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FACTORS INFLUENCING SUSCEPTIBILITY TO VIRAL DISEASES IN PLANTS AND POLLINATORS AND THE EFFECTS OF PLANT VIRUS INFECTION ON BEE ATTRACTION AND NUTRITIONAL RESOURCES

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ABSTRACT

All types of organisms are susceptible to virus infections. However, many organisms have developed responses to virus infections that allow them to coexist with their parasites. Ultimately, host susceptibility is a result of underlying genetic factors controlling immune responses which lead to different infection outcomes even between closely related hosts. Through genetic bottlenecks and tradeoffs between immunity and traits desirable to humans, artificial selection can have consequences for disease susceptibility. A major goal of this research was to determine how domestication processes can alter host immunity by introducing selection pressures that change host responses and infection outcomes. To address these questions, this research focused on two systems that have long, complex histories of domestication and globalization: the honey bee (*Apis mellifera*) and squash plants in the genus *Cucurbita*. This research leveraged multi-year field experiments to characterize host fitness in natural settings and used molecular techniques to quantify immune responses. The results of this work show virus tolerance—defined as reductions in disease while allowing for pathogen replication,—is common in plant and pollinator systems. Additionally, to provide deeper insight into host responses to virus infection in squash plants, RNA sequencing was performed to identify specific transcriptional responses involved with differences in host immunity.

Because domestication of honey bees may result in strong selection pressures that are different than the selection pressures faced by unmanaged bees in the wild (feral), we hypothesized that feral bees would have higher disease burdens but also higher levels of

immune gene expression. Honey bees from 25 pairs of unmanaged (feral) and managed colonies were collected over two years and assessed for pathogen levels, host immune gene expression, and colony survival. We found that feral bees exhibited higher levels of one of the three pathogens tested and this was associated with increased immune gene expression. We also showed that feral colonies had similar levels of overwintering survival even with higher pathogen burdens, suggesting increased virus tolerance compared to managed honey bees. This work also identified immune genes that were associated with increased overwintering survival in both feral and managed honey bee colonies, providing insight into host-parasite ecology.

As host defenses evolve over time, closely related hosts may share defenses contributing to similar levels of disease susceptibility. Thus, in addition to domestication status, host phylogenetic relatedness may also be an important predictor of host susceptibility to viral disease. The second research aim of this dissertation was to determine if either domestication status or host phylogenetic relatedness contribute to differences in susceptibility between squash plant hosts. We used three pairs of domesticated and wild plants in the genus *Cucurbita* to characterize host phenotypes throughout an entire field season. Plants were inoculated with a combination of zucchini yellow mosaic virus (ZYMV) and squash mosaic virus (SqMV). This study was conducted over three years resulting in a robust dataset with over 15 recorded variables related to plant health. Results indicate that domestication is not a driver of susceptibility in this system, but host phylogenetic relatedness is predictive of infection outcomes for many of the traits measured. Also, it was found that virus infection alters the behavior of a specialist pollinator but not a generalist pollinator in this system. Surprisingly, virus

infection also decreased pollen production and nectar sugar concentration which has implications for pollinator nutrition. Furthermore, we found that although infection outcomes differed greatly between hosts, viral load did not, providing support for the importance of tolerance in this system. This study also provides evidence that tolerant plant hosts can maintain nearly all measures of fitness in the field, demonstrating that tolerance deserves further consideration as a disease management strategy in agriculture.

After thorough characterization of susceptibility of different plant hosts in the field, species with low, medium, and high levels of disease susceptibility were chosen to investigate molecular mechanisms underlying reduced disease susceptibility. We exposed plants to mixed infection of ZYMV and SqMV, a mock-inoculation, or no treatment at three different time points under controlled conditions in a grow room. Tissue from these 81 plants was then sent for RNA sequencing. Results showed that less susceptible hosts had reduced responses in the total number of differentially expressed genes and in the number of defense-related genes. Responses in the least susceptible host also subsided more quickly compared to more susceptible hosts and were effective at reducing the accumulation of ZYMV, while SqMV levels were similar between all hosts. This research suggests that reduced disease susceptibility is characterized by delayed or lower magnitude of responses, which may deprive viruses of necessary factors for replication, and ultimately may limit virus-induced damage. Our results also highlight the importance of early host responses before symptom development for infection outcomes. This dataset provides a valuable resource for further investigating the molecular mechanisms of virus susceptibility in plants, which are largely unknown.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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“Live as if you were to die tomorrow; learn as if you were to live forever” - Mahatma Gandhi.

Chapter 1

Introduction

After a pathogen infection, hosts can defend themselves through either resistance or tolerance. Resistance is defined as the ability to reduce pathogen multiplication and can vary in strength, with complete resistance eliminating all pathogen replication or spread (Cooper and Jones, 1983; Glazebrook, 2005; Seal et al., 2021). While definitions of tolerance can vary widely between and within scientific fields, it is generally described as the ability to reduce the fitness costs of an infection while allowing pathogen accumulation within a host (Ayres and Schneider, 2012; Bengyella et al., 2015; Pagán and García-Arenal, 2018; Paudel and Sanfaçon, 2018). Susceptibility can refer to susceptibility to infection or susceptibility to disease and is often used to encompass both concepts. Susceptibility to infection is described as a lack of resistance and can be defined as the likelihood that a host will become infected after pathogen exposure (Glazebrook, 2005). On the other hand, disease susceptibility is viewed as decline in host fitness as a result of a pathogen infection (Sweeny and Albery, 2022).

Due to the rapidly evolving nature of viruses, host resistance to virus infection may be quickly overcome. Resistance to virus infection limits within-host spread or replication of virus but can be specific to one virus strain or genotype (García-Arenal and McDonald, 2003). This results in high selective pressure for viruses to evolve to subvert host resistance, leading to increased pathogenicity (the ability to cause disease in a host) (Woolhouse et al., 2002). The emergence of highly pathogenic viruses in agriculture negates breeding efforts for resistant plants or animals (e.g., insects), with devastating economic impacts. In contrast, because host tolerance allows for the accumulation of viruses, tolerance may offer a more stable state for hosts and may result in less selective pressure for the emergence of highly pathogenic virus strains (Paudel and Sanfaçon, 2018). Identifying virus-tolerant hosts will allow for the focusing of breeding efforts, decreasing losses caused by viruses in agriculture. Although tolerance is common in wild plants, the mechanisms behind virus tolerance in plant and insect systems remain

poorly understood (Prendeville et al., 2012; Roossinck et al., 2015). Additionally, pathogen defenses are often decreased in domesticated compared to wild hosts (Lenne and Wood, 1991) and, therefore, domesticated species offer an opportunity to study factors influencing the presence of tolerance. Here domestication refers to a process where humans manage survival and reproduction of another species, resulting in phenotypic changes in the domesticate (Purugganan, 2022). The process of domestication may mediate selection pressures for virus-tolerant hosts through cultural practices and selective breeding. For example, the high density of genetically similar hosts and attempts to eradicate viral diseases through vector control in agricultural systems may lead to the loss of tolerance and increased selection pressure for pathogenic viruses (Roossinck and García-Arenal, 2015). These factors may be contributing to increased susceptibility to disease (seen as reductions in fitness upon pathogen infection) in domesticated compared to wild hosts (Singh and van der Knaap, 2022). In addition, due to the codependence of crop plants and insect pollinators, it is important to study how plant virus infections alter ecosystem functions performed by pollinators and how the history of domestication impacts pollination and pollinator health.

My research focuses on understanding mechanisms of virus tolerance in domesticated/wild plants and bee pollinators, but also how viral infections in plants may modify pollinator foraging behavior and plant fitness. I primarily use squash plants (*Cucurbita* spp.) and honey bees (*Apis mellifera*) as models because they have been heavily bred by humans, yet wild or unmanaged populations persist in nature. Additionally, they are interacting directly with each other (due to the reliance of squash on insect pollination) and virus infections can have significant economic impacts in both systems.

1.1 Viruses as Symbionts and the Evolution of Virulence

Symbiosis can be defined as an intimate relationship between two dissimilar organisms that are living in or on one another (De Bary, 1879). Symbiotic relationships are categorized into mutual, commensal, or pathogenic interactions. The nature of this relationship can be dependent on many factors (i.e., environment, phenology, level of infection), leading to the concept of conditional mutualism

(Rodriguez et al., 2008; Bao and Roossinck, 2013; Kone et al., 2017a). An organism may be pathogenic in one host and yet, cause no observable fitness costs in closely related hosts (Provvidenti et al., 1984). Viruses are obligate symbionts, and although all viruses benefit from the acquisition of host resources, symbiotic relationships between viruses and their hosts can span a continuum from pathogenic to mutualistic. The most common host-virus relationship is likely commensal, conferring no observable cost to the host (Roossinck and Bazán, 2017). Viruses are present in every type of lifeform examined and many viruses do not produce symptoms of disease upon infection (Moelling, 2012; Roossinck et al., 2015). However, viruses are often studied in the context of pathogenic relationships when negative consequences for infected hosts can be observed.

Pathogenic viruses can have significant impacts for agriculture by directly affecting crops and decreasing their yields, as well as challenging the health of pollinators vital for crop production (Oerke, 2006; Gallai et al., 2009). As the global human population continues to increase, food security is of utmost importance. Therefore, methods are needed for durable and sustainable control of viral diseases. Understanding the evolution of host-virus relationships can help generate effective management strategies by determining what influences decreased virulence - the degree of damage to the host during infection - compared to virulent viruses with high potential to initiate epidemics (Read, 1994). When investigating these relationships, it may also be helpful to consider human influence. Humans also contribute to host-virus dynamics through domestication and artificial selection of organisms that may be more, or less, susceptible to virus infection or by creating environments that may be favorable for virulent viruses (i.e., large-scale monocultures) (Bull et al., 1991; Roossinck and García-Arenal, 2015; Neumann and Blacquière, 2017).

When referring to the evolution of parasite virulence, the *avirulence theory* predicts that parasites evolve towards low virulence to avoid running out of available hosts and jeopardizing their long-term survival (Burnet and White, 1972). However, it is now clear that even apparently long-evolving interactions between hosts and parasites can still result in virulence, providing evidence against the idea that pathogens always evolve towards low virulence (Møller et al., 1990; Herre, 1993; Read, 1994). Also,

the strategy of limiting acquisition of resources from the host may only be effective when the parasite is not co-infecting with others, and when competing with another parasite for resources, more aggressive genotypes can outcompete and dominate the parasite population (Bose et al., 2016).

Alternatively, the *virulence trade-off hypothesis* posits that virulence evolves to reach an optimal level where parasite fitness is maximized (May and Anderson, 1983). One supporting example is myxoma virus in rabbits, where highly virulent strains quickly kill hosts limiting transmission but strains with low virulence are controlled by hosts, and thus intermediate virulence is favored (Dwyer et al., 1990).

Nevertheless, neither of these theories can account for parasite evolution in all instances and multiple modes of transmission (horizontal and vertical) and interactions with other parasites within a host further complicate predictions of virulence evolution (Lipsitch et al., 1996; Alizon et al., 2009; Bose et al., 2016). For example, it is hypothesized that vertically transmitted pathogens will evolve to have low virulence, as their reproduction depends on the host's ability to produce progeny, but horizontally transmitted pathogens can be highly virulent as they do not rely on host descendants (Ewald, 1987). Yet, it becomes more difficult to model virulence evolution when a parasite undergoes multiple modes of transmission (Lipsitch et al., 1996). Additionally, trade-offs between vertical and horizontal transmission have been demonstrated, and show that virulence is not constant (Restif and Kaltz, 2006). While virulence is often viewed as a trait of the pathogen, virulence is ultimately a result of host-parasite interactions (Casadevall and Pirofski, 2001). Indeed, the evolution of virulence can depend on many factors including host heterogeneity and immune responses (Ganusov et al., 2002; Ganusov and Antia, 2003; Lively, 2006).

While much of the work on the evolution of virulence focuses on pathogen changes due to their quicker generation times, hosts also evolve mechanisms to mitigate the costs of pathogen infections. This research focuses on identifying host-parasite interactions where hosts have evolved to mitigate harmful effects of virus infection. I focus on RNA viruses infecting plants and insects, and their host interactions, due to their impact on U.S. and global agriculture. Plants and pollinators are inextricably linked, with many plants requiring pollination by insects for their reproductive success, and insect pollinators

requiring plant resources to survive. This codependence offers a unique framework to study not only how viruses affect their hosts, but also the consequences of plant virus infection for pollinator health.

1.2 Tolerance as a Stable Strategy for Mitigating Antagonism by Viruses

A successful virus infection is the result of interactions between an invading virus and host cellular machinery (Wang, 2015). After a virus enters an infectible host, the outcome of the interaction allows for classification of hosts as resistant, tolerant, or susceptible. Resistance limits pathogen replication, while tolerance limits the damage from infection (Ayres and Schneider, 2012; Bengyella et al., 2015; Pagán and García-Arenal, 2018; Paudel and Sanfaçon, 2018). Here we refer to susceptibility as the lack of either resistance or tolerance that results in disease manifestation. Resistance or tolerance are often measured in relation to more susceptible hosts, and thus their descriptions are typically relative (Glazebrook, 2005). Exceptions are hosts in which viruses do not replicate or spread, which can be classified as completely resistant, or hosts that sustain pathogen levels equal to a susceptible host but exhibit no fitness costs and would be completely tolerant. Disease susceptibility can also exist at varying levels, with extreme susceptibility referring to hosts exhibiting high levels of disease severity (Glazebrook, 2005).

In many cases, complete host resistance is conferred by the presence of a single allele at a given locus, with the gene product interacting with the pathogen via a singular specific mechanism (Müller and Brem, 1991; French et al., 2016). This can facilitate the identification and introgression of a resistance gene into other hosts and is used in agriculture on a large scale (McDonald, 2010). However, by depending on a gene-for-gene interaction of R genes in the host and Avr genes in the pathogen, the durability of this resistance is often lessened, and pathogens frequently defeat the host and regain the ability to infect plants (Cook, 1998; Montarry et al., 2012). Regardless of the genetic mechanisms, because resistance prevents pathogen replication, it places selection pressure on pathogens to evolve to overcome this defense (Dangl et al., 2013; Pilet-Nayel et al., 2017). Resistant hosts may thus

inadvertently select for viruses with mutations to overcome the effects of a resistance gene, and the ability of viruses to overcome genetic resistance has been demonstrated in several cases (Pink et al., 1992; Crescenzi et al., 2013; Song and Ryu, 2017).

