA was purified using SPRI (Solid Phase Reversible Immobilization) beads at a 1:1.5 DNA/beads ratio and eluted in 20 µL nuclease-free water (NFW). The dsDNA concentration was measured fluorometrically using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was tagmented by adding 10 ng of the dsDNA, 1× Tagment DNA buffer (Illumina, San Diego, CA, USA) and 0.5 µL 10× Nextera Tagment DNA enzyme (Illumina) at a final volume of 12 µL, followed by incubation at 55 °C for 10 min. Tagmented DNA was cleaned with SPRI beads and eluted in 15 µL NFW. The cleaned, tagmented

DNA was used as a template (5.8 μL) for addition of full-length adapters with unique bar-code combinations via PCR. This PCR reaction (25 μL final volume) contained the following: 1× Kapa real-time library amplification mix (Kapa Biosystems, Roche, Basel, Switzerland), 0.33 μM each of the primers 5′-CAAGCAGAAGACGGCATACG-3′ (P1) and 5′-AATGATACGGCGACCACCGA-3′ (P2), and 0.02 μM each of adapter 1 and 2 bar-coded primers [414]. The PCR reaction was run at 72 °C for 3 min, 98 °C for 30 s, and 12 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 3 min. PCR reactions were cleaned using SPRI beads, eluted in 15 μL NFW, and concentrations were measured fluorometrically. Equal masses of DNA from each sample were pooled, cleaned using SPRI beads, and eluted in 60 μL of nuclease-free Tris EDTA pH 8.0 (TE). The pooled libraries were size selected (range of 350–500 nucleotides) using a BluePippin and a 2% agarose Pippin gel cassette (Sage Science, Beverly, MA, USA) according to manufacturer's protocol. Size-selected pools were cleaned using SPRI beads with a 1:1.4 DNA/beads ratio and eluted in 20 μL NFW. Cleaned, selected pools were subjected to a final PCR containing 1x Kapa real-time amplification master mix (Kapa Biosystems), 500 pmol of both P1 and P2, and 10 μL of selected pools at a total volume of 50 μL. Thermocycling conditions were 98 °C for 45 s, followed by varying amounts of cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 2 min. Number of cycles was determined by the amount needed for the fluorescence to pass Kapa standard 1. Amplified pools were cleaned using SPRI beads at a 1:1.5 DNA/beads ratio and eluted in 18 μL TE. Final library quantification was performed using the Illumina library quantification kit (Kapa Biosystems) according to manufacturer's protocol. Libraries were sequenced using an Illumina NextSeq 500 instrument using paired-end 2 × 150 sequencing from a NextSeq 500/550 Mid Output Kit v2 (300 cycles) (Illumina).

4.2.2.4: Sequence Analysis

Metagenomic sequencing datasets were processed to taxonomically assign non-tick reads. First, low-quality sequences and adapter sequences were removed using the cutadapt tool version 1.14 under the following settings: -a AGATCGGAAGAGC -A AGATCGGAAGAGC -g GCTCTTCCGATCT -G

GCTCTTCCGATCT -a AGATGTGTATAAGAGACAG -A AGATGTGTATAAGAGACAG -g CTGTCTCTTATACACATCT -G CTGTCTCTTATACACATCT, -q 30,30, --minimum-length 80, and -u 1 [258]. PCR duplicates were collapsed using the CD-HIT-EST tool version 4.7 with the -c 0.96 parameter [191]. Host tick sequences were removed using Bowtie2 version 2.3.2 [192]. First, a bowtie index was created using the reference genome of *I. scapularis* [415]. Reads were then removed using a local alignment with the parameters --local --sensitive --score-min C,60,0. SPAdes genome assembler version 3.10.1 [416] was used to generate contiguous sequences from the remaining reads. Contigs longer than 150 nucleotides (nt) were taxonomically categorized using the BLASTn alignment tool version 2.6.0+ [190,417]. Contigs were assigned taxonomically to the sequence with the highest alignment score and an expect value less than 10^{-8} [190,418]. In order to taxonomically assess reads that were too divergent to produce a high-scoring nt-nt alignment, DIAMOND version 0.9.9.110 was used to query the NCBI nr database with an expect value of 10^{-3} [419]. The number of reads aligning to individual taxa were tallied by remapping host-filtered reads to SPAdes contigs using bowtie. If a contig aligned equally to multiple taxa, the result was collapsed at the lowest common ancestor of the matches. For several genera of bacteria (*Wolbachia*, *Rickettsia* and *Ehrlichia*), it was difficult to assign contigs at the species level because they aligned equally well to sequences from two or more species with the genus. Rather than equally distributing these reads to the multiple species, and potentially assuming the presence of a species that may not actually be present, we collapsed reads that aligned to these taxa at the genus level. Phage sequences were detected at very low levels (≤17 reads in 6 of the datasets), and phage sequences were not further analyzed.

Virus-mapping contigs were collapsed when possible by de novo assembling contigs that aligned on a protein level to a particular virus in Geneious version 11.0.4 [420]. Gaps were filled using PCR and Sanger sequencing. Draft virus assemblies were validated by remapping reads using Bowtie2 as above. All sequencing datasets have been deposited in NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA477560 [421].

4.2.2.5: Validation of Sequencing by PCR

PCR was used to validate sequencing results from a subset of random ticks that contained at least 4 co-infecting microorganisms. dsDNA remaining from library preparation (see above) was diluted 1:20 in nuclease-free water. Primers were created for viral sequences, while existing primers were used for *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia miyamotoi* [422], and the positive control *I. scapularis* glycerol-3-phosphate dehydrogenase (GPDH) primers [423] (**Supplemental Table 4.1**). PCR reactions contained: 1x Luna Universal qPCR Master Mix (NEB, Ipswich, MA, USA), 10 μM each of forward primer and reverse primer, and 5 μL of DNA template at a final volume of 20 μL. Thermocycling conditions for all microorganisms were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. For gpdh, thermocycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 45 s.

4.2.2.6: Statistical Analysis of Microbial Relationships

To measure associations between microorganisms, a table describing the number of reads mapping to various taxa in individual ticks was imported into R studio version 1.0.153 [424]. Co-occurrence relationships were measured using the 'cooccur' package version 1.3 and the function cooccur [425]. This package uses presence–absence datapoints and a hypergeometric distribution to calculate the probability that one site (an individual tick) contains both species 1 and 2, and whether they occur more or less frequently than expected. Correlation measurements were performed using the 'psych' package version 1.7.8 and the function corr.test with a Pearson method and Bonferroni adjustment [426]. Correlations were only analyzed for ticks that were co-infected with both microbes being analyzed, and Pearson coefficients were only considered significant if the adjusted *p*-value was less than 0.05. Microorganism prevalence by tick sex was statistically assessed using a pair-wise Fisher's exact test with a Bonferroni adjustment. R Code and data matrices are available in Github repository: https://github.com/scross92/coinfection_patterns_ixodes_scapularis. In mean abundance (RPM) calculations, values of 0 were set to NA in order to not factor into the reads per million (RPM) calculation.

4.2.2.7: Phylogenetic Analysis of Novel Microorganisms

For phylogenetic analysis of predicted viral sequences, the NCBI nr protein database was queried using the BLASTX tool, and aligning sequences with an expect value of less than 10^{-3} were downloaded [417]. Sequences were collapsed to a representative subset using the CD-HIT tool version 4.7 using parameter -c 0.9 [191]. These representative sequences were aligned using MAFFT version 7.310 under the --auto mode [193]. Alignments were trimmed with trimAl version 1.4.rev15 in the --strictplus mode [194]. These trimmed alignments were imported into Geneious version 11.0.4 and manually inspected. In case of partial sequences, alignments were trimmed to the length of partial sequence and any remaining sequences that were poorly aligned were removed. Phylogenetic trees were created from these alignments using PhyML version 3.3.20180109 under the LG mode and 100 bootstraps [427]. Phylogenetic trees were visualized using FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Phylogenetic analysis of the novel filarial worm sequence was performed essentially as previously described [428]. Primers were used to amplify the 12S rDNA sequence. The PCR product was Sanger sequenced, and the product was aligned against 12S rDNA sequences derived from other filarial worms using MAFFT version 7.310 under the L-INS-i mode. This alignment was then used to create a phylogenetic tree using PhyML using the HKY85 mode and 100 bootstraps.

4.2.3: Results

4.2.3.1: Taxonomic Assessment of the *Ixodes scapularis* Microbiome

In October 2015, 112 adult *Ixodes scapularis* (61 female; 51 male) were collected in northwest Wisconsin. Ticks were cut in half. One half was stored in mosquito diluent at −80 °C, and RNA was extracted from the other half (**Figure 4.1**). RNA-derived shotgun libraries were sequenced using paired-end 2 × 150 sequencing on an Illumina NextSeq instrument, generating an average of 2.1×10^6 read pairs per dataset. After removing low-quality, adapter and tick-derived reads, an average of 4.8×10^4 read pairs

per dataset remained (2.7%). Remaining reads were assembled and taxonomically assigned by comparison at nucleotide and protein levels to NCBI database sequences. In most cases, contigs shared a high degree of sequence identity with existing sequences (**Table 4.1**). In other cases, contigs were less closely related to database sequences. For instance, we identified contigs that shared between 76.1% and 98.2% nt identity in BLASTN alignments with various nematode sequences (**Table 4.1**). Because these sequences appeared to derive from a previously uncharacterized worm, confident species or genus-level assignment was not possible, and contigs were assigned at the level of the family *Onchocercidae* (nematodes). Similarly, unambiguous species-level assignment was not possible for contigs mapping to certain bacterial taxa (*Rickettsia*, *Ehrlichia* and *Wolbachia)* so contigs mapping to these taxa were assigned at the genus level. We calculated the number of reads mapping to particular taxa per million unique reads (RPM) as a measure of RNA level and taxon abundance. Because we did not clean ticks prior to RNA extraction, it is possible that some sequences derived from microbes present on the surface of ticks. Also, detection of pathogen sequences does not necessarily indicate that that particular tick would be a competent vector for the pathogen. Total HeLa cell RNA and water were processed and analyzed in parallel as positive and negative controls.

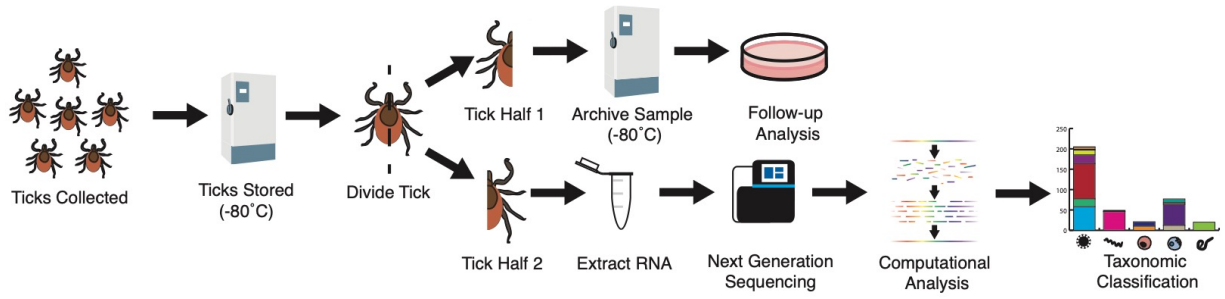

Figure 4.1. Tick Analysis Workflow. Adult *Ixodes scapularis* ticks were collected from northwest Wisconsin. Ticks were then stored at -80 °C in mosquito diluent. Individual ticks were divided in half. The first half was subjected to next-generation sequencing, computational analysis, and taxonomic classification. The second half was archived at -80 °C for future analysis.

We focused our analyses on 18 taxa that accounted for 89% of the assigned non-tick reads in our datasets. These 18 taxa included South Bay virus, Suffolk virus, Blacklegged tick phleboviruses 1–3, Powassan virus, Ixodes scapularis associated viruses 1 and 2, *B. burgdorferi* sensu stricto, *B. mayonii*, *B. miyamotoi*, *Babesia* (*Ba.*) *microti*, *Ba. odocoilei*, *Anaplasma (A.) phagocytophilum*, *Rickettsia*, *Ehrlichia*, *Wolbachia* and *Onchocercidae*. These taxa were selected because they were the most abundant and prevalent in individual ticks and/or because they are human pathogens (**Figures 4.2–4.4; Table 4.1**). Female ticks contained between 1 and 9 of these taxa (female 55) (**Figure 4.3**), while individual male ticks contained between 0 and 6 (male 5, male 29) (**Figure 4.4**).

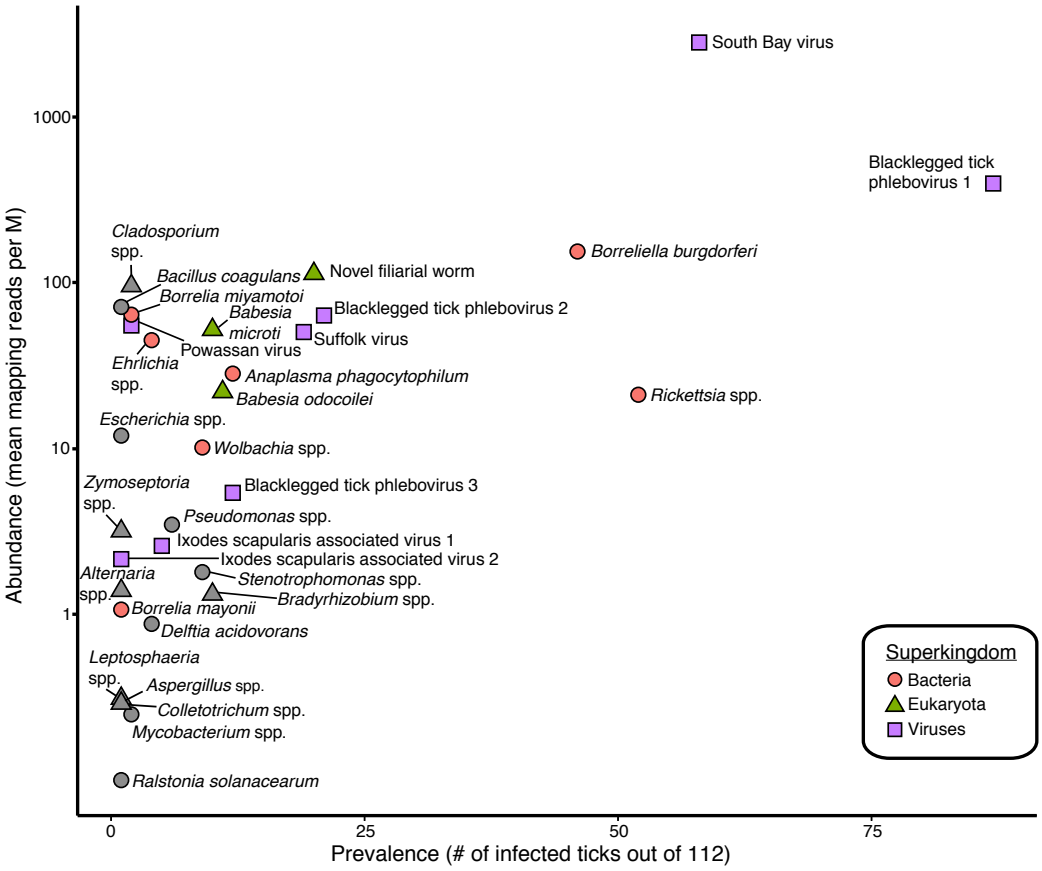

Figure 4.2. South Bay virus and Blacklegged tick phlebovirus are the most abundant and prevalent microorganisms in the sampled *I. scapularis*. The prevalence (number of infected ticks out of 112) and average RNA level (average mapping reads per million unique reads on a log scale) for the indicated taxa are plotted. The superkingdom of each taxa is indicated by shape and color as indicated. The 18 taxa that were selected for focused analysis are colored (other taxa in grey).