In contrast, tolerance is often conferred by processes governed by several genes, making it more difficult to introduce into organisms of interest, but decreasing the likelihood that pathogens will evolve to negate all host defenses (Poland et al., 2009; French et al., 2016; Paudel and Sanfaçon, 2018). As viruses can effectively replicate and persist in tolerant hosts, there is less selection pressure for increased virulence or infectivity compared to host resistance (Rausher, 2001). In some cases, tolerance may also operate using less pathogen-specific mechanisms than resistance and can mitigate the effects of a wider range of pathogen isolates than resistance conferred by a single gene (Korbecka-Glinka et al., 2017). In this regard, tolerant hosts may be less affected by the emergence of new viruses, as they already have mechanisms to limit symptom development. The balance of virus and host fitness in tolerant interactions, therefore, appears to be an evolutionarily stable host defense strategy in cases of moderate virus replication (Restif and Koella, 2003).

It is important to note that tolerance can be measured in different ways. Tolerance can be measured over a dose-response curve and in this case is represented as a slope of a regression between host fitness and pathogen load (Simms and Triplet, 1994; Simms, 2000; Little et al., 2010). When measured as a slope, this can be referred to as range tolerance, but requires the impacts of infection to be measured at multiple pathogen loads in the hosts that are being compared (Pagán and García-Arenal, 2018). Tolerance can also be measured at a single pathogen load, which is described as point tolerance (Little et al., 2010). These descriptions have important implications for conclusions about tolerance in a system. For example, a host may be more tolerant at every level of pathogen measured and have higher point tolerance, but have the same slope and thus, the same range tolerance.

Additionally, tolerance and resistance are not necessarily mutually exclusive, as mechanisms conferring tolerance or resistance have been found to overlap in several systems (Pagán and García-Arenal, 2018). The mechanisms of antiviral resistance attributed to host genes has been thoroughly

investigated for many viral diseases, but the genetic mechanisms of tolerance remain to be explained in many systems (Bengyella et al., 2015; Sanfaçon, 2015; Hashimoto et al., 2016a; Pagán and García-Arenal, 2018). Due to its relevance and potential applications for agriculture, mechanisms of tolerance to virus infection should be explored. While resistance is often the first line of defense when implementing breeding techniques in agriculture, tolerance (or at least the addition of tolerant elements) should be considered to increase the effectiveness and sustainability of disease management strategies. The research proposed here investigates the concept of tolerance in plants (*Cucurbita* spp.) and bee pollinators (*Apis mellifera*), of which RNA viruses are key pathogens.

1.3 RNA Viruses

RNA viruses are the most common viral disease agents in plants (Roossinck, 2003). Invertebrates are also commonly infected with RNA viruses (pathogenic and non-pathogenic) and transcriptomic studies are frequently identifying more viruses in insects (Cook et al., 2013; Li et al., 2015; Shi et al., 2016; Galbraith et al., 2018). RNA viruses are some of the most important pathogens contributing to population declines of managed pollinators including the Western honey bee, *Apis mellifera* (Remnant et al., 2017). Some RNA viruses can infect both plants and insects and cause phenotypic changes in both (Li et al., 2014; Whitfield et al., 2015). Due to the low fidelity of their RNA-dependent RNA polymerases (RdRp), RNA viruses can undergo rapid mutations. Virus evolution rates can be influenced by many factors including intrinsic properties of the pathogen (RdRp fidelity, genome organization favoring reassortment, gene flow), environmental factors (host and vector environment), and transmission events (population bottlenecks, ease of transmissibility, number, and density of susceptible hosts) (Stern and Andino, 2016; Butković and González, 2022). In addition, many viruses are transmitted by insect vectors and the combination of high mutation rates, and the unpredictability of vector populations can make management strategies difficult to design and can lessen the durability of disease control compared to management of other diseases (Perring et al., 1999; García-Arenal and McDonald, 2003).

Additionally, RNA viruses have a diversity of strategies to avoid detection by hosts and inhibit antiviral responses. The genomes of RNA viruses often end with secondary structures such as 3' tRNA-like structures, or poly-adenylated tails (Gallie et al., 1995; Deiman et al., 2000). While these structures do not encode for any proteins, they are commonly used by viruses to initiate translation and to switch between replication and translation, and some of these structures are also needed for initiation of replication (Takamatsu et al., 1990; Takamatsu, et al., 1991). Viruses may also form structures or viral factories where replication and/or assembly can take place. These allow viruses to avoid host detection and prevent host cell death since viral proteins can have cytotoxic effects (Moshe and Gorovits, 2012; Schoelz and Leisner, 2017). In addition to avoiding host detection, viruses compete for host resources or interfere with host physiological processes, which can lead to the manifestation of disease. Virus infection can interfere with a wide range of host cellular functions, including developmental processes and host defense responses (Pallas and García, 2011). Viruses can also reprogram host cells, effectively turning them into viral replication factories, in some cases leading to cell death (Inaba et al., 2011).

1.4 Mechanisms of Antiviral Defense in Plants and Invertebrates

To mitigate the impacts of virus infection, animal and plant hosts rely on a diversity of defense mechanisms. Upon infection, the nonspecific innate immune response acts first. Pathogen associated molecular patterns or PAMPs are conserved patterns recognized by the host via pattern recognition receptors (PRRs) and trigger host pathways, leading to the production of proteins and other metabolites that ultimately limit the replication or spread of the pathogen. This mechanism is similar in plants and insects, with innate immunity playing important roles during virus infection (Kingsolver et al., 2013; Niehl et al., 2016). In plants, innate immune response results in a cascade of events ultimately leading to programmed cell death of the infected area to prevent spread of the pathogen to other cells (Jones and Dangl, 2006). In insects, several antimicrobial pathways exist that limit pathogen infection and spread such as Toll, IMD, JAK-Stat, and autophagy pathways (Kingsolver et al., 2013). These pathways are

well-characterized for their roles in bacterial and fungal infections, with specific receptors for different pathogen types (gram-positive bacteria, gram-negative bacteria, and fungi) (Rosales, 2017). Although they are often activated during virus infection, the mechanisms by which they interact with invading viruses is largely unknown (Galbraith et al., 2015).

To successfully infect their hosts, viruses have ways to suppress or evade these innate immune mechanisms. Therefore, hosts must respond with a second line of defense, and in plants this often takes the form of resistance (R) genes whose products recognize the products of specific avirulence (Avr) genes from the pathogen (Iriti and Faoro, 2007). In plants, some of these R genes encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins which are structurally similar to those found in animals (Lorang et al., 2007). However, NBS-LRR proteins and the pathways they trigger can also be targeted by pathogens, and some pathogen effectors can suppress the resistance conferred by R genes (Caplan et al., 2008). In contrast to dominant R genes, resistance can also manifest from recessive alleles of mutated host susceptibility factors required for host-virus interactions that result in disease (Hashimoto et al., 2016a). In resistant hosts, these genes have mutations that prevent virus interaction, and thus, disease. These recessive resistance alleles are commonly used in virus-resistant crops, but viruses have still been able to overcome this resistance in several cases (Hariri et al., 2003; Kuhne et al., 2003; Kang et al., 2005).

During infection and replication of their genome, RNA viruses can fold into double-stranded RNA (dsRNA) structures which in host cells is often recognized as foreign and targeted by host cellular machinery. This dsRNA may serve as a PAMP, triggering non-specific innate immunity in the host (Flenniken and Andino, 2013; Niehl et al., 2016). RNA interference (RNAi) is the counterpart mechanism of adaptive immunity that acts against dsRNA and viruses in a sequence specific manner, and is quite conserved between plants and insects, using similar enzymes and resulting in similar outcomes (Ding et al., 2004; Pfeffer, 2010). RNAi results in immunological memory, an enhanced response in subsequent encounters with that virus. To counteract RNAi, many viruses have silencing suppressor proteins which can act on different parts of the RNAi pathway in the host (Zsef Burgyá and Havelda, 2011). These

silencing suppressors are often virulence factors and are directly related to the formation of symptoms. Additionally, epigenetic mechanisms can be triggered in both plants and insects in response to virus infection (Galbraith et al., 2015; Wang et al., 2018). Epigenetic reprogramming serves as a way to activate genes involved in the clearance of virus and resistance signaling pathways. While the genetic basis of tolerance is unclear in many systems, it is likely that tolerance involves a combination of many of the mechanisms described above (Paudel and Sanfaçon, 2018).

1.5 The Role of Domestication in the Presence of Antiviral Defenses

Antiviral defenses come at a cost to the host, and when pathogens or their effectors are not present at high frequency, hosts without resistance genes may be selected for (Tian et al., 2003). The durability or persistence of a given defense mechanism in a population may therefore be dependent on the cost associated with that defense. Disease resistance genes are often associated with decreases in host fitness for many reasons. Resistance genes are often closely linked to developmental processes in plants, and many pleiotropic effects of disease resistance genes have been identified (Brown and Rant, 2013). In several cases, yield may be directly reduced by the presence of resistance genes or by the active induction of defenses (Kjær et al., 1990; Heil and Baldwin, 2002; Sharp et al., 2002). The presence of resistance genes to a particular pathogen may also increase susceptibility to other pathogens (McGrann et al., 2014). Therefore, selection of high-yielding plants with high reproductive rates during domestication events may have influenced the loss of costly pathogen defenses in some cases. In a review of trade-offs associated with plant defenses, fitness costs of defenses in crop plants were observed more often than in wild plants, suggesting agricultural practices may increase the strain of defenses in plants, and thus the likelihood these traits will be lost (Bergelson and Purrington, 1996). It has also been hypothesized that with the advent of large-scale monoculture, the lack of biodiversity and use of a single crop genotype on large acreage has facilitated the emergence of severe plant virus diseases (Roossinck and García-Arenal, 2015).

However, many wild plants are infected with viruses known to be pathogenic in crops, but exhibit no symptoms (Roossinck et al., 2015). In fact, there are many examples where virus resistance genes are seen more commonly in wild plants than their domesticated relatives (Lenne and Wood, 1991). Other transcriptomic and genomic studies have also identified overall trends indicating a decrease in disease resistance genes in domesticated compared to wild plants (Hübner et al., 2018; Huang et al., 2019). For this reason, wild cultivars are often used as sources of disease resistance in breeding programs. In addition to resistance, wild plants have also been identified that can tolerate infections of plant viruses (Provvidenti et al., 1984; Pagán and García-Arenal, 2018). While the costs of pathogen defenses in crop plants, and their presence, are often studied in the context of single gene resistance, mechanisms of tolerance may also have been lost during domestication events.

This concept is not exclusive to plants either, as feral populations of honey bees living in the wild have been found to have increased tolerance to parasitic mites and some survive with high levels of virus infections (Locke et al., 2014; Locke, 2016). This has been attributed to small colony sizes in some cases, which may decrease selective pressure for virulent pathogens (Loftus et al., 2016). Indeed, host density can play a vital role in the evolution of pathogen virulence, as increased transmission can mitigate the costs of increased virulence. Thus, densely populated human-managed colonies can allow for more virulent pathogens. In wild conditions, lower virulence (and host tolerance) may be more advantageous for pathogen fitness due to lower density of hosts and fewer opportunities for transmission. For these reasons, tolerance may be quite common in wild ecosystems, where virus infection is prevalent or continuous, and the costs associated with tolerance may be more justified or necessary in hosts under continuous exposure to virus infection.

Outside of environments heavily managed by humans, pathogens and hosts are still evolving together, and information on these relationships is important for learning about the long-term outcomes of host-pathogen interactions. Wild and feral systems can provide valuable insight into host-virus adaptation because in contrast to managed systems they often have higher genotypic diversity, lower host density, and are commonly infected with viruses known to be pathogenic in other hosts. Additionally, wild and

feral species often have mechanisms to limit negative effects of virus infection, which can be utilized in future breeding strategies (Paudel and Sanfaçon, 2018). As single gene resistance may become less durable over time, it is important to identify other outlets for reducing losses caused by viruses.

1.6 Effects of Virus Infection on Plant-Insect Interactions

In pathogenic host-virus interactions, there are definitive fitness costs for the host after virus infection. Disease is characterized by phenotypic changes in the host due to competition for host resources, resulting in the physiological disruption of host function (Pallas and García, 2011). These pathological phenotypes, or symptoms, may be an indirect consequence of infection or they may serve to increase the replication rate and/or transmission efficiency of a given virus (Chesnais et al., 2019). For example, many vector-transmitted plant viruses alter the volatile organic compound (VOC) profile produced by host plants to increase vector attraction to infected hosts (Mauck et al., 2010). These phenotypic changes can differ depending on the lifestyle of the virus, as plant infections with non-persistently transmitted viruses can result in increased probing events and shorter feeding times for aphids, while persistently transmitted viruses can have the opposite effect on plants (Mauck et al., 2012). Symptoms of yellowing in virus infected plants can also increase vector attraction, and thus virus dispersal, with the presence of symptoms directly benefiting the virus (Salvaudon et al., 2013). For viruses that actively replicate in their insect vectors, some may increase the fecundity of the vector or fitness on a given resource (Kuster et al., 2014; Porras et al., 2018).

While plant virus infection is known to have direct consequences for insect vector preference and performance in many cases, the impacts of plant virus infection on plant-pollinator attraction are only recently starting to be discovered (Prendeville and Pilson, 2009; Groen et al., 2016; Mhlanga et al., 2021; Murphy et al., 2023). These impacts would be especially important for viruses that are transmitted vertically through infected pollen grains, as bees could serve as mechanical vectors (Fetters and Ashman, 2023). Some plant viruses may also alter the nutritional content of plants and production of pollen (Blua

et al., 1994; Harth et al., 2016), which can have direct effects on pollinator performance. If tolerance is to be used in breeding strategies for pollinator-dependent crops, its effects on pollinator health should be thoroughly investigated.

1.7 Virus Infection and Host Responses in Honey Bees

Deformed wing virus (DWV) is a positive sense single-stranded RNA virus regarded as the most important honey bee pathogen, resulting in millions of colony deaths, and along with its ectoparasitic mite vector (*Varroa destructor*) is one of the major concerns for beekeepers (Martin, 2001; Martin et al., 2012; Brutscher et al., 2015). While DWV is a globally distributed virus present in most honey bee colonies, in the absence of *V. destructor* infections are often at low levels, and produce no overt symptoms (Dainat et al., 2012; Amiri et al., 2018). DWV can be transmitted horizontally between workers and vertically from queen to offspring within a colony (Amiri et al., 2017). DWV can also be vector-transmitted and is able to accumulate in *V. destructor* which commonly infest honey bee colonies (de Miranda and Genersch, 2010). When transmitted by *V. destructor*, DWV infection in honey bees can result in the appearance of deformed wings and other symptoms such as shortened abdomens, impaired learning ability, abnormal behavior, and reduced lifespan (Iqbal and Mueller, 2007; Gisder et al., 2009; de Miranda and Genersch, 2010; Möckel et al., 2011). Black queen cell virus (BQCV) is another highly prevalent virus in honey bees that can result in arrested development of larvae and host mortality (Mondet et al., 2014; Doublet et al., 2015). Horizontal transmission is mediated via nurse bees feeding healthy larvae after ingesting infected brood food, but the virus can also be vertically transmitted to eggs from infected queens (Allen and Ball, 1996; Chen et al., 2006).