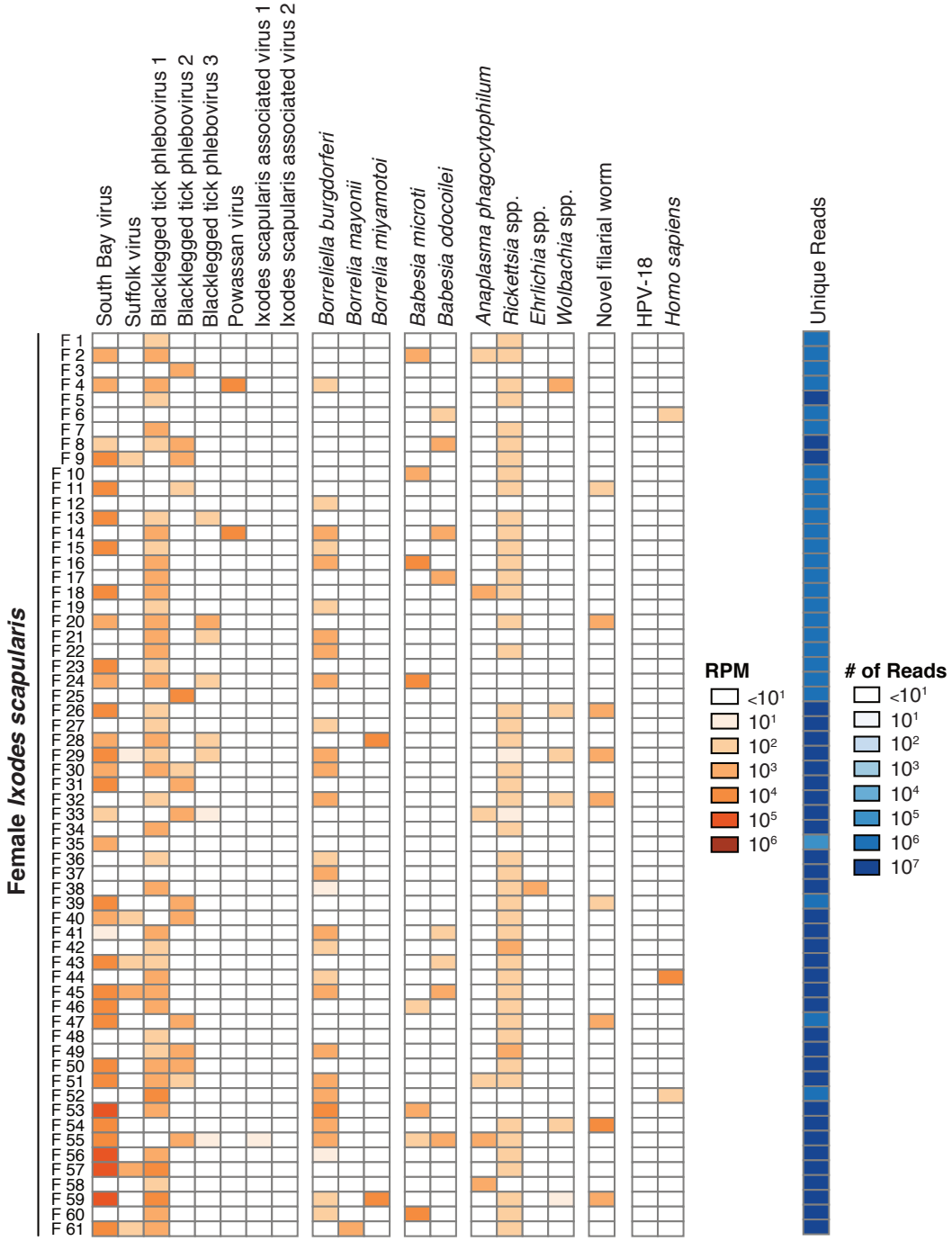

Figure 4.3. Abundance of predominant microbial constituents of female adult *Ixodes scapularis*. 18 taxa of interest were selected for abundance analysis. Between 1 and 9 taxa were detected in female adult ticks ($n = 61$). The number of mapping reads per million unique reads (RPM) is shown, as is the number of unique reads in each dataset. RPM values >10 are shown. HPV-18: human papillomavirus type 18.

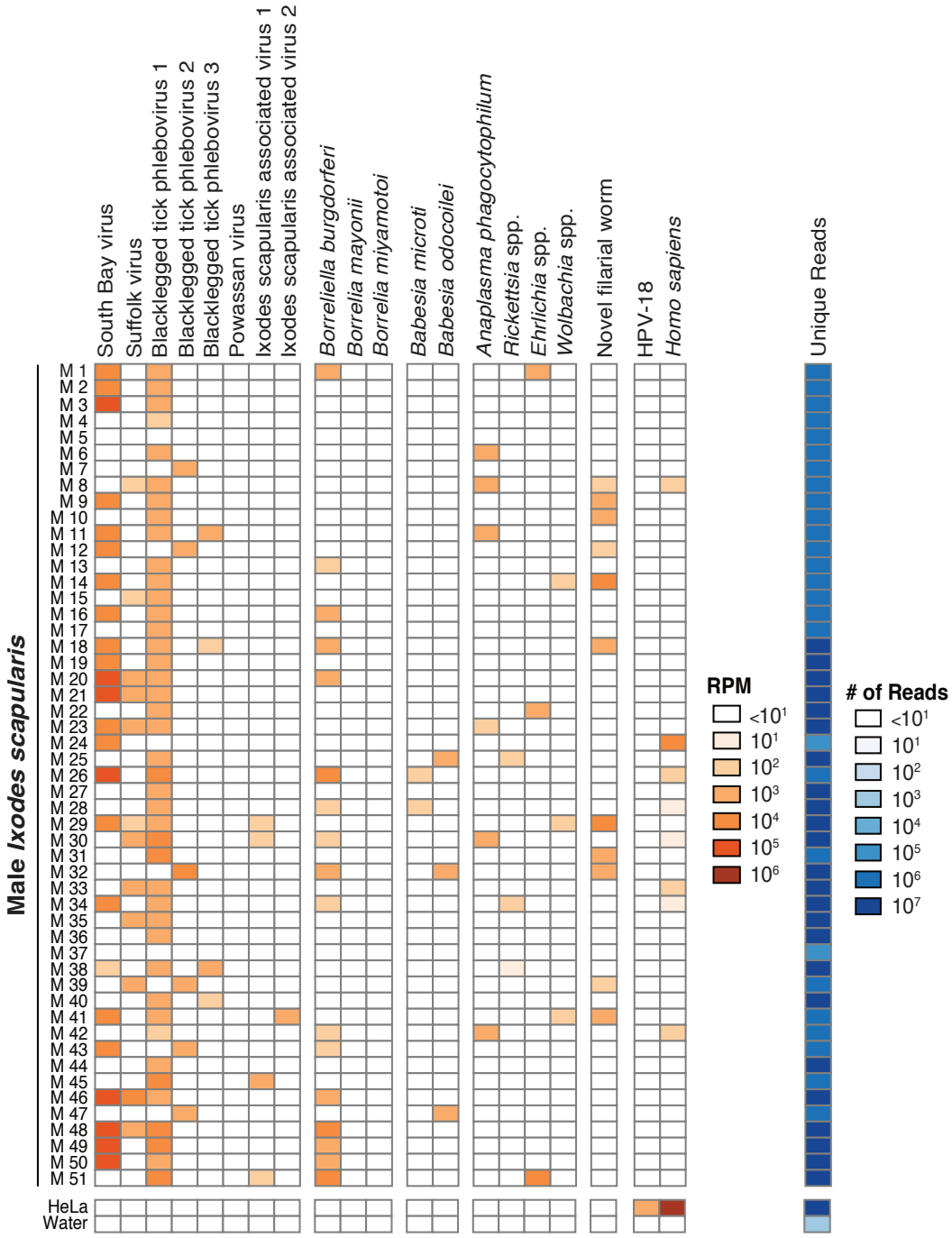

Figure 4.4. Abundance of predominant microbial constituents of male adult *Ixodes scapularis* and controls. 18 taxa of interest were selected for abundance analysis. In male adult ticks (n = 51), between 0 and 5 taxa were detected. The number of mapping reads per million unique reads (RPM) is shown, as is the number of unique reads in each dataset. RPM values >10 are shown. Control datasets generated from HeLa cell total RNA and water are shown. HPV-18: human papillomavirus type 18.

The most abundant and prevalent taxa in individual ticks were blacklegged tick phlebovirus 1 (BLTPV1) and South Bay virus (SoBV), with prevalences of 78% and 52% and mean mapping read levels of 395 and 2796 RPM (**Figure 4.2, Table 4.1**). *Rickettsia* species and *B. burgdorferi*, detected in 46% and 41% of ticks and with mean levels of 21 and 154 RPM, were the next most prevalent.

We evaluated prevalence of taxa by sex and found that *Rickettsia* spp. were detected more often in female ticks (80.3%) than in males (5.9%; **Table 4.1**). This difference was the only statistically significant difference in taxon prevalence by sex (p-value = 3×10^{-15}). This difference has been observed in previous studies [403,429–431]. It has been proposed that the higher prevalence in females may be attributed to an adaption of *Rickettsia* spp. to transovarial transmission [403].

Table 4.1. Taxa prevalence in adult *Ixodes scapularis*.

Taxon	Tick Sex		All Ticks		
	Male (n = 51)	**Female (n = 61)**	**(n = 112)**	**Nucleotide Identity [1]**	**Average Mapping Reads Per Million Unique Reads (RPM)**
Viruses					
Blacklegged tick phlebovirus 1	82.4%	73.8%	77.7%	97.6%	395
South Bay virus	47.1%	55.7%	51.8%	98.0%	2796
Blacklegged tick phlebovirus 2	11.8%	24.6%	18.8%	97.1%	63
Suffolk virus	23.5%	11.5%	17.0%	98.1%	50
Blacklegged tick phlebovirus 3	7.8%	13.1%	10.7%	98.1%	5
Ixodes scapularis associated virus 1	7.8%	1.6%	4.5%	98.3%	3
Powassan virus	0%	3.3%	1.8%	100.0%	55
Ixodes scapularis associated virus 2	2.0%	0%	0.9%	96.4%	2
Bacteria					
Rickettsia spp.	5.9%	80.3%	46.4%	98.8%	21
Borreliella burgdorferi	33.3%	47.5%	41.1%	99.7%	154
Anaplasma phagocytophilum	11.8%	9.8%	10.7%	98.9%	28
Wolbachia spp.	5.9%	9.8%	8.0%	97.5%	10
Ehrlichia spp.	5.9%	1.6%	3.6%	96.6%	45
Borrelia miyamotoi	0%	3.3%	1.8%	100.0%	64
Borrelia mayonii	0%	1.6%	0.9%	99.4%	1
Eukaryotes					
Novel filarial worm	21.6%	14.8%	17.9%	88.7%	113
Babesia odocoilei	5.9%	13.1%	9.8%	100.0%	22
Babesia microti	3.9%	13.1%	8.9%	99.7%	52

4.2.3.2: Validation of Metagenomic Sequencing Results

We used RT-PCR to corroborate sequencing results. Ten ticks that harbored at least 4 organisms were randomly selected. We performed RT-PCR using custom primers and previously published primers for *B. burgdorferi*, *A. phagocytophilum*, *Ba. microti*, and *B. miyamotoi* [422] (**Supplemental Table 4.1**). Primer to amplify *I. scapularis* glycerol-3-phosphate dehydrogenase (GPDH) mRNA were used as a positive control [423]. In all cases where an organism was detected by sequencing, it was also detected by PCR (**Figure 4.5**). However, there were two cases where an organism was detected by PCR but not sequencing: Suffolk virus in tick F2, and Ixodes scapularis associated virus 2 in tick F29 (**Figure 4.5**). We attributed this discrepancy to the fact that PCR is generally more sensitive than sequencing [432].

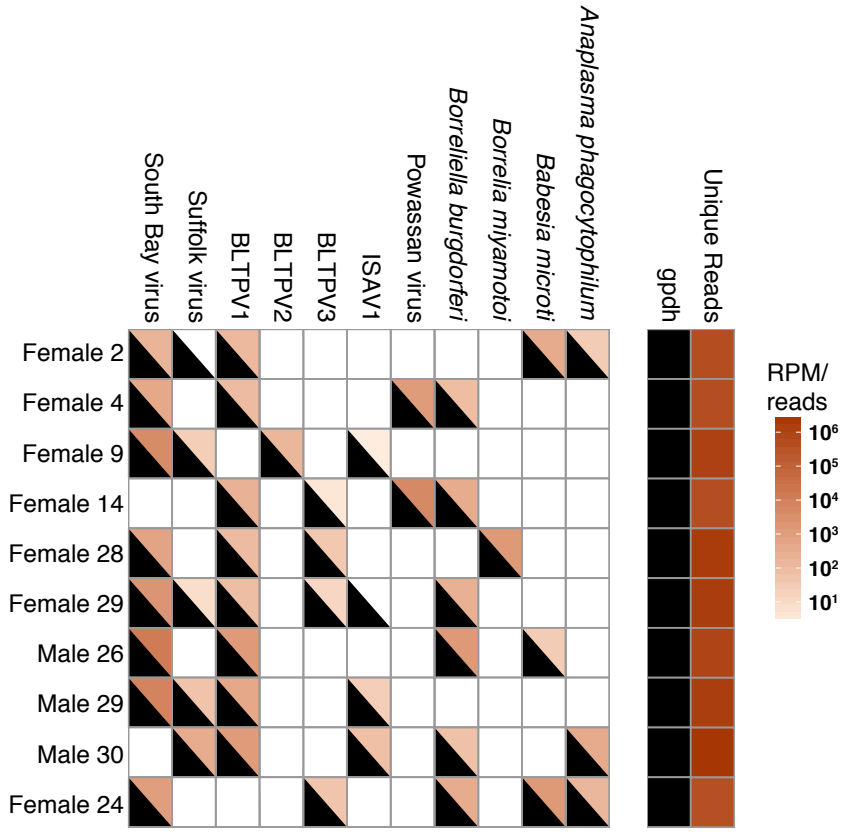

Figure 4.5. PCR detection of microbes was concordant with sequencing-based detection. Ten ticks with at least four organisms detected by sequencing were randomly selected for validation by RT-PCR. PCR positive samples are indicated by a black triangle. *I. scapularis* glycerol-3-phosphate dehydrogenase (gpdh) was used as a positive control for detection of tick RNA. The number of unique reads from each NGS dataset is shown using the same color scale as RPM values. BLTPV: Blacklegged tick phlebovirus; ISAV: Ixodes scapularis associated virus.

4.2.3.3: Detection of New Microorganisms

We also identified new virus or virus-like sequences. These were at relatively low levels in relatively few ticks, and included a mononegavirus sequence most closely related to Norway mononegavirus 1 (**Figure 4.6, Table 4.2**), and sequences related to the S segments of Blacklegged tick phleboviruses and Norway phlebovirus 1 (**Figure 4.7, Table 4.2**). We did not identify phlebovirus L or M segment sequences, and cannot rule out the possibility that the S segment-like sequences correspond to endogenous viral elements. However, the S segment sequences had low coverage, and it may be that the L and M sequences were below the limit of detection. Virus scaffolds ranged from 791 nucleotides to 5020 nucleotides long, and no scaffold was coding complete.

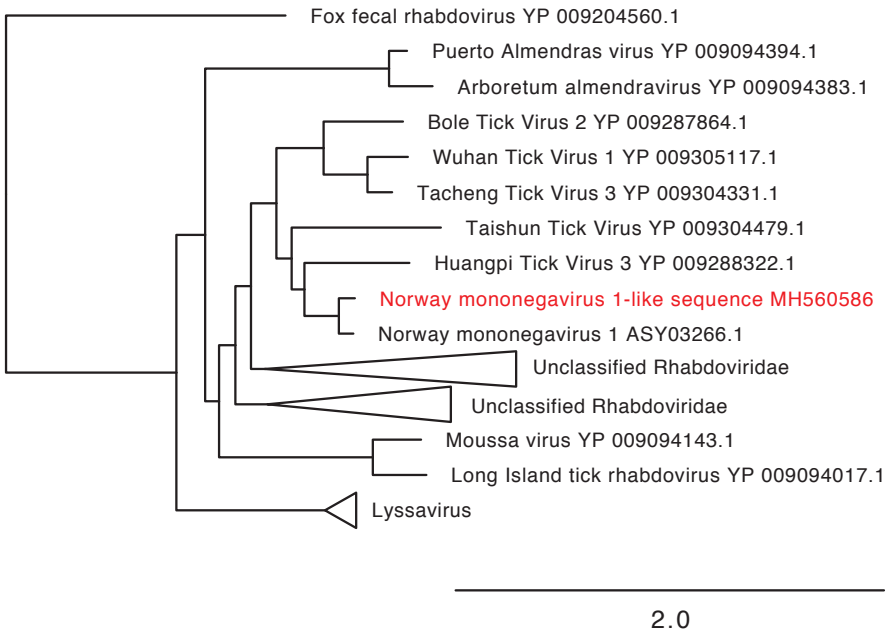

Figure 4.6. **Phylogenetic characterization of new mononegavirus sequence.** Phylogeny based on an alignment of a 508 amino acid region of the viral RNA dependent RNA polymerase (RdRp). This alignment includes mononegavirus reference sequences available through NCBI. Additional closely related unclassified viruses were also included. Triangles indicate collapsed clades. The novel virus sequence is shown in red. Heartland virus was used as an outgroup to root the tree.

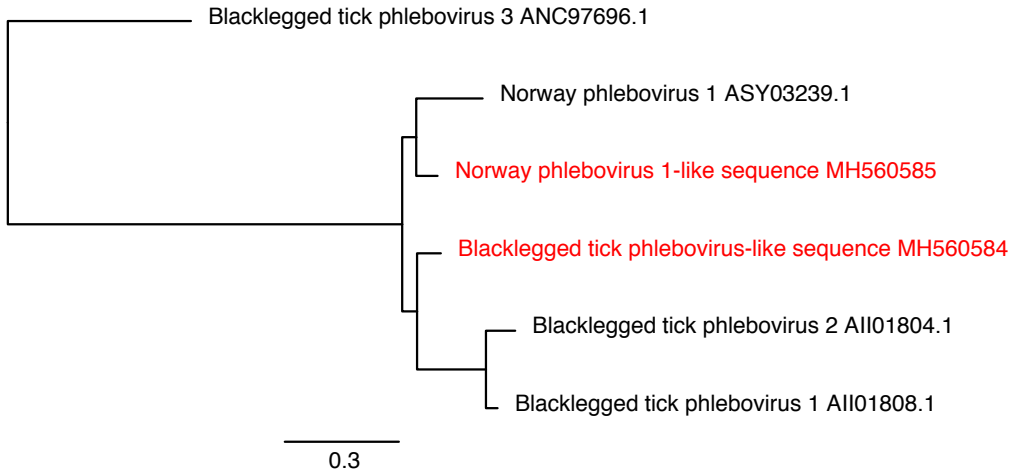

Figure 4.7. Phylogenetic characterization of novel phleboviruses. Phylogeny based on an alignment of a 176 amino acid region of the nucleocapsid protein. This alignment includes phlebovirus reference sequences available through NCBI. Additional closely related unclassified viruses were also included. The novel viruses are shown in red. Phasi Charoen-like phasivirus was used as an outgroup to root the tree.

Table 4.2. New virus-like sequences identified.

	Closest Related Sequence	Contig Length (nt)	Accession	BLAST Percent Identity [1]
Norway mononegavirus 1-like sequence	Norway mononegavirus 1 (MF141072.1)	5020	MH560586	71%
Blacklegged tick phlebovirus-like sequence	Blacklegged tick phlebovirus 1 (KX184201.1)	874	MH560584	77%
Norway phlebovirus 1-like sequence	Norway phlebovirus 1 (MF141061.1)	791	MH560585	78%

[1] *Pairwise identity of BLASTn alignment to highest scoring database sequence.*

We also characterized the phylogenetic placement of the filarial worm (*Onchocercidae* sp. ex *Ixodes scapularis*) that we identified in 20 of the ticks. We used PCR and Sanger sequencing to determine the worm 12S rRNA gene sequence from 6 positive samples and found them to share ≥98.5% pairwise nt identity. A tree based on the alignment of these sequences with related nematode sequences showed them to cluster most closely to other filarial worms recently found in other *I. scapularis* (**Figure 4.8**) [428].

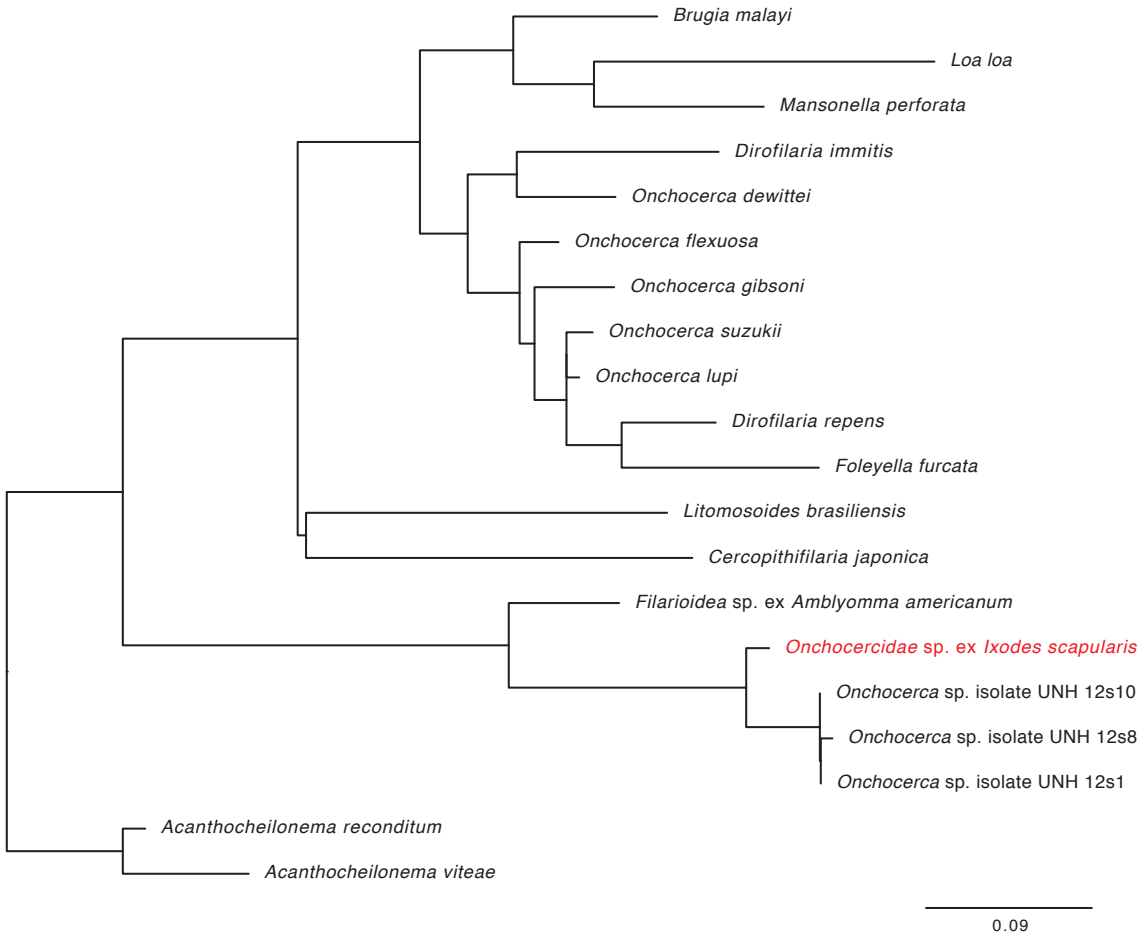

Figure 4.8. Phylogenetic characterization of novel filarial worm. Phylogeny based on an alignment of a 174 base pair region of the 12S rDNA sequence. This alignment included other filarial worm 12S rDNA sequences as used in Namrata, et al. [428]. The novel filarial worm (*Onchocercidae* sp. ex. *Ixodes scapularis*) is shown in red.

4.2.3.4: Co-Occurrence and Correlation Analyses

We next searched for evidence of associations between members of the microbiome of these *I. scapularis* by examining patterns of co-infection. We first evaluated whether pairs of taxa co-occurred more or less than would be expected given their individual infection rates. If co-infection was found more often than expected (positive co-occurrence), it could suggest that infection by one organism could predispose to infection by the second, or that ticks are more likely to acquire both organisms from feeding on a co-infected

vertebrate. If co-infection was found less often than expected (negative co-occurrence), it could suggest that infection by one organism prevents infection by the second.

Five positive and one negative co-occurrence relationships were identified after correcting for multiple hypothesis testing (**Figure 4.9**). The positive relationships were: *Wolbachia* spp. and the novel filarial worm (p-value < 1×10^{-6}), *Wolbachia* spp. and SoBV (p-value = 0.021), *Rickettsia* spp. and SoBV (p-value = 0.041), SoBV and BLTPV3 (p-value = 0.02), and Blacklegged tick phlebovirus 1 (BLTPV1) and *B. burgdorferi* (p-value = 0.039). The sole negative co-occurrence was between BLTPV1 and BLTPV2 (p-value < 1.0×10^{-6}) (**Figures 4.3 and 4.4**).

We then tested whether the abundance of taxa were correlated within individual co-infected ticks, which could suggest that infection by one organism impacts replication of another. After a Bonferroni multiple testing adjustment, three statistically significant positive correlations were identified (**Figure 4.9**, **Supplemental Figure 4.1**). These were *Wolbachia* spp. and the filarial worm (Pearson coefficient = 0.96; p-value = 8.4×10^{-3}), SoBV and *B. burgdorferi* (Pearson coefficient = 0.75; p-value = 1.4×10^{-3}), and SoBV and BLTPV1 (Pearson coefficient = 0.72; p-value = 1.25×10^{-6}).

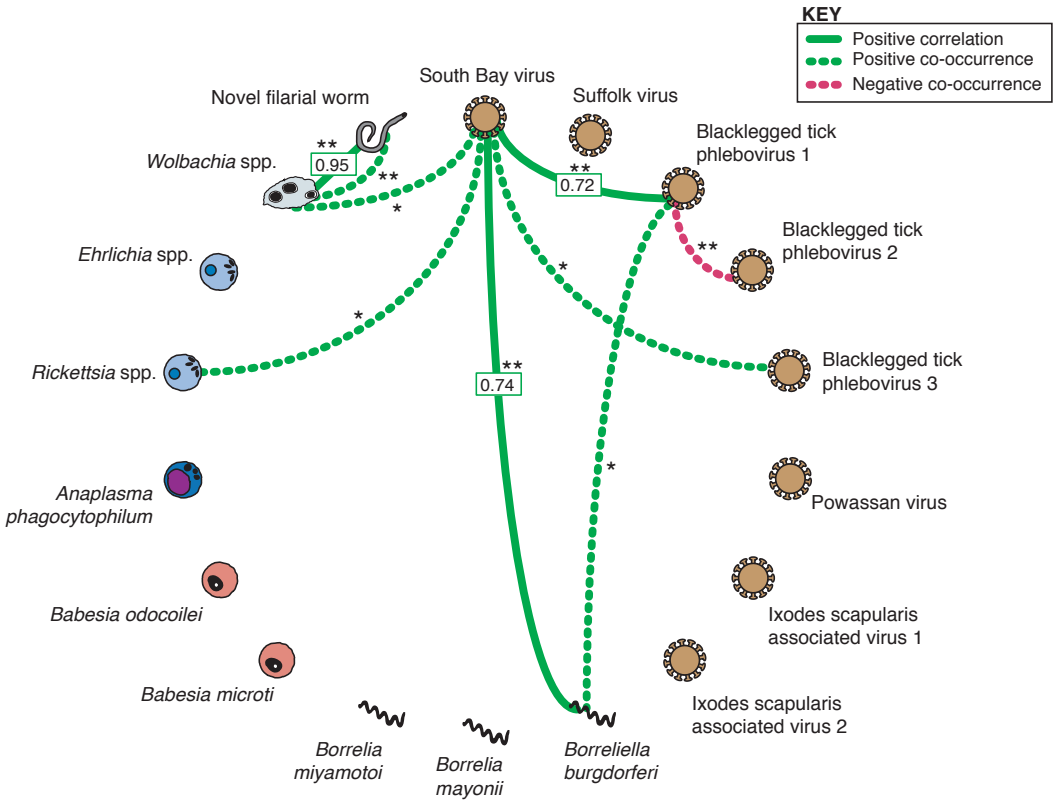

Figure 4.9. Co-occurrence and correlation relationships among tick microbiota. Statistically significant co-occurrence and correlation relationships between microbial constituents within *Ixodes scapularis* ticks. Positive co-occurrence or correlations are depicted in green, while negative relationships are depicted in magenta. Correlation coefficients are shown in boxes. Corrected *p*-values are indicated by: * < 0.05; ** < 0.01.