Nevertheless, honey bee colonies can survive in the wild in the absence of beekeeper management. Due to the importance of *V. destructor* and viruses for honey bee health, several studies have investigated naturally surviving honey bee populations to identify sources of resistance or tolerance. These populations have evolved different mechanisms to limit *V. destructor* infestation such as increased

removal of infested bees (hygienic behavior), removal of mites (grooming behavior), maintaining small colony sizes, and even reducing mite reproduction (Reviewed in Locke, 2016). Interestingly, several mite-resistant populations were shown to have high levels of DWV, similar to mite-susceptible colonies, suggesting that virus tolerance is a common mechanism to mitigate disease in unmanaged honey bee colonies (Locke et al., 2014; Thaduri et al., 2019).

Humoral immune responses are important for combatting microbial pathogens in insects (Rosales 2017). Previous studies of unmanaged (feral) honey bees have shown higher levels of individual immunity that may indicate a more effective response to pathogens in the absence of human management (Youngsteadt et al., 2015). Due to strong selective pressures, variants efficient at dealing with pathogens are likely being selected for in feral populations, while this same selection may not be consistently present in managed honey bee colonies. Previous studies have also shown that increased genetic diversity was associated with increased immunocompetence in feral but not managed honey bee colonies, providing further support for differences in expression of immunity and impacts of human management on host-pathogen dynamics in honey bees (López-Uribe et al., 2017).

1.8 Virus Infection and Host Responses in Cucurbits

The genus *Cucurbita* offers a model system to study the effects of domestication on virus susceptibility, as there are thought to be six independent domestication events resulting in wild progenitors with domesticated descendants and feral escapees (Castellanos-Morales et al., 2018). Cucurbits are prone to yield losses from a variety of viral pathogens, especially members of the *Potyviridae* family, and mixed infections with multiple virus species are common (Ali et al., 2012a, 2012b). Two of the most common viruses found in mixed infections are zucchini yellow mosaic virus (ZYMV, *Potyviridae*) and squash mosaic virus (SqMV, *Secoviridae*). ZYMV is an economically important virus infecting Cucurbitaceae plants in all cucurbit growing regions worldwide (Lisa, 1981). ZYMV and SqMV are transmitted horizontally by aphids and beetles, respectively. Both viruses are also

transmitted through seed at rates high enough to be significant sources of outbreaks (Alvarez, 1978; Simmons et al., 2013).

While the biology, epidemiology, and ecology of SqMV are less well known, ZYMV has been thoroughly investigated for several decades. In addition to yield losses from inhibiting fruit development or causing deformities, ZYMV can also affect paternal fitness of infected plants through reductions in pollen production and performance (Harth et al., 2016; Seda-Martínez et al., 2021). Impacts of ZYMV on host traits can also scale up to affect population dynamics of wild plant populations (Prendeville et al., 2014a). Additionally, ZYMV infection can alter plant-insect interactions with consequences for other pathogens of cucurbits. Specifically, changes in volatile profiles of ZYMV-infected plants have been associated with decreased attraction by cucumber beetles (*Acalymma vittatum*), which serve as vectors of a bacterial pathogen, *Erwinia tracheiphila*, responsible for a fatal wilt disease (Shapiro et al., 2012a). This reduced attraction results in decreased incidence of bacterial wilt in ZYMV-infected plants and could possibly contribute to reduced incidence of other pathogens vectored by cucumber beetles including SqMV (Shapiro et al., 2013). In contrast, changes in leaf color and volatile emission during ZYMV infection can enhance recruitment of aphid vectors, leading to increased transmission of ZYMV, but also can facilitate transmission of other aphid-transmitted viruses (Salvaudon et al., 2013). While impacts of ZYMV infection on plant-insect interactions have mainly been studied in insect vectors, *Cucurbita* spp. rely heavily on bee pollinators (Hymenoptera) for sufficient pollination and subsequent fruit production (Ullmann et al., 2017). Most pollination in the northeastern U.S. is performed by just three main bee groups with different levels of effectiveness (McGrady et al., 2019). Therefore, impacts of virus infection on pollinator behavior could have large implications for crop health in this system and deserve further investigation. Additionally, the diversity of physiological alterations and impacts on plant-insect interactions have mainly been demonstrated in highly susceptible *C. pepo* cultivars or its wild relative, *C. pepo texana*. Thus, it remains unclear if similar impacts are observed in other *Cucurbita* spp.

To avoid crop losses, management of viral diseases in cucurbits focuses on preventing transmission by insect vectors, but also deployment of resistant cultivars (Lecoq and Katis, 2014).

Different levels of susceptibility have been identified between host species (Provvidenti and Gonsalves, 1984). Some wild plants are completely resistant, some exhibit high levels of tolerance, and cultivated plants range from being moderately tolerant to highly susceptible (Provvidenti et al., 1984; Pachner et al., 2015). Both dominant and recessive genes conferring resistance to ZYMV have been identified in wild *C. ecuadorensis* and *C. moschata* cultivars 'Menina' and 'Nigerian Local' (Paris et al., 1988; Paris and Cohen, 2000; Pachner et al., 2011; Nacar Ç et al., 2012). Several of these genes have been bred into highly susceptible *C. pepo* cultivars, but do not confer complete resistance suggesting the importance of minor modifying genes in the original host background (Paris and Cohen, 2000). A previous study introduced a combination of four dominant and two recessive resistance genes to produce strong resistance in *C. pepo*, however, virus accumulation was not quantified in this study, so it is possible resistance was not complete and ZYMV was still able to replicate in these plants (Pachner et al., 2015).

A diversity of antiviral defenses exists in cucurbits but the mechanisms conferring host resistance remain to be elucidated in many cases (Martín-Hernández and Picó, 2021). At least one of the known resistance genes to ZYMV is associated with a recessive mutation in the eukaryotic translation initiation factor (eIF) 4E gene (Ling et al., 2009). Recessive mutation of the eIF4E gene is one of the most well characterized mechanisms of resistance to potyviruses, which inhibits interaction of the viral VpG protein with the host translation machinery, preventing virus genome translation (Moury et al., 2014; Hashimoto et al., 2016b). For another potyvirus, papaya ringspot virus, dominant resistance was identified and attributed to an NBS-LRR gene (Anagnostou et al., 2000). Resistance to SqMV has been identified in several host genotypes, but the mechanisms remain largely unknown (Beloti et al., 2021; Martín-Hernández and Picó, 2021).

In addition to resistance attributed to one or a few dominant or recessive genes, virus resistance can result from efficient activation of antiviral pathways. RNA interference (RNAi) is an effective system for eliminating viruses through silencing after recognizing viral nucleic acids (Soosaar et al., 2005; Bologna and Voinnet, 2014). Phytohormones can also play important roles in antiviral defense, leading to hypersensitive response which prevents virus spread throughout a plant and can lead to systemic acquired

resistance which can last for up to several weeks (Robert-Seilaniantz et al., 2011; Fu and Dong, 2013). These resistance mechanisms may work in concert with each other to achieve overall effective defense against virus infection (Nicaise, 2014). Additionally, some of these defense mechanisms may still allow for virus replication, but could have important roles in mitigating disease manifestation, and thus play critical roles in host tolerance.

1.9 Rationale

Like other host defenses, tolerance may come at a cost, with decreased host fitness in the absence of pathogen infection or increased susceptibility to other pathogens. Yet, in wild/feral systems it may be rare to find hosts that are uninfected, and this constant exposure to pathogens may be driving selection of tolerance mechanisms in these populations. Also, many viruses in wild ecosystems are vertically transmitted, meaning it is advantageous to reduce their costs to host fitness. Deformed wing virus (DWV), black queen cell virus (BQCV), zucchini yellow mosaic virus (ZYMV), and squash mosaic virus (SqMV) can all be vertically transmitted in their insect and plant hosts. Previous research has shown that unmanaged feral honey bees can respond differently to immune challenge and may be facing different pathogen pressures than their human-managed counterparts. Also, it is known that several wild *Cucurbita* spp. are tolerant or resistant to ZYMV infection. However, the systematic investigation of the role that human selection through domestication has had on host susceptibility is lacking. To answer questions about virus tolerance, my research uses squash plants (*Cucurbita* spp.) and honey bees (*Apis mellifera*) as models because they have been heavily bred by humans, yet wild populations persist in nature. Hymenopteran pollinators and plants have an intimate relationship with each other. Squash plants rely on animal pollination and bees acquire nutritional resources from these plants. With plant-pollinator relationships being foundational to agriculture and global food security, it is desirable to take an interdisciplinary approach to study how anthropogenic factors can influence disease susceptibility in both plant and pollinator systems.

The goal of this research was to identify virus tolerant honey bees and *Cucurbita* spp. and attempt to characterize the mechanisms of tolerance in these agriculturally relevant organisms. Comparing wild and feral species to domesticated species offers an opportunity to learn how genetically similar individuals respond differently to pathogen pressures. Additionally, the genetic mechanisms of virus tolerance and its effects for plant-pollinator interactions have not been well studied. Genetic traits associated with tolerance are clearly desirable for breeding purposes, but we must also determine the effects of tolerance on plant fitness via pollination success, plant reproduction, and yields to assess its viability as a management strategy.

1.10 Research Aims

Aim 1: **Determine if feral honey bees are more tolerant to virus infection and if this is mediated by immunological mechanisms** by characterizing virus levels and immune gene expression of feral and managed bees in the same landscape.

Aim 2a: **Determine if wild *Cucurbita* spp. species have higher tolerance to virus infection** by assessing virus accumulation, disease severity, and plant fitness of different wild, feral, and domesticated species during virus infection.

Aim 2b: Determine the consequences of plant virus infection for plant-pollinator interactions. Pollinator visitation on virus-inoculated and uninoculated plant hosts with varying disease susceptibility were compared in field settings.

Aim 3: **Investigate molecular mechanisms of virus susceptibility in *Cucurbita* spp.** Species identified with high, medium, or low levels of disease susceptibility were characterized by analyzing gene expression differences in infected and uninfected plants to describe molecular signatures and potential mechanisms of reduced susceptibility.

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Chapter 2

The Role of Pathogen Dynamics and Immune Gene Expression in the Survival of Feral Honey Bees

Chapter 2 includes a published paper (Hinshaw et al., 2021) that is reproduced here with some modifications. This paper can be found at the following URL: https://doi.org/10.3389/fevo.2020.594263

Studies of the ecoimmunology of feral organisms can provide valuable insight into how host–pathogen dynamics change as organisms transition from human-managed conditions back into the wild. Honey bees (*Apis mellifera* Linnaeus) offer an ideal system to investigate these questions as colonies of these social insects often escape management and establish in the wild. While managed honey bee colonies have low probability of survival in the absence of disease treatments, feral colonies commonly survive in the wild, where pathogen pressures are expected to be higher due to the absence of disease treatments. Here, we investigate the role of pathogen infections [*Deformed wing virus* (DWV), *Black queen cell virus* (BQCV), and *Nosema ceranae*] and immune gene expression (*defensin-1*, *hymenoptaecin*, *pgrp-lc*, *pgrp-s2*, *argonaute-2*, *vago*) in the survival of feral and managed honey bee colonies. We surveyed a total of 25 pairs of feral and managed colonies over a 2-year period (2017–2018), recorded overwintering survival, and measured pathogen levels and immune gene expression using quantitative polymerase chain reaction (qPCR). Our results showed that feral colonies had higher levels of DWV but it was variable over time compared to managed colonies. Higher pathogen levels were associated with increased immune gene expression, with feral colonies showing higher expression in five out of the six examined immune genes for at least one sampling period. Further analysis revealed that differential expression of the genes *hymenoptaecin* and *vago* increased the odds of overwintering survival in managed and feral colonies. Our results revealed that feral colonies express immune genes at higher levels in response to high pathogen burdens, providing evidence for the role of feralization in altering pathogen landscapes and host immune responses.

2.1 Introduction

Feralization is the process by which previously domesticated organisms establish populations in the wild in the absence of anthropogenic influence (Gering et al., 2019a). The outcomes of feralization are typically studied in an evolutionary context, examining how environmental and genetic factors affect the fitness of feral organisms compared to their domesticated sources. It has been suggested that because of genetic bottlenecks and artificial selection during the domestication process, fitness decreases outside of captivity, making feral organisms exhibit reduced fitness upon reintroduction to the wild (Araki et al., 2009; Baskett and Waples, 2013; Meyer and Purugganan, 2013). However, feral organisms often thrive (e.g., cats, dogs, pigs) and do not always revert back to the 'wild-type' (Taylor et al., 1998; Bellard et al., 2017). Indeed, feral organisms often outnumber their wild counterparts, and can lead to shifts in community composition at the ecosystem level by increasing predation pressure on available prey and potentially spilling over pathogens to wild species (Leiser et al., 2013; Bevins et al., 2014; Maeda et al., 2019; Lepczyk et al., 2020). These ecological effects of feralization are gaining more attention due to their implications for conservation biology and ecosystem management.

Feral organisms frequently interact with both domesticated and wild species, and play a critical ecological role in the dynamics of pathogens shared among these closely related groups. The increased fecundity and expanded geographic ranges that result from the domestication processes, often result in large uncontrolled populations of feral organisms harboring infections and serving as a bridge between domesticated and wild hosts (Bevins et al., 2014). This is the case of the feral swine (*Sus scrofa domestica*), a species that has high rates of reproduction, high pathogen loads, and overlaps in range with domestic pigs (*S. scrofa domestica*) and wild boars (*S. scrofa*) (Taylor et al., 1998; Hill et al., 2014). Transmission of several pathogens, including viruses and bacteria, have been documented from feral swine to domestic pigs and wild boars, posing concerns for the role of feral organisms as reservoirs of pathogen transmission to both wild and domesticated populations (Cvetnic et al., 2003; Le Potier et al., 2006; Meng et al., 2009).