4.2.4: Discussion

Recent studies have made significant inroads characterizing the microbiome of ticks, which is a rich mixture of viruses, eukaryotes and bacteria [364,366–369,400–402,408,409,429,433]. These have for the most part characterized the bacterial and viral microbiomes separately [364,366,369,400–402,409–411]. Many have also analyzed pools of ticks. To get a holistic and fine-grained picture of the *I. scapularis* microbiome, we performed unbiased metagenomic sequencing on individual ticks from Wisconsin and quantified the levels of microorganisms using RNA abundance as a proxy for taxon abundance.

We validated our NGS results by PCR and also found good concordance between our results and those of previous studies of ticks from the same region. The pathogens we detected had all been previously

observed in Wisconsin, and our measures of prevalence were in the ranges previously reported [390,434–440]. For instance, we detected Powassan virus in 1.8% of ticks, while previous studies have detected this virus in 1.3 and 4.6% of ticks [434,435]. We detected *A. phagocytophilum* in 10.7% of ticks, and previous studies have detected this organism in 2.5–14% of ticks [436,438,440].

Perhaps the most striking finding of our study was the high prevalence and high relative RNA levels of SoBV and BLTPV (**Figure 4.1**). These viruses were both originally identified in *I. scapularis* in New York State, and have been found to be highly prevalent in ticks in several states in the northeastern USA [364,366,369]. Related viruses have also been identified in *I. ricinus* ticks in several European countries [367,368,433]. These studies did not compare levels of these viruses to that of non-viral microbes, and we found that SoBV and BLTPV1 were in fact more abundant and more prevalent than any other members of the microbiota of the ticks we sampled (**Figures 4.2–4.4, Table 4.1**).

We searched for statistically significant associations in order to identify potential functional interactions between members of the tick microbiota, and also found that SoBV and BLTPV1 were involved in the majority of associations with other organisms. These included a positive correlation between SoBV and *B. burgdorferi* (**Figure 4.9**, **Supplemental Figure 4.1**). The mechanism by which these viruses might be promoting the replication of other microbes remains unclear. Most studies of the impact of viruses in other arthropods have focused on their ability to interfere with the replication of other viruses [66,441]. Experimental studies will be required to validate these findings and to uncover their mechanistic underpinnings.

Nevertheless, several associations reassured us that our analyses had the potential to detect meaningful relationships. The sole negative interaction was a negative co-occurrence between BLTPV1 and BLTPV2, which were rarely observed in the same tick (**Figures 4.2 and 4.3**, **Figure 4.9**). We speculated that this is an instance of superinfection exclusion between relatively closely related viruses (their L segments share ~70% pairwise nucleotide identity). Superinfection exclusion has been documented in other viruses [442–446], including bunyaviruses infecting *Aedes* mosquitoes [447]. We also observed both a positive co-occurrence and a positive correlation between the new filarial worm and *Wolbachia* (Figure 4.9,

Supplemental Figure 4.1). *Wolbachia* has been detected previously in *Ixodes* ticks, including a case where the *Wolbachia* was traced to an infection by an infected endoparasitoid wasp [448]. It is possible that some of the *Wolbachia* sequences we detected were from infections of the tick or some other organisms. However, *Wolbachia* are well-characterized endosymbionts of nematodes [449], and we interpreted the positive co-occurrence and correlation relationships to mean that these were sequences from *Wolbachia* that were infecting worms that were infecting the ticks. As is the case for all hypotheses generated by genomic approaches, these interpretations require experimental validation. Nevertheless, this is an example where shotgun metagenomics provides a richer picture of microbial diversity than would, for example, 16 S sequencing alone [450].

Finally, as has been noted, SoBV, BLTPV, and their relatives have characteristics of mutualistic symbionts [366,451,452]. First, these viruses are highly prevalent in *Ixodes* populations on multiple continents [364,366–369]. Mutualistic symbionts often manipulate their host's replication or fitness to favor their own replication, which has the effect of increasing their prevalence in the population [452–454]. Second, these viruses have lost their M genome segments, and thus appear to lack a glycoprotein, a typical prerequisite for extracellular enveloped virus infectivity. This has occurred independently in the two lineages, and such genomic contraction has been commonly observed for bacterial endosymbionts during their transition from free-living organisms to obligate heritable symbionts [455]. Lastly, these viruses can be transmitted vertically [366]. Whether these viruses are indeed genuine mutualists remains to be validated experimentally. In fact, apart from their genome sequences, phylogenetic placement, and geographical range, little is known about these viruses. A more in-depth characterization of their biology and biological impact, and that of the tick virome in general, is clearly warranted.

4.3: Follow-up Analyses on Virome Constituents in Individual *Ixodes scapularis* Ticks Collected in New York

4.3.1: Introduction

In our initial exploration of these viral constituents of *I. scapularis* ticks, we evaluated a population of ticks collected from a single location (Spooner, WI, USA). To further understand how these relationships may be conserved across diverse *I. scapularis* tick populations, additional ticks were collected from a field expedition in the areas surrounding Albany, New York, USA. An additional 54 individual ticks were screened for South Bay Virus (SoBV), blacklegged tick phlebovirus-1 (BLTPV-1), and *Borrelia burgdorferi*. We focused on these constituents specifically because of their positive correlative and co-occurrence patterns previously observed, and because *B. burgdorferi* is the most important tick-borne pathogen in the USA (**Figure 4.9**). In an effort to understand the biology of these viral constituents, we additionally attempted to isolate SoBV through cell culture using both tick and mammalian cell lines.

4.3.2: Materials and Methods

4.3.2.1: Tick Collection and Sample Preparation

Adult *I. scapularis* ticks were collected near Saratoga Springs, New York, USA by dragging in May 2019 (**Figure 4.10**). Adult ticks were transported to the laboratory and kept alive by rearing at 4 °C in a dessicator. This temperature specifically slows down metabolism of ticks for extended lifespan, while the dessicator provided sufficient humidity.

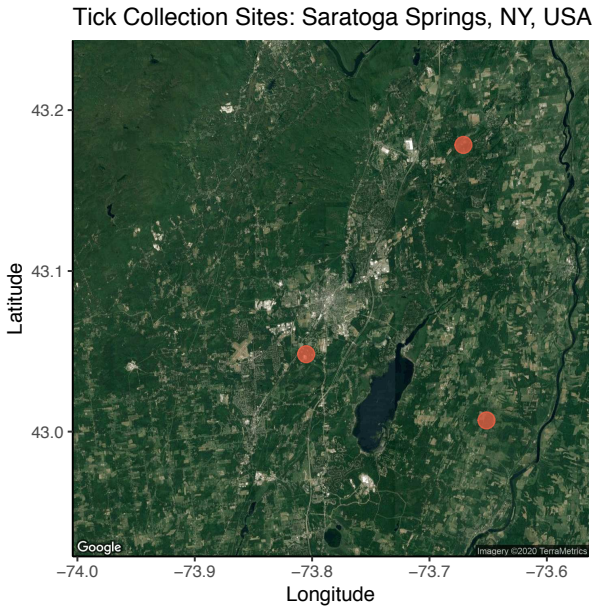

Figure 4.10. Field site locations for tick collection. Ticks were collected at several locations surrounding the Saratoga Springs, NY, USA region via dragging. Collection locations are highlighted by red dots on the map.

Previous species identification performed in the field was verified before sample processing. Individual ticks were surface sterilized by rinsing with 70% ethanol, then added to 200 µL mosquito diluent (20% FBS, 1× PBS, 1× Penicillin/Streptomycin) along with a sterile BB bead, and homogenized at 30 Hz for 3 min. 100 µL of this homogenate was used for RNA extraction and downstream qRT-PCR. Remaining tick homogenates were stored in this preservation medium at -80 °C for future virus isolation attempts. 41 female and 13 male ticks were analyzed.

4.3.2.2: RNA Extraction and qRT-PCR

100 µL of the homogenized tick was subjected to total RNA extraction. RNA was extracted using a bead-based method as previously described [2] (see also: Section 2.2.4). cDNA was synthesized by adding 5.5 µL of RNA to 200 pmol of a random 15-mer oligonucleotide and incubated for 5 min at 65 °C, then set on ice for 1 min. A RT mixture containing the following was added (10 µL reaction volume): 1× SuperScript III (SSIII) FS reaction buffer (Invitrogen), 5 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleotide triphosphates (dNTPs) (NEB), and 100 U SSIII reverse transcriptase enzyme (Invitrogen), then incubated

at 42 °C for 30 min, 50 °C for 30 min, then at 70°C for 15 min. 90 µL of nuclease-free H_2O was added to dilute the cDNA to a final volume of 100 µL.

Following cDNA synthesis or DNA extraction, qPCR reactions were set-up using Luna qPCR Master Mix (NEB) following the manufacturer's protocol. The qPCR reaction was performed on LightCycler 480 (Roche) under the following protocol: 95°C for 3 min, 40 cycles of 95°C for 10s, then 60°C for 45s, and then followed by a melting curve analysis of 95°C for 5s, 65°C for 1 min, and an acquisition starting from 97°C with a ramp rate of 0.11 °C/s and 5 acquisitions per degree. The following primers were used: South Bay virus: 5′- AAGCCAAGAGCAAACCTGACC-3′, 5′- CCTGTCGCATTTCCCTTTTCCT-3′; blacklegged tick phlebovirus-1 virus: 5′- CTTGGAGCCACAACCTCACT-3′, 5′- GTTTTCTTGACCGCCAGGTA-3′; fliD (*B. burgdorferi* primer): 5′- TGGTGACAGAGTGTATGATAATGGAA-3′, 5′- ACTCCTCCGGAAGCCACAA-3′; *Ixodes scapularis* tick Actin: 5′- GCCCTGGACTCCGAGCAG-3′, 5′- CCGTCGGGAAGCTCGTAGG-3′.

4.3.2.3: Cell Culture and Attempted Isolation of Viruses

Two cell lines were reared for attempted isolation. The first was the Vero African green monkey kidney epithelial cell line. These cells were cultured in Dulbecco's Modified Eagle Media (DMEM; Millipore Sigma) with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin at 37°C and 5% CO_2. The second cell line was an embryo-derived tick cell line, ISE6 (ATCC). ISE6 cells were reared in cL15B300 media, made as previously described [456], and grown at 34°C.

Tick homogenates used for virus isolation were selected following qRT-PCR screening and were identified as positive for SoBV. Two homogenates were used for ISE6 cells, and one homogenate was used for Vero cells (total of 3 homogenates used). Homogenates were filtered (0.22 µm filter) to remove any eukaryotic or bacterial microorganisms. Homogenates were added to cells in a 6-well plate and incubated overnight for ~12 hours. Following incubation, media was collected and refreshed. For Vero cells, 600 µL of the supernatant was collected at 7, 10, 15, and 21 days post-inoculation and media was completely refreshed at 7 and 15 days. Vero cells were split at 15 days post-inoculation. For ISE6 cells, 600 µL of the

supernatant was collected at 9, 12, 17, and 23 days post-inoculation, and media was completely refreshed at 9 and 17 days. ISE6 cells were not split during the entire duration of the experiment. On the last day supernatant was collected, cells were collected as well. All sampled supernatants and cells were stored at -80°C until RNA extraction occurred. RNA was extracted and screened via qRT-PCR as described above.

4.3.2.4: Statistical Analysis of Microbial Relationships

Statistical analyses were performed as previously described (see section 4.2.2.6). Co-occurrence relationships were measured using the cooccur R package version 1.3 and the function cooccur [425]. Correlation measurements were performed using the psych R package version 1.9.12.31 and the function corr.test with a Pearson method and Bonferroni adjustment [426]. Correlations were only analyzed for ticks that were co-infected with both microbes being analyzed, and Pearson coefficients were only considered significant if the adjusted *p*-value was less than 0.05.

4.3.3: Results

4.3.3.1: Statistical associations between microbial constituents are population dependent.

Of the 54 ticks screened, 35 were positive for SoBV, 45 were positive for BLTPV-1, and 34 were positive for *B. burgdorferi*. Previous analyses showed positive correlative interactions existed between SoBV and BLTPV-1 and SoBV and *B. burgdorferi* (**Figure 4.9**). These relationships were further examined within the 54 ticks from NY. Correlative testing was performed using the relative microbial RNA levels (relative to the housekeeping gene Actin). Only ticks that were co-infected were used in this analysis. No correlations between SoBV and BLTPV-1 (Pearson coefficient = 0.024, p = 0.9007, **Figure 4.11**) or SoBV and *B. burgdorferi* (Pearson coefficient = -0.071, p = 0.7421, **Figure 4.11**) were found. Previously, a positive cooccurrence relationship between BLPTV-1 and *B. burgdorferi* was noted (**Figure 4.9**). When a cooccurrence analysis was performed within these NY ticks, no relationship was identified between BLPTV-1 and *B. burgdorferi* (28 expected cooccurrences, 27 observed) or any other combination of

microbes (SoBV and BLTPV-1, 29 expected cooccurrences, 29 observed; SoBV and *B. burgdorferi*, 22 expected cooccurrences, 24 observed).

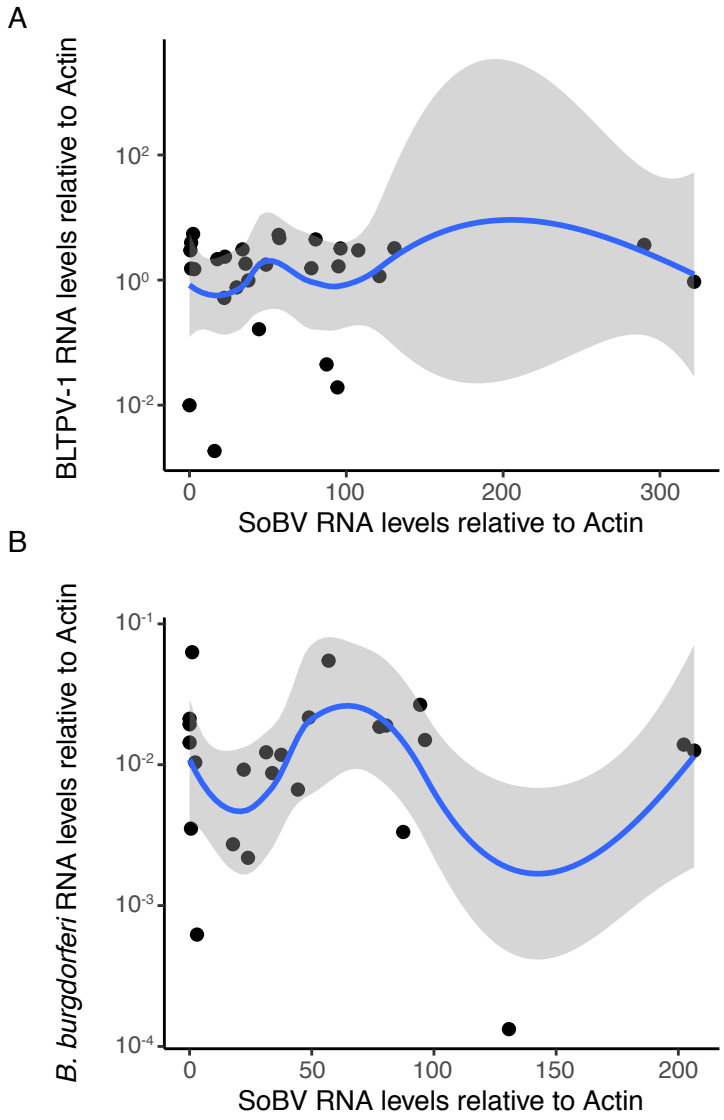

Figure 4.11. No correlations seen within multiple microbial pairs by abundance. Scatterplots of microbial loads relative to the housekeeping gene Actin. Plots of (A) South Bay virus (SoBV) vs blacklegged tick phlebovirus-1 (BLTPV-1) and (B) SoBV vs Borrelia burgdorferi. Lines of best fit using the loess model are depicted. No significant correlative relationships were observed.

4.3.3.2: Unsuccessful isolation of tick-associated bunyaviruses via cell culture.

A critical step for further biological characterization of viruses is their isolation. Attempted isolation of SoBV occurred by inoculating a mammalian cell line (Vero cells) and a tick-derived cell line

(ISE6) with filtered homogenates from individual ticks. Harvesting of cells and supernatant at various time points was performed to measure changes in SoBV RNA levels. These individual homogenates had tested positive for SoBV via qRT-PCR. SoBV RNA levels in cells and supernatant decreased to undetectable levels and did not rise again above detectable levels steadily over 21-23 days for both cell lines (**Table 4.3**). Attempted isolation of BLTPV-1 using ISE6 cells also failed, following the same pattern of decreasing RNA levels to undetectable levels as seen with the SoBV isolation attempt (data not shown).

Table 4.3. Attempted isolation of South Bay virus on tick and mammalian cell lines. Various SoBV-positive homogenates derived from individual ticks were inoculated on either ISE6 cells (tick) or Vero (mammalian) cells. Supernatant and cells were collected at various time points and screened for virus presence via qRT-PCR. nd: not detectable.

Cell Line	Inoculum #	Day	Sample Type	C_t Value
ISE6	Homogenate 1 Tick 4	0	Inoculum	23.93
		1	Supernatant	28.43
		9	Supernatant	31.39
		12	Supernatant	nd
		17	Supernatant	31.70
		23	Supernatant	nd
		23	Cells	nd
ISE6	Homogenate 2 Tick 18	0	Inoculum	21.33
		1	Supernatant	27.89
		9	Supernatant	31.75
		12	Supernatant	nd
		17	Supernatant	nd
		23	Supernatant	nd
		23	Cells	nd
Vero	Homogenate 3 Tick 7	0	Inoculum	23.62
		1	Supernatant	27.55
		7	Supernatant	30.48
		10	Supernatant	nd
		15	Supernatant	nd
		15	Cells	nd
		23	Supernatant	nd
		23	Cells	nd

4.3.4: Discussion

In an effort to identify how interactions between microbial constituents within *I. scapularis* ticks are conserved across populations, we collected additional ticks from the region surrounding Saratoga Springs, NY, USA. In 54 individual ticks, no correlative or cooccurrence relationships were identified between 3 microbes of interest: South Bay virus, blacklegged tick phlebovirus-1, and *Borrelia burgdorferi* (**Figure 4.11**). Viruses are nested within a niche of environmental and ecological interactions [381]. How tick behaviors and resources vary between geographic location may influence these microbial interactions.

Behaviors, ecology, and resources have already been suggested as important factors for the bacterial tick microbiome composition [457,458]. In this follow-up exploration, only one additional population of ticks was evaluated, but another study has also indicated that these associations were not seen in populations of ticks they screened (New York and Connecticut, USA) [459]. Given the variance of environments, and expansion of ticks northward due to climate change [460], it may still be of value in identifying *I. scapularis* populations with these relationships. By doing so, additional factors (e.g. ecologies, genetics) that may attribute to these possible functional interactions can be identified. Additional screening of ticks from Spooner, WI, USA is of interest to understand how these relationships are either maintained or change through time and seasons.

In an attempt to isolate a tick-associated virus, SoBV, we were unsuccessful using several different cell lines (**Table 4.3**). This may not be surprising as these tick-associated bunyaviruses are missing the M segment found in a classic bunyavirus genome. This segment encodes the glycoprotein, which is typically necessary for extracellular enveloped virus infectivity. For continued study of these viruses, several options may be feasible. First, a reverse genetics system would allow for the transfection of RNA into cells bypassing the need for the glycoprotein for *in vitro* culturing. Whether RNA alone is infectious in an *in vivo* system would need to be explored. Another alternative is to use a similar iterative breeding scheme performed for the isolation of galbut virus ([163] and see Chapter 2). SoBV is suggested to be vertically transmitted making this approach a potential option [366].

CHAPTER 5: CONCLUDING REMARKS

Throughout each chapter, in depth discussions on the major findings of my research have been presented. In these concluding remarks, I will address how my dissertation findings have contributed to the growing field of virome studies and future directions of my findings.

5.1: Biological Understanding of Partitiviruses; Common Constituents of Arthropod Viromes

Metagenomic sequencing has revealed a depth and diversity of viruses that is almost incomprehensible. Viruses are being discovered at an unprecedented rate, and virus families are being reshaped. *Partitiviridae* is one of these families that has undergone an expansion with partitivirus-like sequences identified within arthropods samples, including important insect model organisms (i.e. *Drosophila melanogaster*) and disease vectors (*Ae. aegypti, An. gambiae, Culex spp.*). But it was not clear that these virus-like sequences represented true viruses. For example, the uncertainty of galbut virus is reflected within its name: the Lithuanian word galbūt means “maybe”. Furthermore, it was unclear whether these partitivirus-like sequences even infected arthropods, as until recently, partitiviruses were only known to infect plants, fungi, and protozoa [76]. Despite the widespread occurrence of partitiviruses in these important insects, nothing had been published on the characterization of these viruses or their biological impact on their hosts.

My thesis research has aimed to go beyond where metagenomic-based studies often stop. Rather than simply characterizing viral sequences, I sought to characterize the biology of these viruses and their interactions with the host. I have focused on partitiviruses, as they appear to be common constituents of arthropod viromes. The subsequent investigation into these partitiviruses has drastically improved our knowledge on this relatively new field and sets the stage for valuable insights for partitiviruses as models of persistent infection and potential biocontrol use. The following are key contributions highlighted from my thesis research.

Partitiviruses have now been established as *bona-fide* arthropod-infecting viruses. Using galbut virus, we identified viral proteins and RNA across *D. melanogaster* tissues strongly supporting that *D. melanogaster* was the true host. Subsequent microinjection of flies with a galbut virus positive homogenate showed adult flies can support active replication of galbut virus, that it can be transmitted to the offspring, and that infection can be maintained for multiple generations. Establishing an infection system is essential for understanding the biology of virus-host interactions.

Successful partitivirus maintenance within arthropod populations appears to be supported by their efficient vertical transmission. Both galbut virus and verdadero virus were vertically transmitted by both parents at ~100% efficiency. Vera virus appears to not be as efficiently transmitted as we were able to "purge" detectable levels of vera virus from *D. melanogaster* in our iterative breeding scheme. However, transmission efficiency may be influenced by co-infection and additional host factors. Further investigation into partitivirus co-infection is warranted.

Galbut virus, when infecting *D. melanogaster*, appears to have minimal impacts on host fitness. This is supportive of the canonical ideology that partitiviruses have little to no impacts on host fitness, hence being called cryptic viruses. Observed fitness phenotypes associated with galbut virus infection were altered by sex and strain, highlighting the importance of these factors in infection outcomes. Further investigation into additional lines of flies to identify conserved phenotypes across strains is warranted. Additionally, galbut virus can infect other *Drosophila* species, and how fitness changes across the hosts should be investigated. How verdadero alters the fitness of *Aedes aegypti* is also justified and can set precedence for its utility as a biocontrol agent.

5.2: The Role of the Virome in Biocontrol Measures

Verdadero virus is not an isolated case of a partitivirus identified in mosquitoes. Partitiviruses are being discovered at a high rate in various mosquitoes, including in other important mosquito vectors [75,78,161,162]. In RNA metagenomic sequencing of chikungunya virus (CHIKV) infected *Ae. aegypti* mosquitoes, verdadero virus RNA was found in the saliva alongside CHIKV RNA. Furthermore, the

RNA levels of verdadero virus were much higher than CHIKV (**Figure 5.1**). Can mosquito-infecting partitiviruses (e.g. verdadero virus), be used as biocontrol agents? Verdadero virus, for example, already shows promising features such as the highly efficient, biparental vertical transmission. Two subsets of experiments need to be performed to understand the future use of partitiviruses as biocontrol agents. First and foremost, experiments testing the vector competence of partitivirus-infected and uninfected mosquitoes needs to be done. If these partitiviruses contain an innate ability to block or decrease arbovirus transmission, like several insect-specific viruses (see Section 4.1), this could be highly promising for biocontrol use. Second, experiments testing fitness of partitivirus-infected mosquitoes, similar to that performed for galbut virus (see Chapter 3), need to be performed. Galbut virus appears to have minimal impacts on fitness in *D. melanogaster*, but in armyworm moths, partitiviruses were quite detrimental [184]. Useful biocontrol agents minimally or positively affect host fitness, otherwise despite high vertical transmission rates, they likely will never reach fixation within a population or possibly will be purged completely.

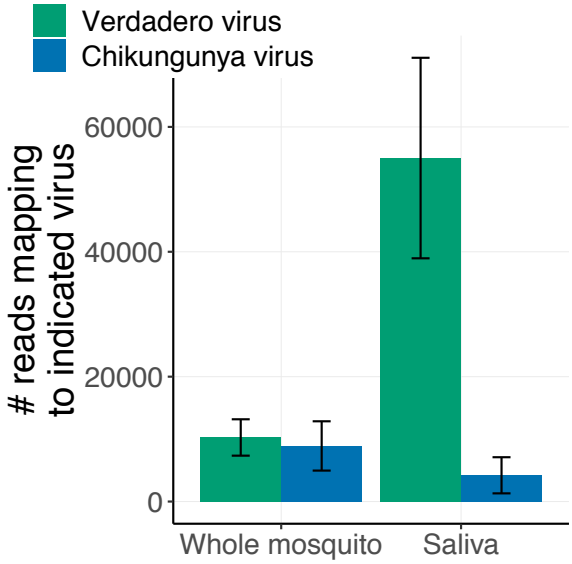

Figure 5.1. Verdadero virus RNA levels are enriched in saliva compared to chikungunya virus in *Aedes aegypti* mosquitoes. *Ae. aegypti* mosquitoes were infected with CHIKV and their entire bodies and saliva were screened for CHIKV. Verdadero virus, originally identified in this strain of *Ae. aegypti*, was also present in these samples. The number of mapping reads to each virus in replicate pools of 15 CHIKV-infected mosquitoes or saliva is depicted.

Major constituents of the *Ixodes scapularis* tick microbiome are South Bay virus (SoBV) and blacklegged tick phlebovirus-1 (BLTPV-1). In one population of ticks, these viruses had possible functional interactions with pathogens. Of particular interest were the interactions with *Borrelia burgdorferi*, the causative agent of Lyme disease. Although these interactions were not supported in a second population of ticks, important insights were gained. First, the interactions of SoBV and BLTPV-1 initially described showed potential enhancement, not suppression, of *B. burgdorferi*. This raises an important aspect that not all virome constituents will necessarily be useful in reducing vector competence. Furthermore, because these interactions differed in populations, this study raises a key concern in using microbes as biocontrol agents. Careful consideration must be taken to ensure that manipulated interactions caused by microbes will be conserved across various ecological environments and host genetics.

5.3: Looking Forward: Important Questions to Pursue in the Field of Arthropod-infecting Partitiviruses

Although the virome is comprised of many different interactions, the goal of this dissertation was start unraveling the biology and impact of key players. As such, I have focused predominately on arthropod-infecting partitiviruses. As a new field of virology research, there are a plethora of questions and directions to pursue in this research. In closing, I layout a few questions I find relevant for the advancement of this field and the use of partitiviruses as models in virus-host interactions:

1. *What is the major driving force keeping galbut virus at ~60% prevalence in wild D. melanogaster populations?* Despite having highly efficient vertical transmission (Chapter 2) and having minimal impacts on host fitness (Chapter 3), galbut virus maintains a worldwide prevalence of ~60%. A hypothesis is that there are innate antiviral alleles within these populations that function against galbut virus [140,206,207]. In this dissertation, only 3 of >200 lines from the *Drosophila* Genetic Reference Panel (DGRP) were examined. A genome

wide association study (GWAS) using a larger panel of flies from the DGRP could help decipher the answer. Using galbut virus susceptibility as the mapping phenotype, a GWAS can identify genetic loci associated with this resistance at a high power [140,462]. A secondary hypothesis is that other microbial interactions could be at play, leading well into a second important question to answer.