The ecological conditions of feralization may facilitate tolerance or resistance to disease. Pathogens of domesticated species are usually managed by humans to avoid the rapid spread of diseases among domesticated animals. On the contrary, pathogen transmission in feral populations is uncontrolled. Under these conditions, host–pathogen interactions in feral populations may therefore facilitate the rapid evolution of natural mechanisms of disease tolerance or resistance (LeConte et al., 2007; Locke, 2016). Thus, the maintenance of traits associated with disease resilience may be relaxed in domesticated species, while greater ability to mitigate the negative effects of pathogens is critical for the survival of feral organisms (Moreira et al., 2018).

Feral colonies of the honey bee (*Apis mellifera* L.) provide an ideal model to investigate the hypothesis that host–pathogen dynamics during feralization favor higher expression of defenses and disease tolerance in feral organisms. *Apis mellifera* is a eusocial bee species that has undergone extensive domestication efforts for traits such as increased honey production, decreased aggressive behavior and reduced frequency of swarming (Lecocq, 2018). Managed honey bee colonies frequently colonize wild environments and become feral because colonies reproduce through swarming (Winston, 1991). Both domesticated and feral honey bees face serious challenges due to a large number of pests and pathogens (Calderone, 2012; Mcmahon et al., 2016). One of the major drivers of disease and colony losses among honey bees is the ectoparasitic mite *Varroa destructor*, which acts as a vector for multiple bee-infecting RNA viruses that significantly weaken colonies and decrease their overwintering survival (Gisder et al., 2009; Martin et al., 2012). Varroa mites and associated viruses are considered major antagonists of honey bee health and, because of their strong effects on honey bee survival, managed colonies are often treated with chemical acaricides multiple times per year to decrease mite numbers. If untreated, most managed honey bee colonies die within the first year (Kraus and Page, 1995; LeConte et al., 2010). Nevertheless, feral honey bee colonies have been documented as surviving long-term in the wild in the absence of beekeeper management, where Varroa mites and viruses are not artificially controlled, and can therefore pose high selective pressure on colonies (Locke, 2016).

Previous studies have indicated that feral honey bee colonies may exhibit higher immune responses than managed colonies (Youngsteadt et al., 2015, but see Lowe et al., 2011). However, it is unclear how the expression of different immune phenotypes in managed and feral conditions is associated with colony survival and resistance or tolerance to parasites. Honey bees rely on both individual and social mechanisms of immunity to protect the colony from pests and pathogens, and management likely has an influence on both types of defenses (Neumann and Blacquière, 2017; Taric et al., 2020). While behavioral responses, such as hygienic behavior, play key roles in protection against pathogens (Simone-Finstrom, 2017), humoral immune responses in individual bees are also critical for pathogen defense and control of infections (Di Prisco et al., 2016; McMenamin et al., 2018). Several immune pathways are involved in the immune response against viruses, bacteria and fungi. For example, antimicrobial peptides (AMPs) produced from several immune pathways (e.g., Toll and Imd) have key general roles in insect immune systems (Yi et al., 2014; Brutscher et al., 2015). The RNA interference (RNAi) pathway is an antiviral defense pathway in insects that targets sequence-specific double-stranded RNA produced during RNA virus replication (Gammon and Mello, 2015).

Here, we investigate the role of pathogen infections and immune gene expression in the survival of feral and managed honey bees to answer the following questions: (1) are feral colonies reservoirs of pathogens with increased levels of pathogens compared to managed colonies?; (2) do increased pathogen levels lead to higher expression of immune genes in feral colonies than in managed colonies?; (3) is immune gene expression correlated with survival of honey bee colonies? Over a 2-year period, we sampled feral and managed colonies in the same landscapes with the participation of beekeepers who reported the location of colonies. We collected individuals from a total of 44 colonies during the spring and fall of the years 2017 and 2018 to compare the ecoimmunology and survival of colonies in apiaries and in the wild. The virus most directly linked to Varroa mite infestations (DWV) was found at significantly higher levels in feral than managed colonies, and the expression of most immune genes was also higher in feral colonies. We also identified two immune genes that are associated with colony survival and that can potentially be used as biomarkers of health in honey bee colonies.

2.2 Materials and Methods

2.2.1 Sample Collection

We established a collaborative community science project to locate feral honey bee colonies across the state of Pennsylvania, United States. Participants reported the locations of feral colonies by submitting GPS locations of colonies into a web portal or via email. To be included in the study, all feral colonies needed to survive at least one winter in wild, unmanaged conditions. We checked each reported colony in early spring to corroborate activity and record overwintering survival (**Figure 2.1**). We paired each feral colony with one managed colony located within a seven-mile radius to control for site variation between colonies located in geographic areas with different landscapes and climates. A total of eight pairs of feral and managed colonies (n = 16 colonies) in 2017 and 17 pairs (n = 34) in 2018 were included in the laboratory analyses (**Figure 2.2**). In the case where a managed colony was not able to be sampled a second time due to death or other reasons, the feral colony was paired with a different managed colony in the same location (between 2017 and 2018, n = 3; between spring and fall 2018, n = 1). This resulted in 20 unique feral colonies and 24 unique managed colonies being sampled over the course of this study (Supplementary Data Sheet 1). Additionally, we were unable to obtain data on the overwintering status of one managed colony in 2018, therefore it was omitted from the analyses of overwintering survival. Due to the death of either a managed or feral colony in a pair, only two pairs of colonies were sampled in both 2017 and 2018.

We sampled approximately 75 forager bees from the entrance of each colony in the spring (March–June) and fall (August– October) (dates in Supplementary Data Sheet 1). Individuals were sampled with aerial insect nets and transferred to 50 ml conical tubes that were placed on dry ice to preserve RNA quality before long-term storage at −80◦C. All sampling sites were on private property and permission was obtained from the land owners. The specific locations and contact information of participants was kept confidential. No protected or endangered species were involved in these studies.

Figure 2.1: Cavities used by feral honey bee colonies included in the study. (**A**) Colony inhabiting a tree cavity with abundant propolis at the entrance of the nest in Saxonburg (PA, United States). (**B**) Feral colony nesting inside the wall of a house in New Bethlehem (PA, United States) (**C**) Entrance of a feral colony nesting in an abandoned shed in Harrison Valley (PA, United States).

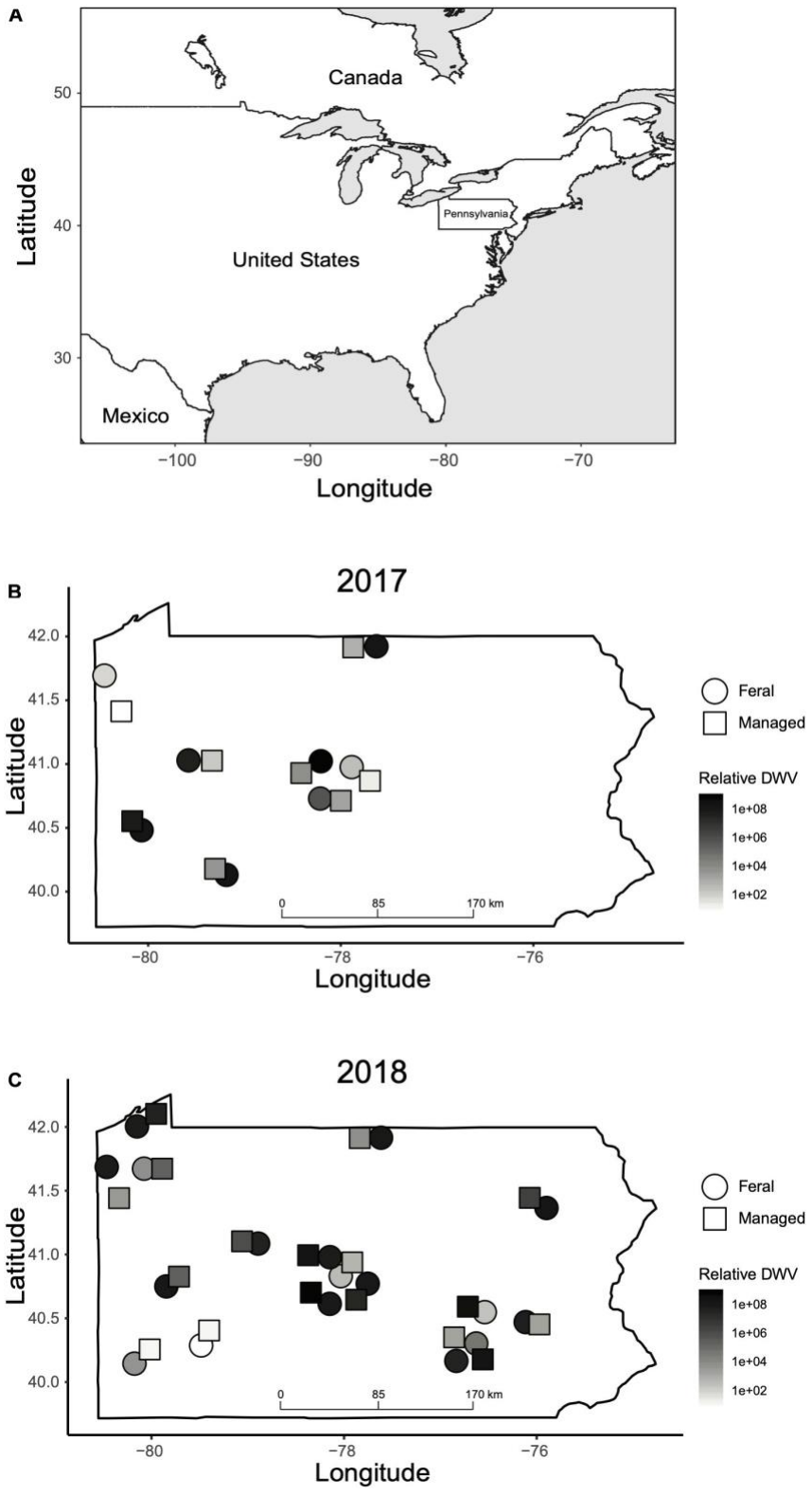

Figure 2.2: Geographic locations of feral and managed colonies sampled in this study. (**A**) Map of North America indicating the location of Pennsylvania in the northeast of the United States. (**B**) Location of feral (circles) and managed (squares) colonies sampled in 2017. (**C**) Location of feral

(circles) and managed (squares) colonies sampled in 2018. The color of each shape corresponds to relative DWV levels ($2^{-\Delta\Delta CT}$ values) recorded in the fall for that colony. Colony locations are approximate and have been adjusted slightly due to overlapping points.

2.2.2 Selection of Pathogens and Immune Genes

To characterize disease dynamics in honey bee colonies, we quantified three pathogens that commonly infect honey bees and negatively impact colony health. *Deformed wing virus* (DWV) is an RNA virus that is considered the most detrimental honey bee pathogen for its ubiquity, global distribution, and role in overwintering losses of honey bees (Martin et al., 2012; Brutscher et al., 2016). It can be transmitted horizontally and vertically within a colony, but is also efficiently transmitted by Varroa mites which can increase the titer of this virus leading to overt, clinically symptomatic infections (Gisder et al., 2009; Möckel et al., 2011). We also quantified *Black queen cell virus* (BQCV), another RNA virus that is highly prevalent in adult honey bees globally, but predominantly affects immature bee stages (prepupae and pupae) (Mondet et al., 2014). BQCV is transmitted vertically and horizontally between adults and from adult bees to developing bees, but has not been shown to be transmitted by Varroa mites (Chen et al., 2006). BQCV may also have synergistic interactions with other pathogens due to its correlation with viruses and the fungal parasite *Nosema ceranae* Fries (D'Alvise et al., 2019). *Nosema ceranae* is a common microsporidian gut parasite of honey bees, contributing to decreased lifespans in infected bees (Higes et al., 2008; Goblirsch, 2018). We quantified this pathogen in all colonies sampled in 2018.

To characterize immune gene expression in feral and managed colonies, we quantified transcript expression of six genes (argonaute-2, vago, pgrp-s2, pgrp-lc, defensin-1 and hymenoptaecin) from several immune pathways. The genes argonaute-2 (ago2) and vago, from the RNAi pathway, have been shown to be upregulated after viral infection (Brutscher et al., 2015). The gene pgrp-s2 encodes for an upstream

recognition receptor involved in activation of the Toll immune pathway, and pgrp-lc encodes a transmembrane protein activator of the Imd (Immune Deficiency) pathway. Both of these genes are upregulated in pathogen-infected honey bees (Evans et al., 2006; Brutscher et al., 2017). Additionally, we quantified genes corresponding to two antimicrobial peptides (AMPs), defensin- 1 (Def1) and hymenoptaecin (Hym), produced by the Toll and Imd pathways. These AMPs have key roles in honey bee immune responses to viruses, bacterial and fungal pathogens (Yi et al., 2014; Brutscher et al., 2015).

2.2.3 RNA Extraction and Quantitative PCR

For total RNA extraction, we dissected abdomens from thirty bees per colony by removing the stingers, hindguts, and midguts. Ten abdomens per sample (three samples per colony) were pooled into 2.0 ml tubes with 2.0 mm BashingBead Lysis Tubes (Zymo Research, Irvine, CA, United States) and homogenized using a BeadBlasterTM24 (Benchmark Scientific, Edison, NJ, United States) at 6.0 m/s for three 30 s intervals. We extracted RNA from homogenate using RNeasy spin columns (QIAGEN, Hilden, Germany), according to the manufacturer's protocol and eluted into nuclease-free water. We assessed the quantity and quality of RNA using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, United States).

We quantified the three pathogens and the expression of six immune genes through quantitative reverse-transcriptase PCR (qRT-PCR) using previously developed primer sequences (Table 1). The three RNA extracts from pooled individuals per colony were individually used as templates to produce cDNA using random primers and MultiScribe RT, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, United States). A total of 2 µg RNA was used for each cDNA synthesis. A total of 40 ng of cDNA was used for each qPCR reaction. We carried out reactions in 384-well plates using a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Each well contained 5 µl Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, United States), 0.25 µl of each of the forward and reverse primers (10 µM), 2.5 µl nuclease- free H_2O, and 2 µl cDNA template. The following

reaction conditions were used: 60 s at 95◦C for initial denaturation, then 40 cycles of 15 s at 95◦C for denaturation, and 30 s at 60◦C for annealing, extension, and data collection followed by a melting curve analysis of 15 s at 95◦C, 60 s at 60◦C, and 1 s at 95◦C to determine the specificity of amplification products. In each plate, we ran all reactions in triplicate and included negative controls of nuclease-free water for each set of primers. After surveying three reference genes (*ef1-alpha, eIFS8, and GAPDH1*), we determined that *elongation factor 1-alpha* (*ef1-alpha*) was a suitable reference gene due to its similar level of expression in all samples, and we used it as the reference gene for these experiments (**Table 2.1**).