2. *How does Wolbachia affect galbut virus susceptibility of flies?* In Chapter 3, galbut virus was shown to change sensitivities to pathogens in a strain and sex specific manner in *D. melanogaster*. Galbut virus did not significantly change core components of the microbiome. A microbe of relevance is *Wolbachia*, which as stated multiple times throughout the dissertation, has known antiviral effects [211]. Understanding how *Wolbachia* interacts with partitiviruses is of high interest. DGRP 360 flies, known to be *Wolbachia*-positive, were more refractory to galbut virus infection, while the susceptible DGRP 399 and 517 lines are *Wolbachia*-negative (see Chapter 2). Contrary, *Wolbachia* was not shown influence the virome of wild *D. melanogaster* [77]. A relatively easy experiment to decipher this would be to introduce *Wolbachia* into the DGRP 399 and 517 lines, removing it from DGRP 360 flies, then challenging all lines again with galbut virus to see how susceptibilities change.
3. *How do responses to galbut virus change across host species?* Here, galbut virus was measured in the model organism *D. melanogaster*, but other species of *Drosophila* are also infected by galbut virus [69,172]. This infers that galbut virus is a generalist. Galbut virus may be an optimal model virus to better understand conserved and distinct innate antiviral immune responses across *Drosophila* species. *Drosophila* C virus has been a useful model in elucidating much of the antiviral immune response of *D. melanogaster* [205], but it has a narrow host range [463], restricting the utility of it for understanding broadly conserved *Drosophila* antiviral responses. By infecting a wide range of fly hosts, viral genetic factors may also be identified that allow for such a broad distribution of *Drosophila*; giving evolutionary insights into virus-host interactions. Additional experiments of interest include identifying whether partitiviruses

can cross larger arthropod species barriers (as performed with the moth-infecting partitiviruses [184]). This could lay a foundation for using "foreign" partitiviruses as biocontrol agents, similar to how the *Wolbachia* strain responsible for antiviral effects in *Ae. aegypti* originates from *D. melanogaster* [343,346].

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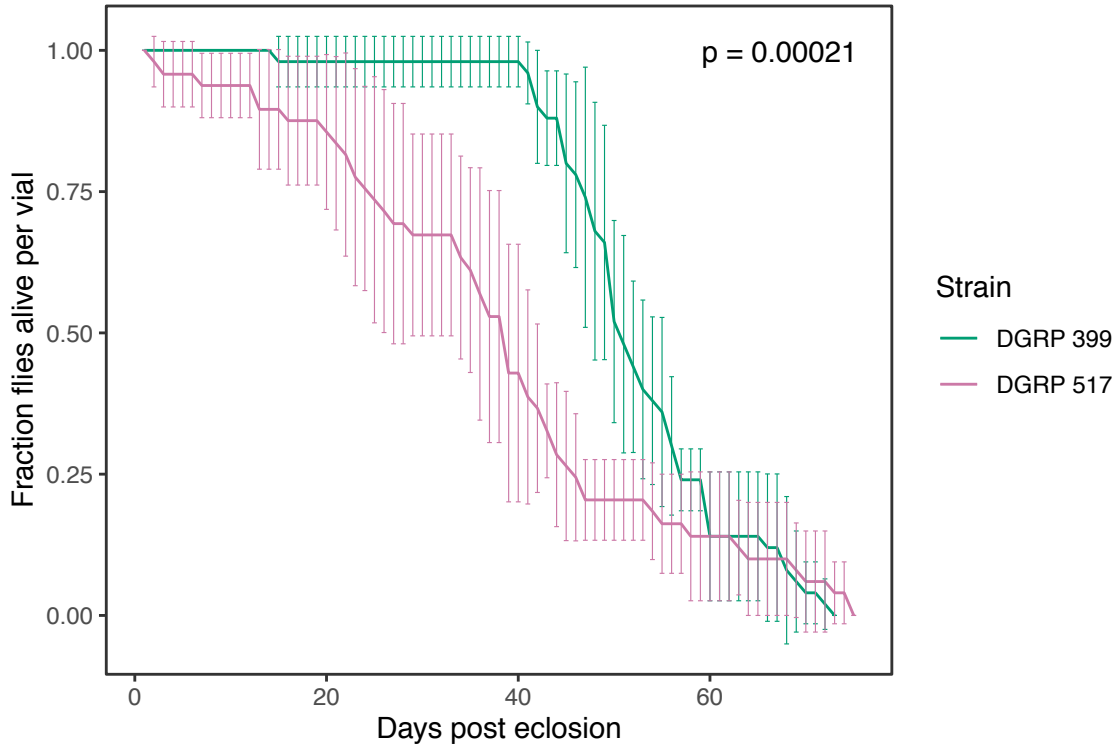
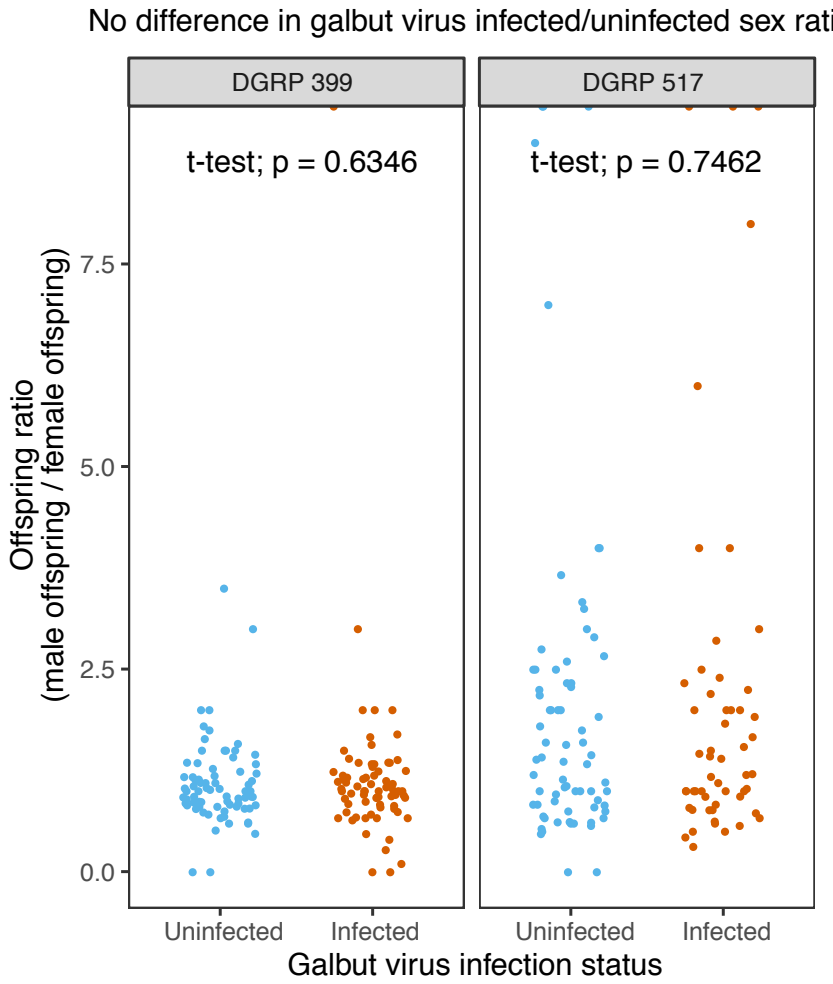
APPENDIX

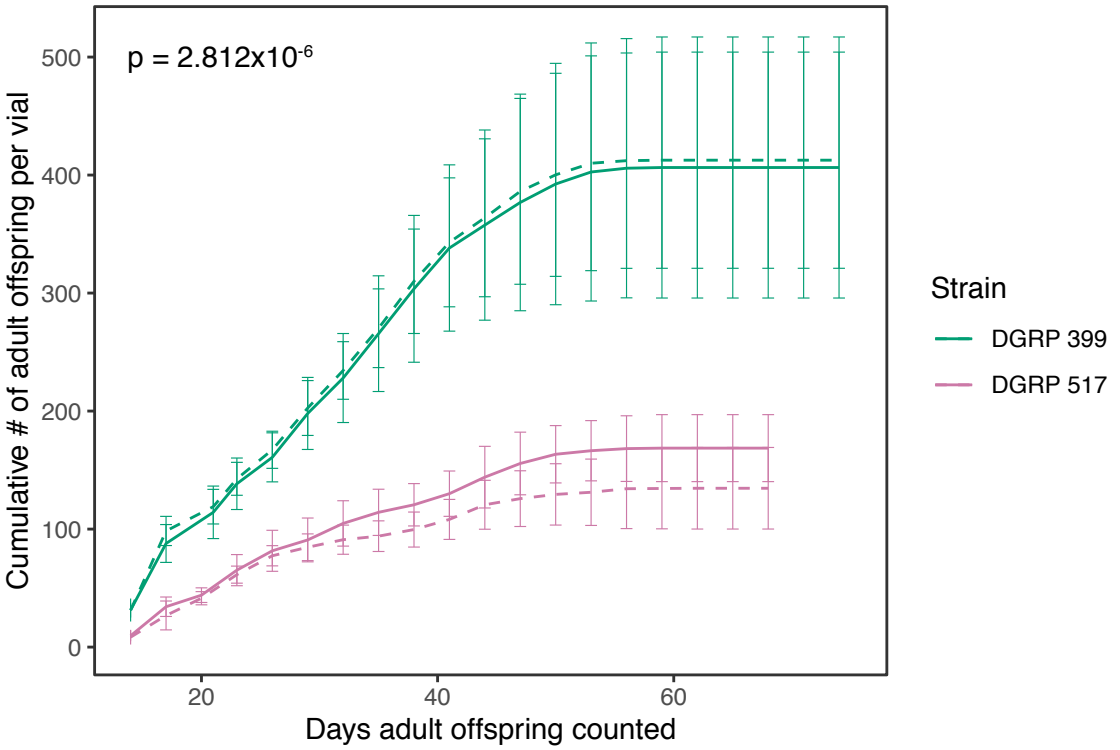
Chapter 3 Supplemental Figures and Table



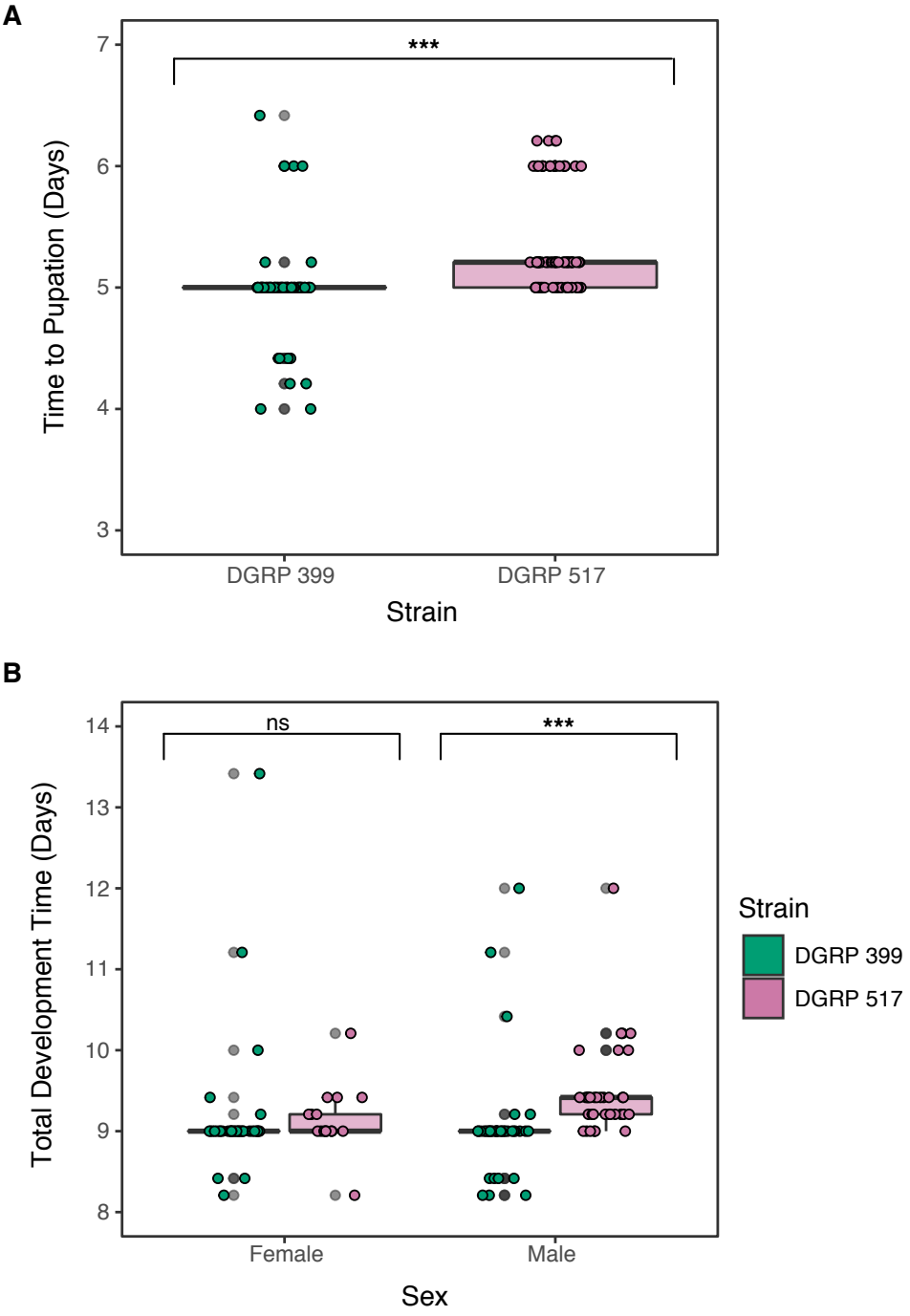
Supplemental Figure 3.1 Lifespan of DGRP strains (uninfected flies). Galbut virus uninfected groups were housed in batches of 10 flies and monitored daily for survival. DGRP 517 flies have a significantly decreased survival (p=0.00021) compared to DGRP 399 flies. DGRP: *Drosophila* Genetic Reference Panel.