We determined the Ct value for each sample by taking the mean of the three technical replicates. We used the Ct value for the reference gene and subtracted this from the Ct value for the target to generate ΔCt values for each sample. These ΔCt values were then normalized to the managed colony with the lowest relative abundance (highest ΔCt) of the target for that sampling period (spring or fall of the year sampled), generating ΔΔCt values. We then calculated the relative amounts of transcripts using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). As each colony had three samples consisting of ten bees each, we took the average $2^{-\Delta\Delta CT}$ value of the three biological replicates and used this for subsequent analyses.

For absolute quantification of DWV copy numbers in samples collected in the fall of each year, we used synthetic DNA corresponding to the sequence of DWV amplified by our primers, in the form of gBlocks gene fragments (Integrated DNA Technologies, Coralville, IA, United States), producing ten-fold dilutions (10^1–10^7 copies of synthetic DNA) run in each qPCR plate. We calculated the copy number using the formula: copy number = DNA concentration (ng/mL) × 6.02×10^{23} (copies/mol)/length (140 bp) × 6.6×10^{11} (Wu et al., 2017). The log DNA copy numbers were then plotted with Ct values, producing linear standard curves for each qPCR plate, allowing for the estimation of DWV copy number in each sample.

Table 2.1: Sequences of forward and reverse primers used for the quantification of pathogens and immune genes through quantitative PCR.

Gene name	Forward sequence (5’–3’)	Reverse sequence (5’–3’)	Category	Source

elongation factor 1-alpha	GGAGATGCTGCCATCGTTAT	CAGCAGCGTCCTTGAAAGTT	Endogenous reference	Lourenço et al. (2008)
DWV	GTTTGTATGAGGTTATACTTCAAGGAG	GCCATGCAATCCTTCAGTACCAGC	Virus	Ryabov et al. (2014)
BQCV	CTGGGCGAACATCTACCTTTCC	GCAATGGGTAAGAGAGGCTTCG	Virus	Luong et al. (2015)
Nosema ceranae	CAATATTTTATTATTTTGAGAGA	TATATTTATTGTATTGCGCGTGCA	Fungal parasite	vanEngelsdorp et al. (2009)
ago2	TTGGTGCAGACGTGACTCAT	TTGGATCGTGACTTGCTGCT	Immune gene (RNAi)	This study
vago	TTTTCGCTGCCGAGGAGAAG	GCACATACCGGGAAAATCGC	Immune gene (RNAi)	This study
hymenoptaecin	ACAATGGATTATATCCCGACTCGT	CAATGTCCAAGGATGGACGAC	Immune gene (AMP)	Vannette et al. (2015)
defensin-1	GGCTGCACCTGTTGAGGAT	TGTCCTTTGAATGAGAGAAGGTCA	Immune gene (AMP)	Vannette et al. (2015)
pgrp-lc	TCCGTCAGCCGTAGTTTTTC	CGTTTGTGCAAATCGAACAT	Immune gene (Imd)	Evans et al. (2006)
pgrp-s2	TTGCACAAAATCCTCCGCC	CACCCCAACCCTTCTCATCT	Immune gene (Toll)	Li et al. (2016)

2.2.4 Statistical Analysis

Due to non-normality, $2^{-\Delta\Delta CT}$ values were log-transformed and analyzed through generalized linear models (GLM) with a Gaussian distribution using the glm function in R package ‘stats’ (R Core Team, 2019). The relative expression of each target was analyzed separately using management (feral or managed), sampling time (spring or fall of 2017 or 2018), and their interactions as fixed effects. Two-way ANOVA was used to test for overall effects of management and sampling time on target expression. The estimated marginal means were then calculated with the emmeans function in R package ‘emmeans’ (Lenth, 2020). These values were then used for post hoc tests with a Tukey adjustment for multiple comparisons to determine differences in relative target abundance between feral and managed colonies at each timepoint. To evaluate associations between pathogen levels and immune gene expression among all

samples, we calculated the Spearman's rank correlation coefficients using the cor.test function in R package 'stats.'

To assess the role of immune gene expression, pathogen levels, and management in overwintering survival, we used a log linear generalized linear mixed model (GLMM) with a binomial distribution using the glmer function in R package 'lme4' (Bates et al., 2015). The original full model included data from the fall of both years with overwintering survival as the response variable, and the relative levels of DWV and BQCV, relative expression of all six immune genes, and management as fixed effects, and year of sampling as a random effect. We used a backward model selection to identify the fixed effects that contributed significantly to the model. We calculated the variance inflation factors (VIF) for the model using the vif function in R package 'car' (Fox and Weisberg, 2019). All VIF values were less than 3, and thus all fixed effects were kept in the final model. Ultimately, the model with the lowest AIC value was chosen. All analyses were conducted in R version 3.6.2.

2.3 Results

2.3.1 Pathogen Levels

Our results indicate that mean DWV levels were significantly higher in feral colonies compared to managed colonies in fall of 2017 ($P < 0.05$, $z = -2.470$), but not at other timepoints ($P > 0.05$) (**Table 2.2** and **Figure 2.3**). Levels of BQCV and *N. ceranae* did not differ between groups ($P > 0.05$). All 44 colonies tested positive for the presence of DWV and BQCV at all timepoints, while N. ceranae presence was more variable over time (**Figure S2.1**). Out of the 34 colonies tested, *N. ceranae* was detected in eight feral and 11 managed colonies in the spring (47.1% and 65%, respectively), and 12 feral and 10 managed colonies in the fall (76% and 59%, respectively).

Table 2.2: Results of two-way ANOVA on generalized linear models (GLM) assessing the effects of time, management, and their interaction on pathogen levels and immune gene expression. Values show

the degrees of freedom (D.F.), effect sizes (*F* statistic), and probability that the null hypothesis is correct (*P*-value) using an alpha of 0.05.

Transcript	Terms	D.F.	*F*-value	*P*-value
DWV	Time	3	8.766	< 0. 0001
	Management	1	7.603	0.007
	Time*Management	3	0.874	0.4578
BQCV	Time	3	8.677	< 0. 0001
	Management	1	0.339	0.562
	Time*Management	3	0.342	0.795
N. ceranae	Time	1	0.002	0.967
	Management	1	0.191	0.663
	Time*Management	1	0.086	0.77
ago2	Time	3	36.982	< 0. 0001
	Management	1	3.439	0.0669
	Time*Management	3	1.226	0.3046
vago	Time	3	4.259	0.0073
	Management	1	10.185	0.0019
	Time*Management	3	0.779	0.5087
hymenoptaecin	Time	3	6.75	0.0004
	Management	1	33.42	< 0. 0001
	Time*Management	3	12.25	< 0. 0001
defensin-1	Time	3	23.23	< 0. 0001
	Management	1	25.34	< 0. 0001
	Time*Management	3	11.38	< 0. 0001
pgrp-lc	Time	3	44.425	< 0. 0001
	Management	1	5.245	0.0243
	Time*Management	3	1.73	0.1663
pgrp-s2	Time	3	10.717	< 0. 0001
	Management	1	24.374	< 0. 0001
	Time*Management	3	6.096	0.0008

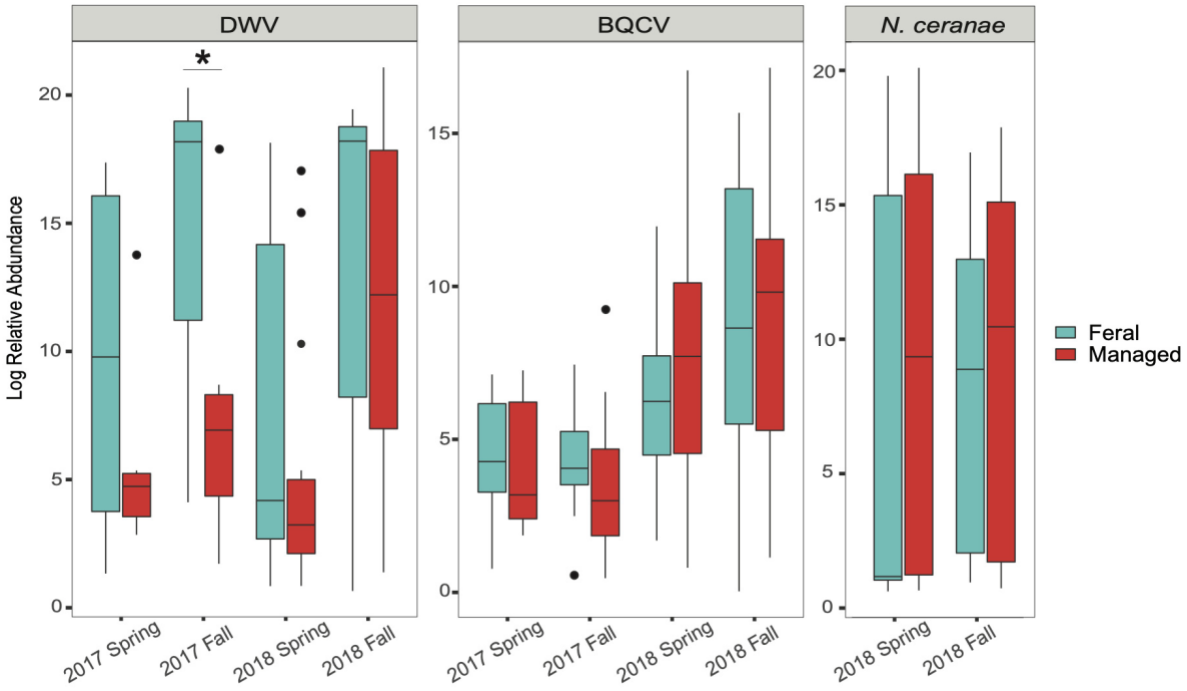

Figure 2.3: Boxplots showing relative abundance of pathogens in feral (blue) and managed (red) honey bee colonies for each sampling period. Relative abundance = $2^{-\Delta\Delta CT}$ values for each colony (calculations described in main text). In 2017, $n = 8$ pairs of colonies. In 2018, $n = 17$ pairs of colonies. Horizontal line of each box represents the median and circles represent outliers. Asterisk denotes statistical significance ($*P < 0.05$).

2.3.2 Immune Gene Expression

In the spring of 2017, feral colonies exhibited higher average expression of five out of the six immune genes tested (*defensin- 1*, *hymenoptaecin*, *pgrp-lc*, *pgrp-s2*, and *ago2*), although pathogen levels were not significantly different between managed and feral colonies (**Table 2.2** and **Figure 2.4**). In the fall of 2017, expression of *defensin-1*, *hymenoptaecin*, *pgrp-s2* remained higher in feral colonies, while the gene *vago* had higher average expression in managed colonies. In 2018, immune gene expression was similar between feral and managed colonies, yet feral colonies had higher average expression of *hymenoptaecin* and *pgrp-s2* in the spring, regardless of the similar levels of pathogens in feral and

managed colonies. No significant differences in average gene expression between feral and managed colonies were observed in the fall of 2018. Interestingly, pathogen pressures were also similar at this time point.

Spearman's correlations (ρ) revealed that DWV levels were positively correlated with the expression of *hymenoptaecin* in the spring ($\rho = 0.32$), but not significantly correlated with any other pathogen levels or immune genes, while BQCV levels were positively correlated with *N. ceranae* levels in the spring ($\rho = 0.48$). *Nosema ceranae* levels were also positively correlated with the expression of *defensin-1* and *pgrp-s2* in the fall ($\rho = 0.39$; $\rho = 0.35$, respectively). However, all correlation coefficients between pathogen levels and immune gene expression were low ($\rho < 0.4$) (Mukaka, 2012), suggesting factors other than pathogen levels likely contribute to immune gene expression (**Table S2.1 & Table S2.2**).

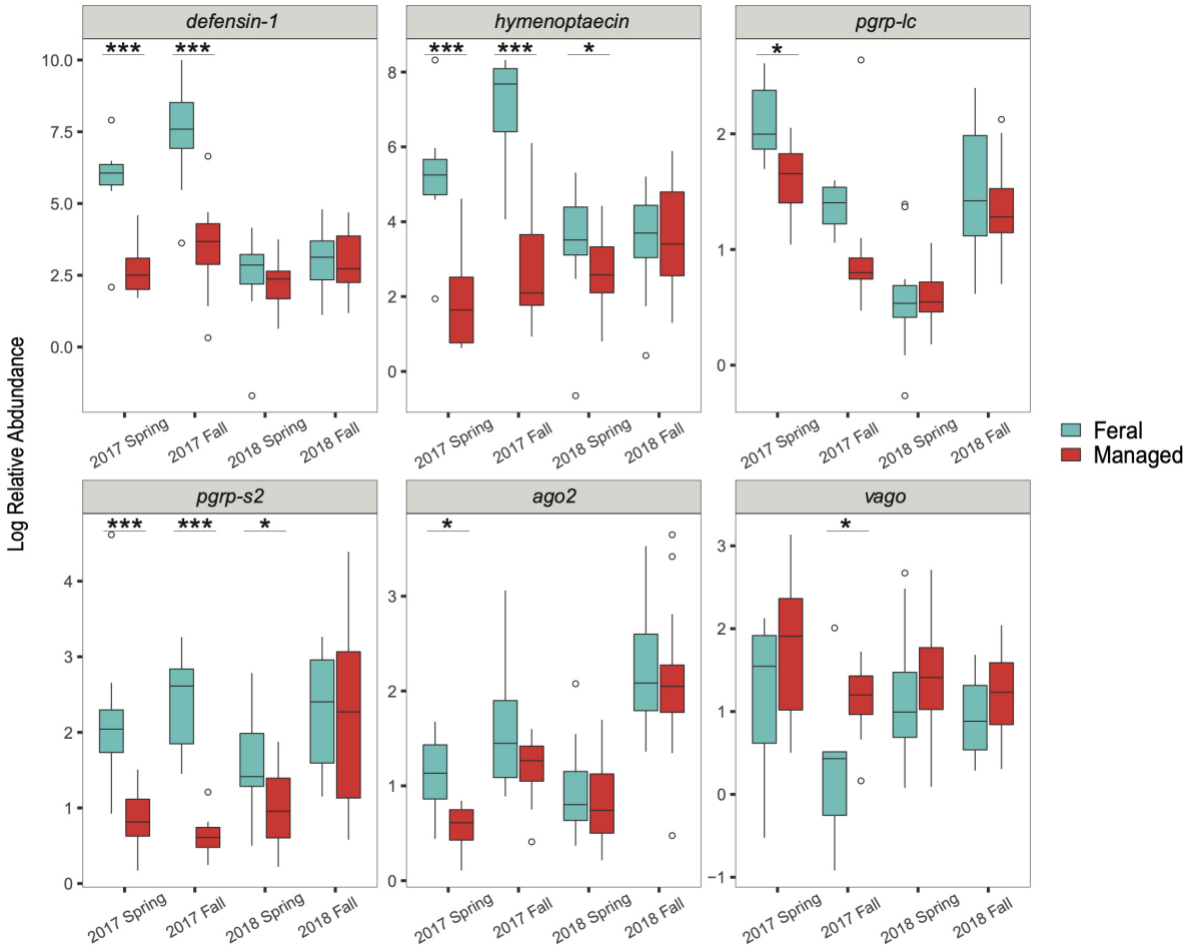

Figure 2.4: Boxplots showing immune gene expression of feral (blue) and managed (red) honey bee colonies for 2017 (n = 8 pairs of colonies) and 2018 (n = 17 pairs of colonies). Relative abundance = $2^{-\Delta\Delta CT}$ values for each colony (calculations described in main text). Horizontal line of each box represents the median and circles represent outliers. Asterisks denote statistical significance (*P < 0.05, ***P < 0.001).

2.3.3 Overwintering Survival

Total survival of colonies over the 2017–2018 winter was 63% for both feral and managed colonies. For the 2018–2019 winter, survival was 47% and 38% for feral and managed colonies, respectively. Of the five feral colonies that survived the 2017– 2018 winter, two also survived the 2018–2019 winter. Two managed colonies were sampled in both years, and one of these also survived the

2018–2019 winter. Despite the similar overall survival between managed and feral colonies, more feral than managed colonies survived with high copy numbers of DWV ($>10^7$) (**Figure S2.2**).

The best predictive model for overwintering survival included management, DWV levels, and expression of *hymenoptaecin*, *vago*, and *pgrp-s2* as fixed effects (AIC = 65.5). The level of DWV in a colony was negatively correlated with overwintering survival (DWV: $P < 0.05$, standard estimation error of coefficient = 0.06). Management and *pgrp-s2* expression were also negatively correlated with overwintering survival, although neither were significant predictors (Management: $P = 0.105$, s.e. = 0.877; *pgrp-s2*: $P = 0.062$, s.e. = 0.51) (**Figure 2.5**). The expression of *hymenoptaecin* and *vago* were both significantly positively correlated with survival (*hymenoptaecin*: $P < 0.05$, s.e. = 0.265; *vago*: $P < 0.05$, s.e. = 0.757) (**Figure 2.5**).

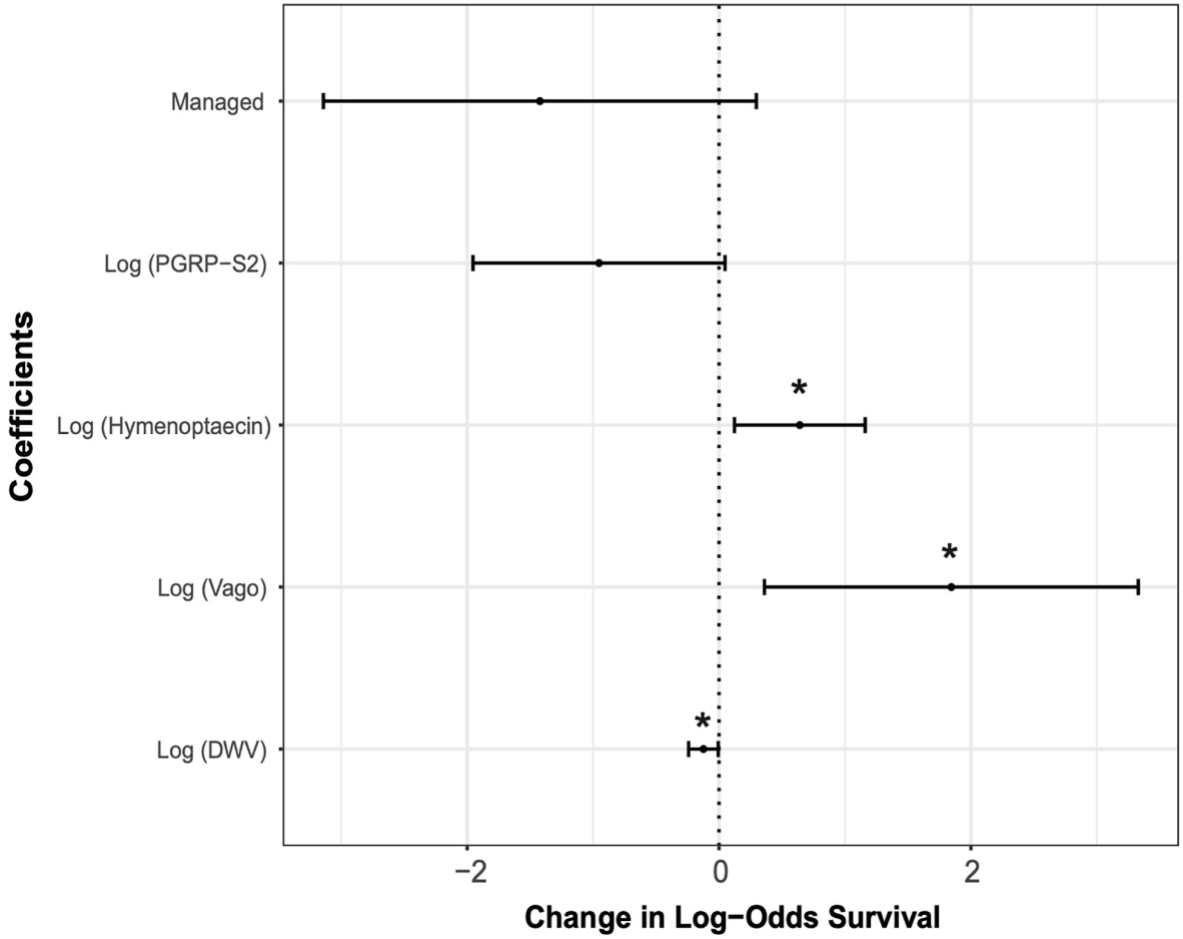

Figure 2.5: Effect sizes of the generalized linear mixed model (GLMM) showing estimates of each variable along with 95% confidence intervals. Asterisks denote variables that were statistically significant predictors of overwintering survival (*P < 0.05). Estimates of fixed effects are: Management = –1.422; Log (*pgrp-s2*) = –0.953; Log (*hymenoptaecin*) = 0.641; Log (*vago*) = 1.845; Log (DWV) = –0.124.

2.4 Discussion

In this study, we investigated the ecoimmunology of feral and managed bees over a 2-year period. Our results show that overall feral honey bee colonies have higher levels of DWV despite yearly and seasonal variation. Alternatively, BQCV and *N. ceranae*, which were constant across seasons, did not differ with management and were not linked to decreased survival in this study. While we did not investigate the transmission efficiencies of pathogens from feral to managed honey bees, our results provide support for the ability of feral colonies to serve as reservoirs of DWV. We also found evidence of higher immune gene expression in feral colonies, even at timepoints when DWV levels were similar between managed and feral colonies. Further analysis of all colonies revealed that levels of DWV infection were positively correlated with the expression of *hymenoptaecin* in the spring and *N. ceranae* levels were correlated with *defensin- 1* and *pgrp-s2* in the fall. The strength of correlations was low suggesting additional factors such as genetic background and environmental conditions play an important role in immune phenotypes. Last, we found significant associations between the expression of two immune genes (*hymenoptaecin* and *vago*) and survival in both feral and managed colonies. These genes have been previously identified as differentially expressed in virus- infected honey bees, but this is the first report of expression being correlated with reduced host mortality (Kuster et al., 2014; Ryabov et al., 2014). Feral and managed colonies also had similar probabilities of survival, despite higher DWV titers in feral than managed colonies.

The survival of feral colonies in the presence of high viral titers suggests that feralization may facilitate the expression of traits that confer virus tolerance. While host resistance limits pathogen replication, tolerance reduces the fitness costs of infection while allowing for pathogen accumulation (Ayres and Schneider, 2012). Higher DWV levels in feral colonies indicate a lack of resistance in these hosts, but the combination of higher accumulation of DWV in feral colonies and similar survival to managed colonies indicates the presence of tolerance. Detectable levels of DWV in all tested colonies suggests that all hosts are susceptible to infection but susceptibility to disease (i.e. overwintering mortality) differs between colonies. Virus tolerance to DWV has previously been observed among mite-resistant honey bees in several locations across many years suggesting that it is heritable (Locke et al., 2014; Russo et al., 2020). Additionally, even without miticidal treatment, several feral colonies had low levels of DWV, suggesting these colonies may exhibit additional traits such as hygienic behavior to reduce the number of *Varroa* mites, and thus viruses present (**Figure S2.2**) (Wagoner et al., 2019). Natural selection is a primary driver of evolution in host–pathogen dynamics and allows organisms to potentially respond to many stressors (Papkou et al., 2019). Management practices of domesticated organisms change the fitness landscape and can alter their evolutionary trajectories (Wilkins et al., 2014; Nygren et al., 2015; Milla et al., 2018). Managed honey bees are typically under different environmental conditions compared to their feral counterparts, and while beekeeping practices often aim to limit pathogen abundance, these practices may relax the selective pressures that pathogens exert on managed colonies, potentially delaying host-parasite coevolution (Neumann and Blacquière, 2017). While low input beekeeping management reduces pathogen burdens and oxidative stress compared to commercially managed colonies, highly invasive beekeeping practices may cause added stresses (Taric et al., 2019). Even though the degree of disease management varies greatly among beekeepers (Underwood et al., 2019), *Varroa* mite management may inhibit adaptations for decreased susceptibility to mites and viruses in managed honey bee colonies (Blacquière et al., 2019). In addition to virus tolerance, other traits such as small colony sizes, frequent swarming, and increased hygienic and grooming behavior appear to be critical for the survival of feral colonies in the presence of high pathogen pressure

(Gramacho and Spivak, 2003; Seeley, 2007; Locke, 2016; Loftus et al., 2016; Russo et al., 2020). Future studies should focus on providing information about the molecular mechanisms of disease tolerance in these feral colonies to directly test for the role of feralization in altering selective pressures and leading to different immune phenotypes in honey bees.

Although mechanisms of virus tolerance are still unknown for insects, one possible explanation is the ability of highly infected bees to limit the overreaction of immune responses. Immune effectors, such as pro-phenoloxidase, produced by insects can have cytotoxic effects that work to limit infections, but also cause damage to host tissues (Sadd and Siva- Jothy, 2006; Hillyer, 2016). This self-damage can lead to increased aging and higher mortality as a result of increased inflammation (Alaux et al., 2010; Khan et al., 2017). Virus-tolerant colonies may therefore have mechanisms to limit inflammation-induced damage. A second possible mechanism includes transgenerational immune priming, the development of immune memory via vertical transmission of immunological experiences, and its effects have been demonstrated in several invertebrates (Tetreau et al., 2019). Vertical transmission of DWV and the fact that queens may preside over a colony for several years would favor immune-primed offspring in feral colonies, in contrast to beekeeping operations where queens are replaced frequently. Another potential mechanism includes changes in the virulence of DWV and *Varroa* mites in feral colonies. Genotypes of DWV are known to differ in virulence and *Varroa* mite transmission can favor certain strains of the virus (Ryabov et al., 2019). While our study did not assess *Varroa* mite levels or the genetic diversity of DWV, previous work showed that *Varroa* mites from managed colonies had increased population growth compared to mites from feral colonies (Dynes et al., 2020), providing evidence for the role of management in selecting for mites with greater reproductive rates. While the specific mechanisms are unclear, the absence of human management and the process of feralization may lead to changes in virus virulence and host tolerance in honey bees.

In addition to different selective pressures experienced by feral and managed colonies, feral colonies can also have different genetic backgrounds, contributing to differences in immune phenotypes

and disease outcomes. Previously, López-Uribe et al. (2017) showed that feral and managed honey bees exhibit some genetic differentiation even at short geographic scales. Although we provide evidence for differential immune gene expression between feral and managed colonies, the role of ancestry and genetic diversity in this difference remains unclear. Immune gene expression is heritable in honey bees and virus tolerance may have a heritable basis as well, making it plausible that different genetic backgrounds play an important role in the different immune phenotypes observed between feral and managed colonies (Decanini et al., 2007; Thaduri et al., 2019). The underlying variability and heritability of immunological traits combined with different selection regimes may lead to different evolutionary trajectories for feral and managed honey bees.

We found higher expression of several immune genes in feral colonies compared to managed colonies, suggesting that feralization has led to increased pathogen defenses, although this was not true for all genes and differed with time of sampling. Specifically, we found that the expression of *hymenoptaecin* was higher in feral colonies, while the expression of *vago* was higher in managed colonies. However, both genes were associated with increased colony survival. Hymenoptaecin is a general AMP involved in responses to many pathogens including DWV and *Varroa* mites (Evans et al., 2006; Kuster et al., 2014; Brutscher et al., 2015; Wu et al., 2020). Other studies have shown that this gene is consistently upregulated in response to pathogens and during wounding events in honey bees and may be a potential biomarker to quantify honey bee health (Galbraith et al., 2015; Brutscher et al., 2017; Doublet et al., 2017; Zanni et al., 2017). We also identified the expression of *vago* as important for the overall survival of colonies. This transcript is expressed upon activation of the RNAi pathway, which recognizes the dsRNA of viruses and leads to the increased expression of *vago* or its orthologs in mosquitoes, fruit flies, bumble bees, and honey bees (Deddouche et al., 2008; Paradkar et al., 2012; Ryabov et al., 2014; Niu et al., 2016). In DWV-infected honey bees, *vago* expression has been shown to be significantly increased, providing evidence for its role in antiviral responses (Ryabov et al., 2014). To our knowledge this is the first report of *vago* expression being directly linked to increased survival in honey bees. These two genes could be considered biomarkers of honey bee health that can be used to

predict the ability of a colony to survive the winter (López- Uribe et al., 2020). Additionally, genome-level studies looking for signatures of selection at regulatory regions of immunity in honey bees may also provide important information on mechanisms of pathogen resilience.