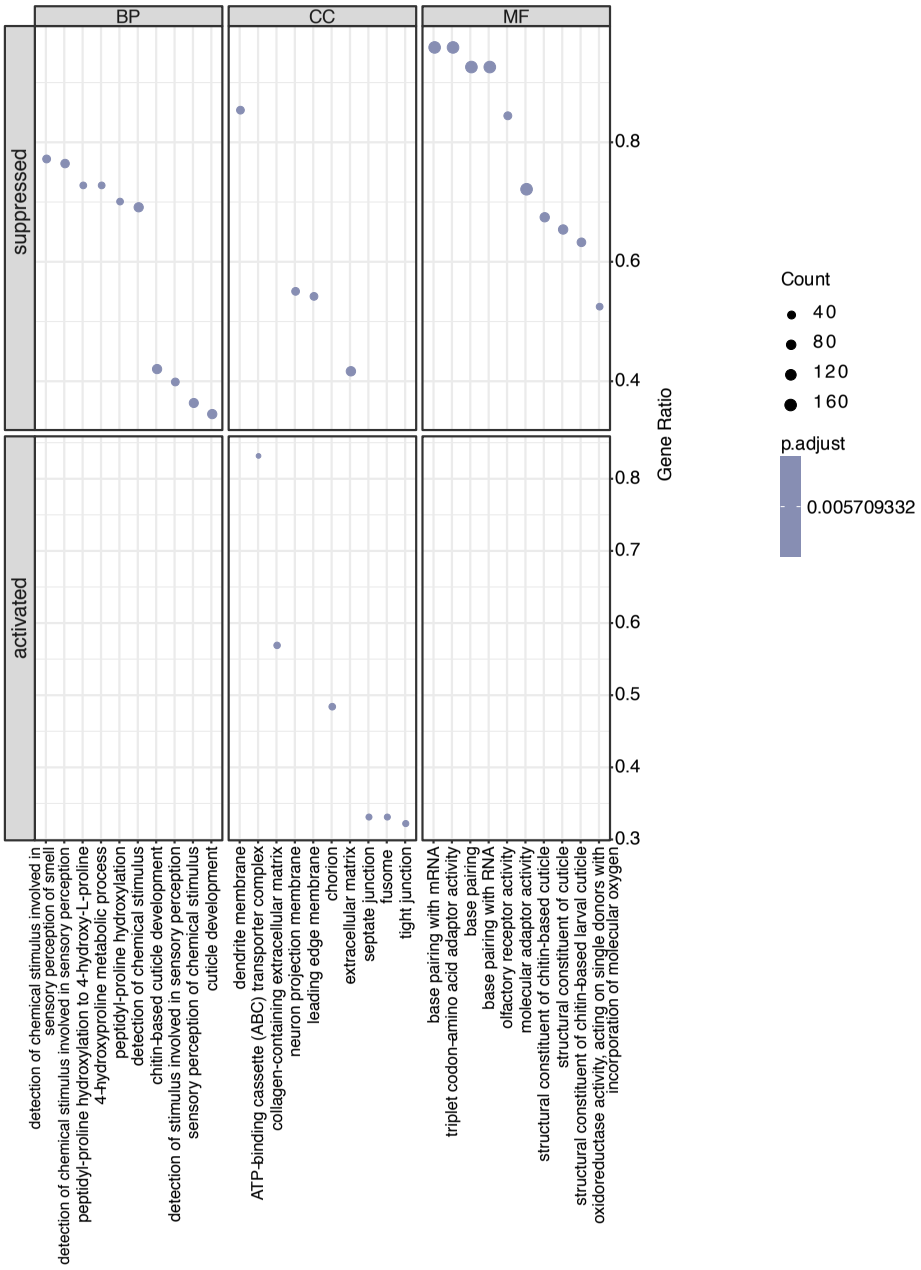
Supplemental Figure 3.2. Galbut virus infection does not influence adult offspring sex ratio. Offspring collected from groups of galbut virus infected or uninfected parents from DGRP 399 and 517 strains every 14 days (see **Fig 2**). Offspring sex ratios from each time point were calculated by dividing total male offspring by total female offspring. No statistical significance was measured in either strain (t-test).



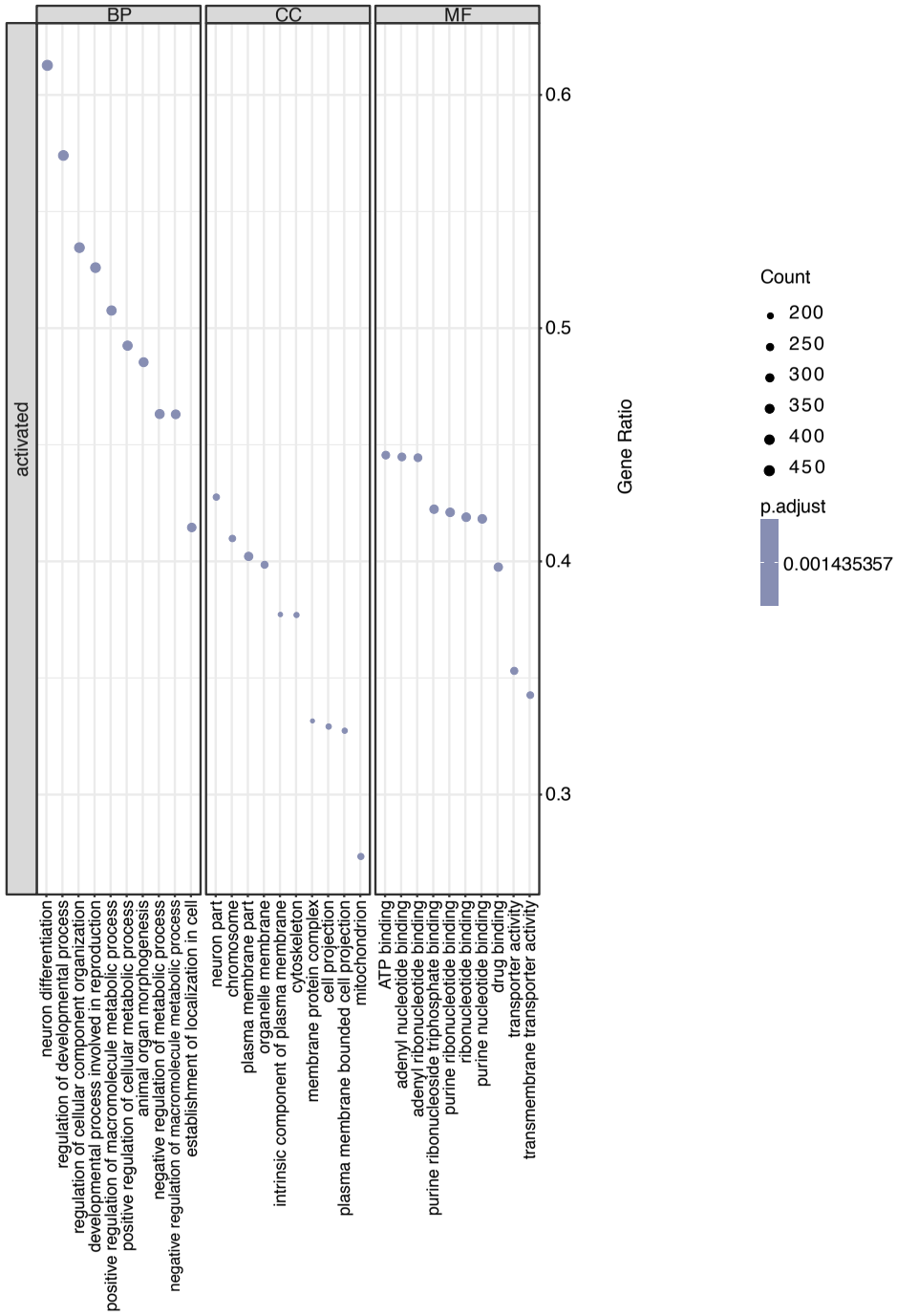
Supplemental Figure 3.3. Drosophila fecundity of two DGRP strains as determined by cumulative adult offspring count. Galbut virus-uninfected flies were housed in batches of 10 flies (same groups used in the lifespan survival analysis; see **Fig 1**) and cumulative number of female and male offspring were calculated. DGRP 517 flies have a significantly decreased total offspring count compared to DGRP 399 flies (t-test; $p=2.812x10^{-6}$) DGRP: *Drosophila* Genetic Reference Panel.



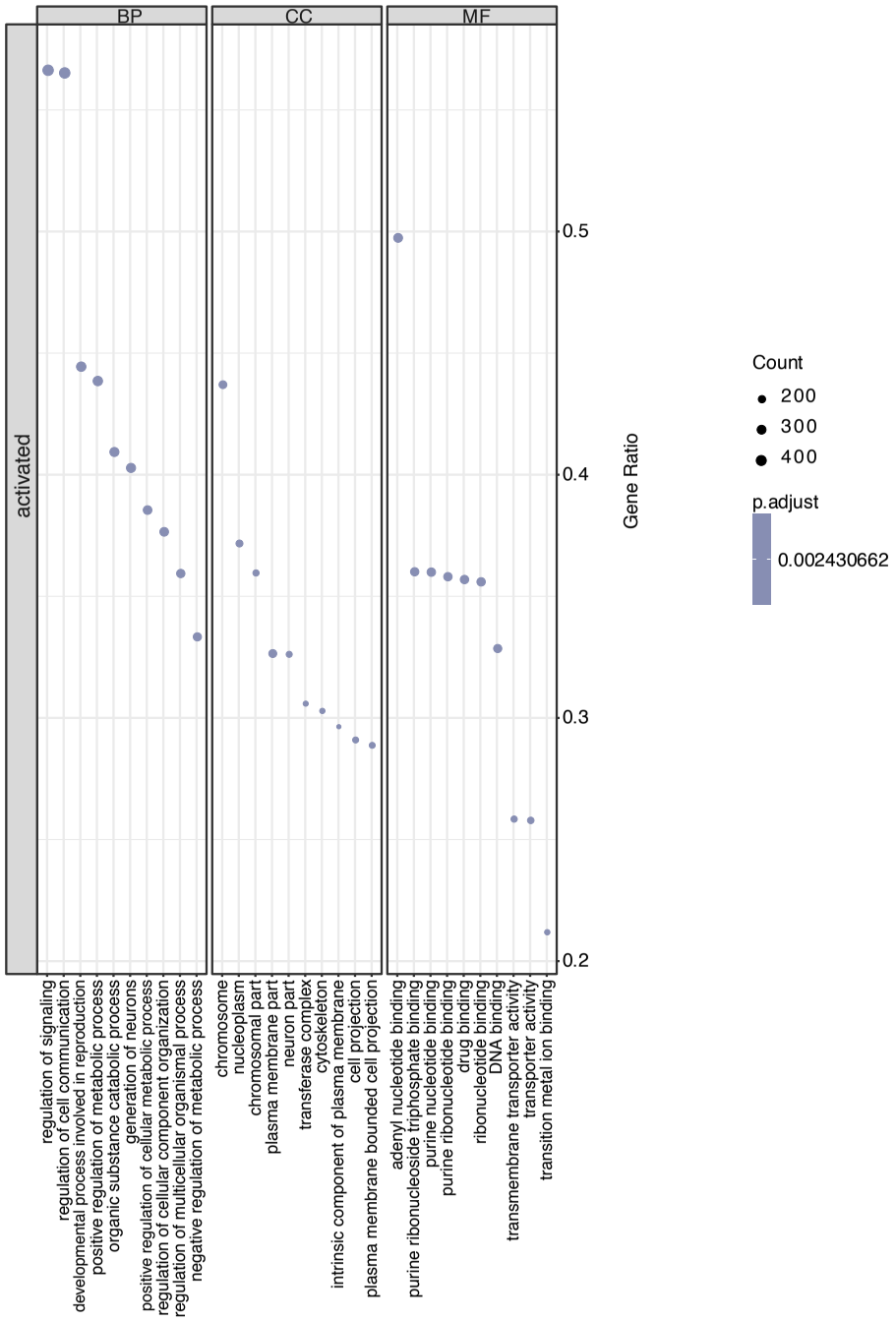
Supplemental Figure 3.4. Developmental speeds are altered by strain and sex. (A) Eggs were collected, reared, and checked daily for until pupation began. Once pupation began, flies were checked approximately every 5 hours (morning, midday, evening). DGRP 517 flies pupated significantly slower than DGRP 399 flies (Wilcoxon, $p=6.6x10^{-10}$) (B) Flies were continually monitored until flies eclosed, and this time was recorded as the total developmental time. Once flies began to eclose, flies were checked approximately every 5 hours (morning, midday, evening). Sex of adult flies was recorded and total development time between infected and uninfected flies was compared by sex. There is no significant difference in total development speeds between DGRP 399 and DGRP 517 female flies. DGRP 517 male flies reached adulthood significantly slower (Wilcoxon, $p=1.13x10^{-13}$). ns: not significant; ***: $p < 0.001$



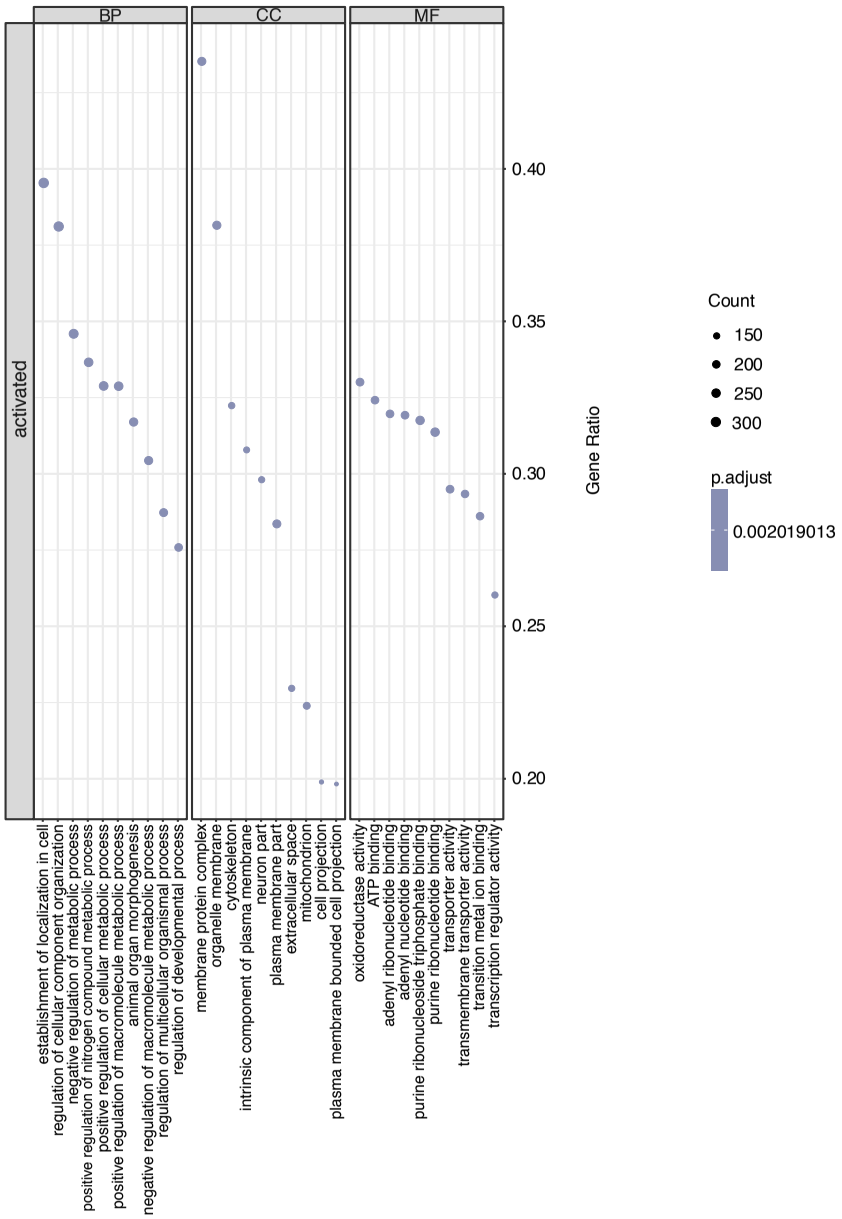
Supplemental Figure 3.5. Dot plot of differentially regulated gene ontology (GO) pathways in infected DGRP 399 female flies. A dot plot representation of the top differentially regulated GO pathways in galbut virus-infected DGRP 399 female flies as determined by gene set enrichment analysis (GSEA) using the R package "clusterProfiler". Top 10 differentially regulated pathways are plotted in each GO category (biological function, BF; cellular component, CC; molecular function, MF). Differentially regulated pathways for these flies were either upregulated (activated) or downregulated (suppressed). Size of dots corresponds with number of differentially regulated genes (DEG; counts) identified in each specified GO pathway. Percentage of DEGs in a given GO pathway (number of DEGs divided by total number of genes listed under the specified GO pathway) is plotted as gene ratio.