Feral organisms offer valuable systems to study potential negative consequences of domestication and anthropogenic influence by examining host–pathogen interactions of organisms that recently escaped managed conditions (Burdon and Thrall, 2008; Gering et al., 2019b). Previous studies of feral honey bees have examined levels of mite infestation, pathogen pressures, or the combination of pathogen pressures and immune gene expression, but the association of host–pathogen dynamics with colony survival was not previously investigated (Seeley, 2007; Thompson et al., 2014; Youngsteadt et al., 2015). Here, we quantified pathogen levels, immune gene expression, and linked this to overwintering survival in managed and feral honey bee colonies. This allowed for the identification of specific genes associated with overwintering survival of honey bees and evidence of virus tolerance in feral colonies, linking immunity, infection, and survival under natural conditions. Further identification of the genetic mechanisms of virus tolerance and biomarkers of bee health can help breeding efforts to focus on increasing these traits in selected honey bee stocks (e.g., Robertson et al., 2020), thus decreasing overall colony losses for the beekeeping industry. Future studies should assess the role of feralization on pathogen dynamics and ecoimmunology in other domesticated species.

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2.6 Supplemental Material

Supplementary Data Sheet 1 can be found at:

https://www.frontiersin.org/articles/10.3389/fevo.2020.594263/full#supplementary-material

Supplemental Tables

Table S2.1: Spearman's correlations of pathogens and immune genes for combined data from feral and managed colonies sampled in 2017 and 2018, sampled in the Fall. Asterisks denote statistical significance ($^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.001$).

Fall	DWV	BQCV	N. ceranae	Def-1	Hym	PGRP-LC	PGRP-S2	Ago2
BQCV	0.23							
N. ceranae	0.1	0.25						
Def-1	0.06	-0.1	0.39**					
Hym	0.14	-0.02	0.32*	0.75***				
PGRP-LC	0.12	0.18	0.33*	0.09	0.23			
PGRP-S2	0.17	0.19	0.35**	0.39***	0.67***	0.32**		
Ago2	0.25*	0.25*	0.24	-0.01	0.11	0.37**	0.54***	
Vago	-0.19	0.09	0.25	-0.13	-0.22	0.1	0.02	0.2

Table S2.2: Spearman's correlations of pathogens and immune genes for combined data from feral and managed colonies sampled in 2017 and 2018, sampled in the Spring. Asterisks denote statistical significance ($^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.001$).

Spring	DWV	BQCV	N. ceranae	Def-1	Hym	PGRP-LC	PGRP-S2	Ago2
BQCV	0							
N. ceranae	0.21	0.48**						
Def-1	0.19	-0.1	0.09					
Hym	0.32**	0.1	0.12	0.7***				
PGRP-LC	0.25*	-0.19	0.1	0.54***	0.31**			
PGRP-S2	0.18	0.06	0.07	0.71***	0.9***	0.35**		
Ago2	-0.2	0.33*	0.09	0.35**	0.29**	0.14	0.39**	
Vago	0.05	0.16	0.29	-0.12	-0.22	0.19	-0.19	0.34**

Supplemental Figures

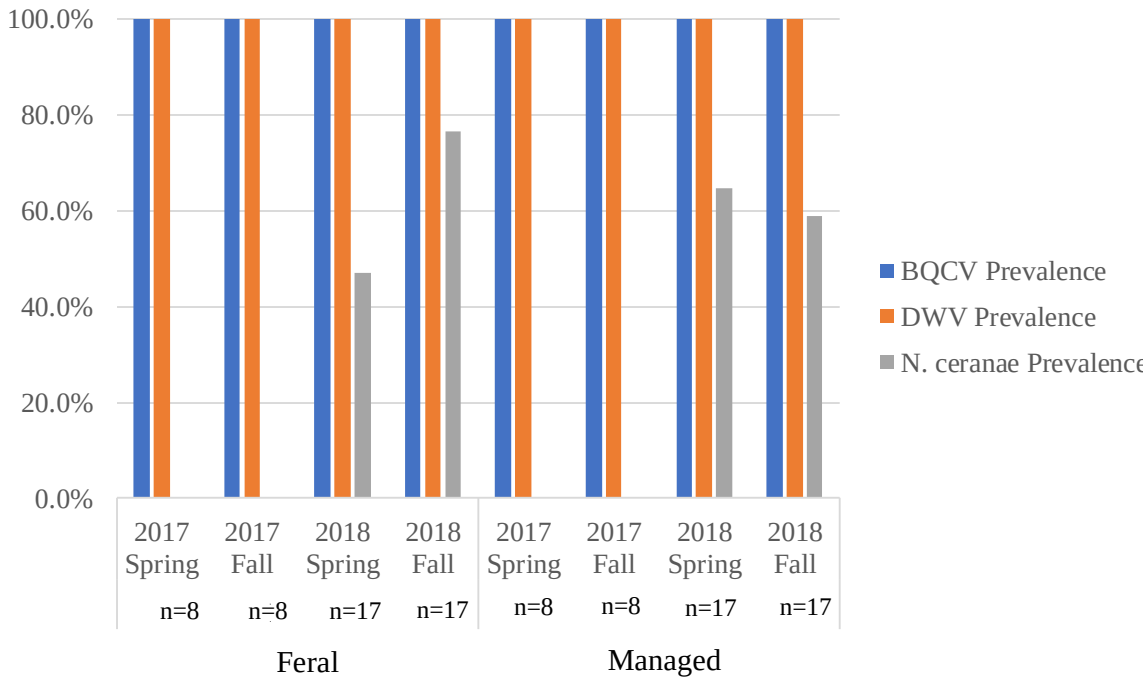

Figure S2.1: Prevalence of pathogens in feral and managed colonies. Deformed wing virus (DWV) and Black queen cell virus (BQCV) were detected in all colonies at all times of sampling. Presence of *Nosema ceranae* was tested for in colonies sampled in 2018, but not in 2017. *Nosema ceranae* was found in 47.1% and 76.47% of feral colonies in spring and fall, respectively, while 64.7% and 58.8% of managed colonies tested positive for *N. ceranae* in spring and fall, respectively.

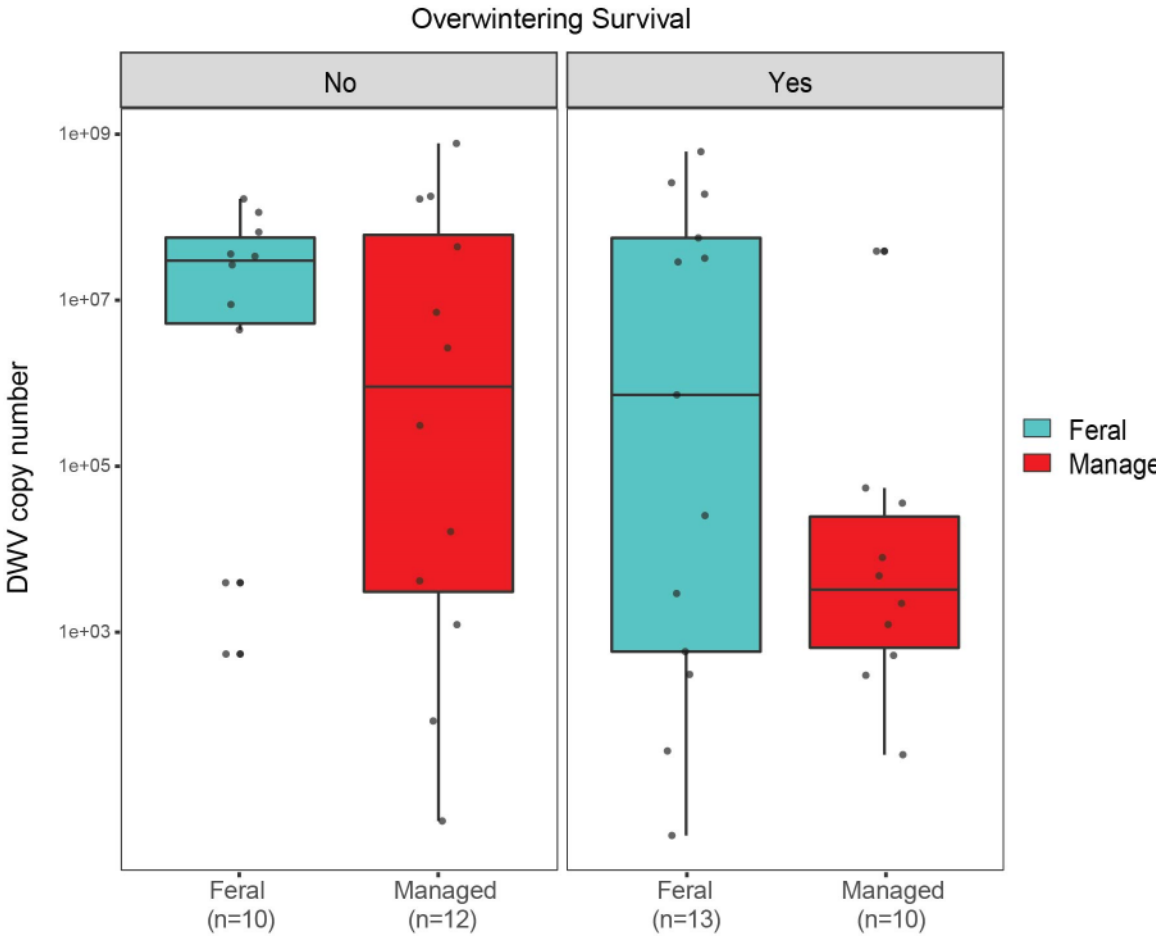

Figure S2.2: DWV copy number in feral and managed honey bee colonies in colonies that died (No) and survived (Yes). The effects of management, overwintering survival, and their interaction were analyzed using GLM, followed by ANOVA to assess their influence on DWV copy number (management: df = 1, F = 2.895, P = 0.096; overwintering survival: df = 1, F value = 4.281, P = 0.045).

Chapter 3

Plant Virus Impacts on Yield and Plant-Pollinator Interactions are Phylogenetically Modulated Independently from Domestication.

Chapter 3 is currently in preparation for submission to *Phytopathology*.

Plant defenses are conserved in phylogenetically related species. Domestication can alter host genotypes through artificial selection with potential losses in host defenses. Therefore, both domestication and host phylogenetic structure may influence plant virus infection outcomes. Here, we examined the association of phylogeny and domestication with the fitness of infected plants. We inoculated three pairs of domesticated and wild/non-cultivated squash (*Cucurbita* spp.) with a combination of zucchini yellow mosaic virus (ZYMV) and squash mosaic virus (SqMV) and recorded fitness traits related to flowers, pollination, fruit and seed viability in the field over two separate years. In a separate field experiment we also recorded foliar symptom severity and relative abundance of both viruses. We found a gradient of susceptibility across the 6 tested lineages, and phylogenetic relatedness, but not domestication, contributed to differences in infection outcomes and impacts on several fitness traits. The traits most strongly impacted by infection were fruit number, fruit weight and germination. Virus infection also impacted the quantity and quality of floral rewards and visitation rates of specialist bee pollinators. There were no detectable differences in viral load between the 6 host taxa suggesting differences in susceptibility were attributable to tolerance not resistance. Our results highlight the importance of phylogenetic relatedness in predicting host susceptibility to disease across wild and domesticated plants and the ability of tolerant hosts to maintain fitness in the field. Broader consequences of

plant pathogens for beneficial insects, such as pollinators, should also be considered in future research.

3.1 Introduction

With the devastating impacts caused by plant pathogens, many studies have investigated the evolution of resistance genes in plants, which inhibit pathogen replication or spread within a host (McDowell and Woffenden, 2003). This has contributed to a growing body of knowledge on the coevolutionary nature of plant-pathogen relationships and how resistance genes are gained and lost within and between taxonomic groups of plants (Pan et al., 2000; Zhang et al., 2010; Jia et al., 2015; Menz et al., 2020). Yet, comparatively far fewer studies have focused on competent hosts to determine how continuous responses of disease severity might differ with phylogenetic relationships or an evolutionary history of domestication (Gilbert et al., 2015; Ferrero et al., 2019).

Among crops, the domestication processes can drastically alter how plants respond to pathogens. The loss of traits related to plant defenses to pathogens and herbivores is often one of the unintended consequences of domestication as cultivars lose genetic diversity through breeding (Lenne and Wood, 1991; Moreira et al., 2018; Singh and van der Knaap, 2022). Evidence from genome analyses in sunflower and tomato shows that domestication has resulted in decreased numbers of disease resistance genes (Hübner et al., 2018; Gao et al., 2019). Crops also show increased susceptibility to bacterial pathogens (Córdova-Campos et al., 2012; Jaiswal et al., 2020). One possible explanation is the presence of fitness costs associated with maintaining resistance genes, including decreased fecundity (Tian et al., 2003). However, in some cases domesticated plants have moderate or no differences in susceptibility compared to wild relatives (Nygren et al., 2015; Ferrero et al., 2019). Agricultural production focuses on strategies that

prevent the exposure of crops to pathogens and thus, reduced encounters with viruses may lessen the benefits of defenses in domesticated hosts (Lecoq and Katis, 2014). Yet, eliminating pathogen exposure is rarely possible and pathogens have caused agronomic damage since the dawn of agriculture. Thus, selection for less susceptible varieties is likely constantly occurring, contributing to mixed evidence of increased susceptibility in domesticated plants.

Studies of plant viruses often differ in how they define fitness, which may be measured differently in wild and crop plants. Plant virus infections are usually non-lethal but can have effects on many traits which may be relevant for wild ecosystems or crop production (Malmstrom and Alexander, 2016). Individual plant fitness ultimately refers to fecundity or viable progeny production but for crops the most meaningful fitness traits are yield and vigor which are not always directly related to reproduction (Abbai et al., 2020). Pathogenic plant viruses often induce mosaic symptoms in the leaves or other alterations in the appearance of vegetative structures. Thus, visible leaf symptoms are often used as proxies of plant fitness even without empirical evidence of their relationship with fecundity or population-level fitness (Prendeville et al., 2014b; Bock et al., 2022). While distortions in plant appearance are relatively easy to assess, visual symptoms do not always have a strong relationship with either viral load or plant fitness, and thus many assessments of susceptibility are incomplete (Rodriguez-Cerezo et al., 1991; Gal-On, 2007; Handford and Carr, 2007; Pagán et al., 2007). Additionally, many wild and crop plants rely on insects for pollination and marketable yields or seed set. Therefore, declines in plant fitness can be driven by a lack of insect visitation but plant-pollinator interactions are rarely studied in the context of plant virology (Prendeville and Pilson, 2009; Groen et al., 2016; Murphy et al., 2023).