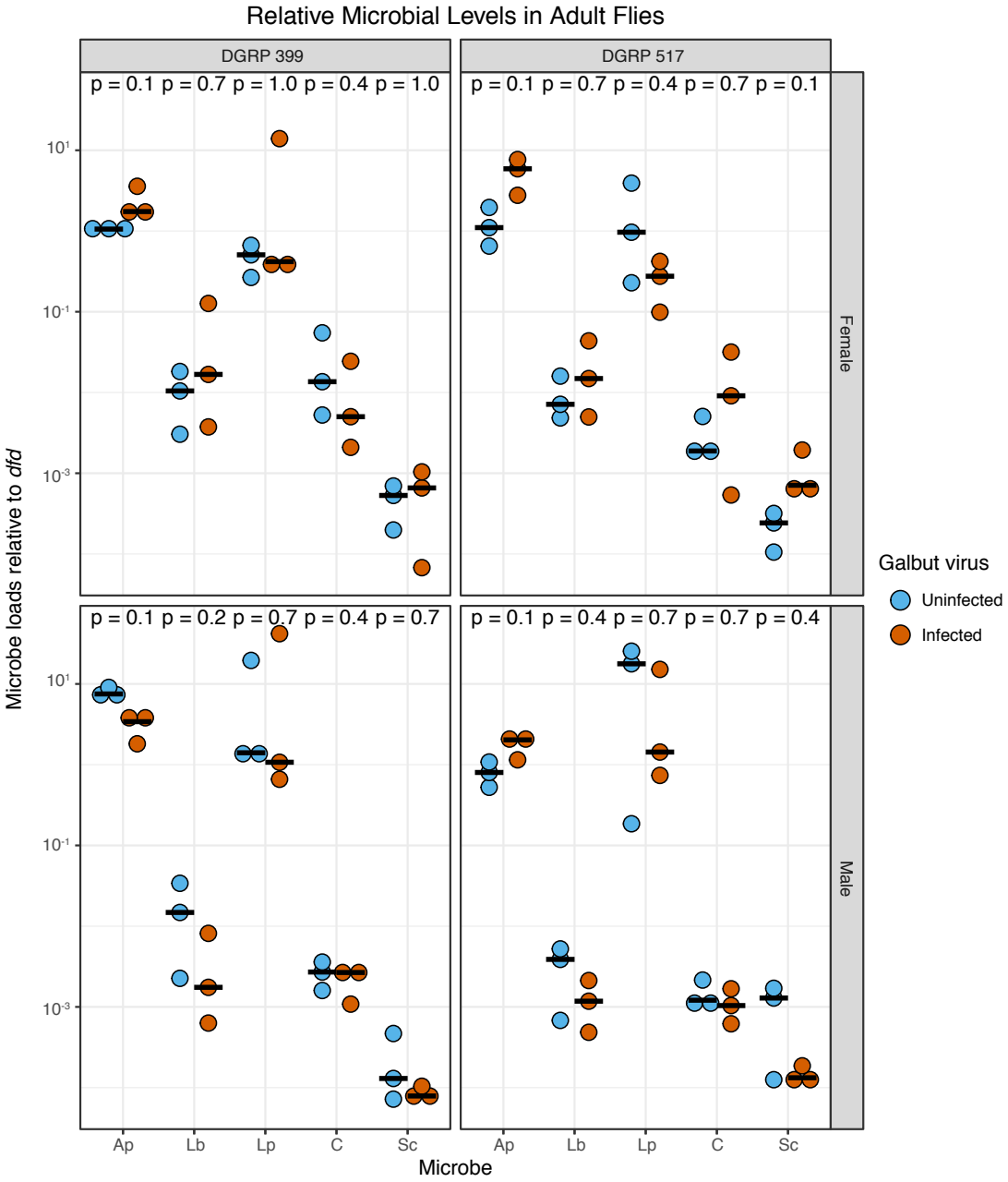
Supplemental Figure 3.6. Dot plot of differentially regulated gene ontology (GO) pathways in infected DGRP 399 male flies. A dot plot representation of the top differentially regulated GO pathways in galbut virus-infected DGRP 399 male flies as determined by gene set enrichment analysis (GSEA) using the R package "clusterProfiler". Top 10 differentially regulated pathways are plotted in each GO category (biological function, BF; cellular component, CC; molecular function, MF). All top differentially regulated pathways for these flies were upregulated (activated). Size of dots corresponds with number of differentially regulated genes (DEG; counts) identified in each specified GO pathway. Percentage of DEGs in a given GO pathway (number of DEGs divided by total number of genes listed the specified GO pathway) is plotted as gene ratio.



Supplemental Figure 3.7. Dot plot of differentially regulated gene ontology (GO) pathways in infected DGRP 517 female flies. A dot plot representation of the top differentially regulated GO pathways in galbut virus-infected DGRP 517 female flies as determined by gene set enrichment analysis (GSEA) using the R package "clusterProfiler". Top 10 differentially regulated pathways are plotted in each GO category (biological function, BF; cellular component, CC; molecular function, MF). All top differentially regulated pathways for these flies were upregulated (activated). Size of dots corresponds with number of differentially regulated genes (DEG; counts) identified in each specified GO pathway. Percentage of DEGs in a given GO pathway (number of DEGs divided by total number of genes listed under the specified GO pathway) is plotted as gene ratio.



Supplemental Figure 3.8. Dot plot of differentially regulated gene ontology (GO) pathways in infected DGRP 517 male flies. A dot plot representation of the top differentially regulated GO pathways in galbut virus-infected DGRP 517 male flies as determined by gene set enrichment analysis (GSEA) using the R package "clusterProfiler". Top 10 differentially regulated pathways are plotted in each GO category (biological function, BF; cellular component, CC; molecular function, MF). All top differentially regulated pathways for these flies were upregulated (activated). Size of dots corresponds with number of differentially regulated genes (DEG; counts) identified in each specified GO pathway. Percentage of DEGs in a given GO pathway (number of DEGs divided by total number of genes listed under the specified GO pathway) is plotted as gene ratio.

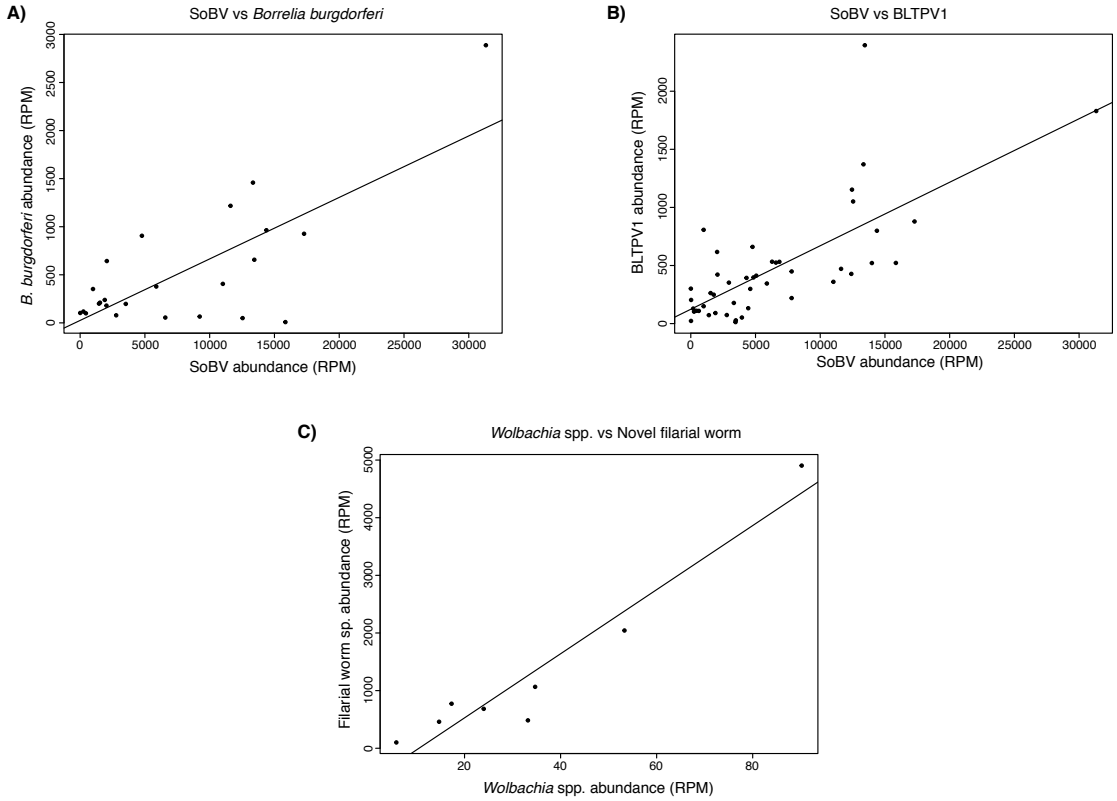


Supplemental Figure 3.9. Galbut virus infection does not alter relative levels of major microbiome constituents in flies. Predominant microbiome constituent levels of DGRP flies in our lab were measured via qPCR. Microbial loads were normalized to the single copy, housekeeping gene *deformed* (*dfd*; $2^{-\Delta Ct}$). Levels were quantified for each experimental group from 3 pools (10 flies per pool). Median values are plotted as crossbars. No significant differences in relative loads between galbut virus infected and uninfected flies were observed. Ap: *Acetobacter persici*; Lb: *Lactobacillus brevis*; Lp: *L. planatarum*; C: *Corynebacterium* spp.; Sc: *Saccharomyces cerevisiae*.

Supplemental Table 3.1: Primers used for detecting galbut virus RNA levels and microbiome constituents.

Product	Forward (5`->3`)	Reverse (5`->3`)	Original Citation
Galbut virus	CCGTGAAGCAAGGAATCAAT	TGCCGATTTTCTGCTCTTTT	NA
RpL32	TGCTAAGCTGTCGCACAAATGG	TGCGCTTGTTCGATCCGTAAC	NA
Acetobacter spp.	TAGCTAACGCGATAAGCACA	ACAGCCTACCCATACAAGCC	AM Early et al: https://doi.org/10.1371/journal.pone.0170332
Lactobacillus brevis	TCAGTTTTGAGGGGCTTACCTCTCT	GGCATCCACCATGCGCCCTT	AM Early et al: https://doi.org/10.1371/journal.pone.0170332
Lactobacillus planatarum	TGCGGCTGGATCACCTCCTTTC	ACTGGTTCGGTTCCAATGGGCC	AM Early et al: https://doi.org/10.1371/journal.pone.0170332
Corynebacterium spp.	AAACGGGTACGCATCACG	GGGTTGATATTCCCGTACCC	NA
Saccharomyces cerevisiae	AGGAGTGCGGTTCTTTG	TACTTACCGAGGCAAGCTACA	H Chang et al: https://doi.org/10.1016/j.mimet.2007.08.013
deformed (*dfd*)	GTAGCGAAGAAACCCACCAA	ACGCTCCACTCACCTCATTC	AM Early et al: https://doi.org/10.1371/journal.pone.0170332

Chapter 4 Supplemental Information



Supplemental Figure 4.1. Positive correlations are seen within multiple microbial pairs by abundance. Scatterplot of RNA levels (mapping reads per million unique reads; RPM) for (a) South Bay virus (SoBV) and Borrelia burgdorferi, (b) SoBV and blacklegged tick phlebovirus 1 (BLTPV1), and (c) Wolbachia spp. and the novel filarial worm specie. Linear regression lines are also plotted.

Supplemental Table 4.1: Primer sequences used for validation of metagenomic sequencing results.

Primer Name	Sequence (5' to 3')	Melting Temp (℃)	Product Size
fliD-Fa	TGGTGACAGAGTGTATGATAATGGAA	60	~100
fliD-Ra	ACTCCTCCGGAAGCCACAA	60	~100
msp2-Fb	ATGGAAGGTAGTGTTGGTTATGGTATT	60	77
msp2-Rb	TTGGTCTTGAAGCGCTCGTA	60	77
18S-F	CGACTACGTCCCTGCCCTTTG	60	77
18S-R	ACGAAGGACGAATCCACGTTTC	60	77
glpQ-F	GACCCAGAAATTGACACAACCACAA	60	126
glpQ-R	TGATTTAAGTTCAGTTAGTGTGAAGTCAGT	60	126
SoBV L-seg For	AAGCCAAGAGCAAACCTGACC	60	276
SoBV L-seg Rev	CCTGTCGCATTTCCCTTTTCCT	60	276
Blacklegged Tick Phlebovirus 1 F	CTTGGAGCCACAACCTCACT	60	152
Blacklegged Tick Phlebovirus 1 R	GTTTTCTTGACCGCCAGGTA	60	152
Blacklegged Tick Phlebovirus 2 F	AGAAGACCCTGCTGAGACCA	60	212
Blacklegged Tick Phlebovirus 2 R	CACTATCCATGGCGTGTTTG	60	212
Blacklegged Tick Phlebovirus 3 F	GCAAAGCCAAACTGAGGAAG	60	202
Blacklegged Tick Phlebovirus 3 R	GCCTTCTTGGCAGTGAACTC	60	202
Powassan Virus F	ACCCTGAATTGGTCAACGAG	60	176
Powassan Virus R	TGCTCCCATTGTCACCAATA	60	176
Suffolk Virus F	CCGGGAACAACTGAAGTCAT	60	182
Suffolk Virus R	AGTGTGTTTGGCACAATGGA	60	182
Ixodes scapularis associated virus 1 F	TGCTTGAGGGAAGTCTCGAT	60	189
Ixodes scapularis associated virus 1 R	TACACTCCACACCGTTCCAA	60	189
I. scap gpdh F	AGCATAGCGAGCGTTGTG	55	161
I. scap gpdh R	TCGGCATTTGTAGAGACTGG	55	161
Onchocerciadea 12S rDNA F	TGACTGACTTTAGATTTTTCTTTGG	56	~180
Onchocerciadea 12S rDNA R	AATTACTTTCTTTTCCAATTTCACA	56	~180