Here, we characterize viral infection outcomes in wild and domesticated plants in the genus *Cucurbita* to determine how disease susceptibility is impacted by domestication or phylogeny. We inoculated plants with two viruses that commonly coinfect cucurbits: zucchini yellow mosaic virus (ZYMV; *Potyviridae*) and squash mosaic virus (SqMV; *Secoviridae*). In

2019 and 2021, we assessed 15 traits related to plant fitness (in the general categories encompassing flowers, pollination, fruits, seeds) on infected and healthy plants of three wild and three domesticated *Cucurbita.* We hypothesized that domesticated lineages are more susceptible to disease than wild plants because of losses of key defense traits and genetic diversity during the domestication process. Interestingly, members of Cucurbitaceae contain a limited number of resistance genes compared to other plant families, suggesting that mechanisms of tolerance could be important for host defenses (Lin et al., 2013). In 2020, using a similar experimental design we quantified virus levels and foliar symptoms to provide insight on whether differences in susceptibility are attributed more to tolerance or resistance across the different focal lineages. Our results show that host taxa exhibit a gradient in their degree of susceptibility to viruses, but domestication was not associated with susceptibility. In contrast, we found that infection outcomes are associated with host phylogenetic relatedness for several plant traits suggesting that the ability of plants to respond to these viruses is evolutionarily constrained. Our results also indicate that plant virus infections can have off-target effects that alter pollinator behavior and have consequences for pollinator nutrition.

3.2 Materials and Methods

3.2.1 Study System

The genus *Cucurbita* offers a model system to study the effects of domestication on virus susceptibility as this group of plants has been domesticated at least 6 independent times, and was one of the earliest cultivated plant groups in the Americas approximately 10,000 years ago (Smith, 1997; Castellanos-Morales et al., 2018). The most widely cultivated *Cucurbita* spp. in

North America are *Cucurbita pepo*, *C. moschata*, and *C. maxima*, which comprise hundreds of cultivated varieties of squash, pumpkins and gourds (Ferriol and Picó, 2008; Hernandez et al., 2023). Seeds for one of the wild plants used in this study are commercially available (*C. maxima andreana*), but they are not cultivated on a large scale, whereas all the domesticated taxa are commonly cultivated (**Table S3.1**). Our choice to plant taxa in a field setting allowed us to use a rigorous experimental design and collect large amounts of data from plants in the same field. None of the taxa used in our experiments are known to contain genes for qualitative resistance to viruses and represented competent hosts for both viruses of interest. Our choice of host taxa was also aided by the availability of a comprehensive phylogeny of *Cucurbita*, allowing us to perform phylogenetic comparative analyses when investigating responses to virus infection (Kates et al., 2017).

We investigated the fitness effects of two viruses endemic to the study area that co-infect cucurbits. Zucchini yellow mosaic virus (ZYMV) and squash mosaic virus (SqMV) produce similar symptoms and are transmitted horizontally by aphids and beetles, respectively, which are pests frequently infesting these plants, and were observed on all plant taxa in our experiments. Both viruses are also transmitted through seed at rates high enough to be significant sources of outbreaks (Alvarez, 1978; Simmons et al., 2013).

3.2.2 Field Experimental Design

In preparation for planting in the field, seedlings from all six taxa were inoculated with a combination of ZYMV and SqMV and grown in a greenhouse with 16:8hr light:dark setting. Plants were inoculated at the three true leaf stage using as inoculum source coinfected leaves from symptomatic *C. pepo* ssp. *ovifera* var *texana* collected from a natural field epidemic in PA, USA (Harth et al., 2018). Leaves used for inoculum had ZYMV and SqMV infection confirmed

by ELISA based Immunostrips® (Agdia Inc., Elkhart, IN) tests. Symptomatic leaves were then homogenized in phosphate buffer (0.1 M Na2HPO4·2H2O + 0.1 M KH2PO4) using a mortar and pestle containing activated carbon and carborundum. The pestle was dipped in the inoculum and rubbed on the adaxial leaf surface of each seedling. The rest of the plants were mock-inoculated using the same procedure but using buffer without leaf tissue to serve as controls. Approximately 14 days post inoculation (dpi), we transplanted 16 plants of each treatment (12 total treatments) into the field at Russel E. Larson Agricultural Research Station at Rock Springs, PA, USA. We used a split-plot design with four replications, each plot containing six rows. Each row contained one taxon chosen at random with four virus-inoculated plants next to four mock-inoculated plants. We randomized the orientation of inoculated and mock-inoculated plants for each taxon in each block. In total, we grew 32 plants of each taxon (16 virus-inoculated, 16 mock-inoculated). This design was replicated in 2019, 2020, and 2021 at three field sites located within 1km of each other. After transplanting, we did not control for additional pathogen transmission in the field and by the end of the experiments many uninoculated plants had symptoms of virus infection. However, timing of infection is important for viral symptom development (Blua and Perring, 1989) and natural infections of uninoculated plants in the field happened later in the season, allowing for the detection of differences in host traits between originally inoculated and uninoculated plants.

3.2.3 Categorization of Susceptibility

To determine if differences in infection outcomes were attributed to differing levels of virus accumulation in each taxon, we recorded foliar symptom severity and viral load of individual plants in the field during the 2020 field season. Symptom severity was recorded ~60 dpi and ranked on a scale of 0-4 based on visual symptoms using a modified protocol from Kone

et al., 2017. Each plant was assessed individually for symptoms and assigned a value: 0 = No disease symptoms; 1 = Mild mottling on ≤ 10% of leaf area; 2 = Mottling on ≤ 50% of leaf area/light downward cupping; 3 = Pronounced downward or up cupping of leaf/chlorosis/75–100% leaf mottling; 4 = Severe mottling/severe distortion of leaf/crinkled leaf/stunting of entire plant/leaf bunching. (**Figure S3.1**). Only a subset of plants that were rated for symptom severity were analyzed for viral load.

To estimate viral load per plant, we collected leaf samples ~60 dpi from symptomatic leaves on three separate branches of the same plant using ~1cm metal hole punchers and combined samples into 2ml screw-cap tubes containing 300µl of RNAlater (Sigma-Aldrich, St. Louis, MO, USA). Samples were then stored at –20°C until RNA could be extracted using a *Quick*-RNA™ Plant Miniprep kit (Zymo Research, Irvine, CA, USA). A total of 2µl RNA from each sample was used as template to produce cDNA using random primers and MultiScribe RT, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, United States). We then performed virus quantification via duplex probe RT-PCR using Eukaryotic 18S rRNA Endogenous Control primer/probe set (ThermoFisher, Waltham, MA, USA) and custom primers and probes for either virus. Each 10µl reaction contained 5µl of iTaq Universal Probes supermix, 2µl cDNA, and rRNA endogenous control mix at a final concentration of 150nM primers and 250nM probe, and primers and probe for either virus at a final concentration of 200nM primers and 150nM probe (Primer sequences in **Table S3.2**). Reactions testing for ZYMV and SqMV were run in separate wells. We carried out reactions in triplicate in 384-well plates using a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Reaction conditions were as follows: 2 minutes at 95°C for initial denaturation, then 40 cycles of 15 s at 95°C for denaturation, and 60 s at 60°C for extension, and data collection. We determined the cycle threshold (Cq) value for each sample by taking the mean of the three technical replicates.

Amplification of both primer/probe sets was still detected in virus-free control plants. However, we determined a limit of detection (ΔCq value ≥ 20 for ZYMV; ΔCq value ≥ 15 for SqMV) and removed samples that produced ΔCq values above this limit or samples from asymptomatic plants before analysis of viral load. This was deemed acceptable, as we were only interested in quantifying the relative abundance of viruses between plants. We then used the Pfaffl method to calculate the relative expression ratio of either virus (Pfaffl, 2001). The taxon with the lowest average virus gene expression (highest average ΔCq) was used as the calibrator and calculations were performed separately for each virus. We then calculated the ratio of the expression of the viral gene of interest divided by the expression of the housekeeping gene, producing a value of fold change compared to the control group.

3.2.4 Pollinator Behavior and Floral Traits

To identify the impacts of virus infection on pollination, we quantified visitation rates of the two main pollinators in the study area: bumble bees and squash bees. Bumble bees such as the common eastern bumble bee (*Bombus impatiens*) are generalist pollinators that collect resources from many different families of plants and consume nectar but not pollen from cucurbits due to defenses in cucurbit pollen (Brochu et al., 2020). In contrast, squash bees, *Eucera* (*Peponapis*) *pruinosa*, collect pollen exclusively from cucurbits, which is required for their development, and acquire nectar mainly from cucurbits but also other plants. To quantify pollinator visitation to flowers, we performed in-person observations on a group of four plants from the same treatment during ten-minute intervals. We recorded the type of bee, the type of visitation, and the sex of the flower visited. We defined landing events as those where bees landed on a flower but left within one second and were not seen collecting any resources, while all other events were recorded as resource (nectar or pollen) acquisition events. We avoided counting the same bee multiple times

when possible and recorded the total number of staminate and pistillate flowers in each observed plot. We used these flower counts as an estimate of daily flower production. We visited plots in a randomized order each day and always observed both virus-inoculated and mock-inoculated plants of a taxon from a block in the same day. All observations took place from 6:30am-10:30am when pollinators are most active. In 2019, we conducted pollinator observations and flower counts over 19 days between July 25 and August 23, and in 2021 observations were over 17 days between July 15 and August 14. For statistical analysis, we divided the number of landing or nectaring events by the total number of flowers to get an estimate of visitation type per flower for a plot. We did not have enough observations of pollen collection events to perform statistical analyses.

In 2021, we quantified floral rewards from infected and uninfected plants of all taxa. The day before anthesis, we covered female flowers with a mesh bag and placed twist ties on male flowers to prevent access by pollinators the following day. The next morning, we removed flowers and immediately collected pollen by scraping the anther with the wooden end of sterilized cotton swabs into 1.7ml microcentrifuge tubes. We then used microcapillary tubes to collect nectar from flowers into microcentrifuge tubes and stored samples at -20°C until analyses could be performed. For this sampling, we paired each inoculated plant with a mock-inoculated plant of the same genotype and sampled them at the same time of day. We pooled pollen and nectar from three flowers of each plant and sampled a minimum of five inoculated and mock-inoculated plants per taxon.

To obtain an estimate of pollen production per flower, we first lyophilized samples until all water was removed using a Labconco FreeZone 18L Lyophilizer (Labconco, Kansas City, MO, USA). We then recorded dry weight of samples using an XP26 microbalance (Mettler Toledo, Columbus, OH, USA). We then performed a Bradford assay with 1mg of pollen per sample using Bio-Rad Protein Kit Assay I (Bio-Rad, Hercules, CA, USA). We performed all

reactions in triplicate in 96 well plates and included standard curves of known quantities of protein standard (bovine γ-globulin) in each plate. For nectar analysis, we recorded the volume of nectar from each sample using microcapillary tubes. To measure the concentration of sucrose, glucose, and fructose in each nectar sample we used the Megazyme Sucrose/D-Fructose/D-Glucose Assay Kit (Neogen®, Lansing MI, USA). Nectar was diluted 1:10 using Milli-Q water and sugars were analyzed according to the manufacturer's protocol. The protocol was modified to support smaller volumes in 96 well plates. We used one µl of nectar for each reaction and ran reactions in triplicate with a standard curve of known sugar quantities in each plate.

3.2.6 Statistical Analysis

3.2.6.1 Viral load and symptom severity:

We conducted all analyses in R version 4.2.2 (R Core Team 2022). We characterized the differences in relative amounts of virus across taxa using a generalized linear mixed model (GLMM) with the package 'lme4' (Bates et al. 2015) using relative viral load as the response variable, taxon as a fixed effect, and block in the field as a random effect. We ran separate models for the relative abundance of each virus and the ratio of ZYMY:SqMV in plants sampled at approximately 60 dpi. We checked that the data met model assumptions by assessing the normality of the residuals and equal variance. In cases where these assumptions were not met, data were log transformed or log(x+1) transformed for traits which contained zeros in the dataset. To answer whether average symptom severity is different between taxa, we ran a cumulative link mixed model (CLMM) using the 'ordinal' package (Christensen 2016) as these models are appropriate for ordinal categorical data. The model included taxon as fixed effect and block as a random effect. Before analysis, we converted severity ratings to categorical data as they represent

broad categories, not continuous measurements. For all models, we then calculated the estimated marginal means with the 'emmeans' package (Lenth 2023) that summarize the mean response for each category of a factor and are adjusted for other variables in the model while avoiding imbalances in the data by giving equal weight to each comparison in the reference grid. We used the estimated marginal means for *post hoc* tests with a Šidák adjustment (Šidák 1967) for multiple comparisons to determine differences between taxa (**Figure 3.1**; **Figure S3.3**). For both symptom severity and viral load variables, we then ran analyses to determine if domestication status was a significant predictor. Models were run as above, except with domestication status instead of taxon as the fixed effect. We also tested for general relationships between foliar symptom severity and viral load using CLMMs with severity as the response variable, each viral load variable as a fixed effect, and taxon and block as random effects.

3.2.6.2 Fitness traits:

To determine if virus infection impacted the different trait values and how domestication status or phylogenetic distance are associated with these responses, we ran generalized least squares using the 'nlme' package (Pinheiro et al. 2023). This allowed us to fit regressions for each trait looking at the interaction between virus infection and domestication status. Before analysis, we calculated the average trait value per block separately for infected and uninfected plants of each taxon. These per-block averages were used as the response variable in subsequent analyses, accounting for variation between blocks in the field. We also ran GLMMs on datasets that were not averaged over blocks using block as a random effect in the models and received similar results to GLS, so we focus our reporting on GLS methods.

To assess if there was phylogenetic signal in the response to infection, we ran models with or without phylogenetic correlation structure (Pagel's lambda and Ornstein-Uhlenbeck)

(Symonds & Blomberg 2014). We used a phylogeny generated in a previous study on multiple *Cucucrbita* spp., and we trimmed the tips of the phylogeny to only include only taxa used in our study (Kates et al. 2017). We then performed model comparison based on the small-sample size corrected Akaike Information Criterion (AICc) score to determine which models performed best based on a delta > 4 (Burnham & Anderson 2002). In cases where the delta between the two best models was < 4, we used the model that included Pagel's lambda correlation structure. When the best model included phylogeny, we also report alpha or lambda as a measure of phylogenetic signal for Ornstein-Uhlenbeck and Pagel's lambda models, respectively.

After model comparison, we used ANOVA on the output of the best model to determine if virus infection or the interaction between virus infection and domestication status was a significant predictor of trait values at $p < 0.05$. Since the interaction between infection and domestication was not significant for any trait in either year, the models used for further analysis only include virus infection as a fixed effect. We then used the package 'emmeans' (Lenth 2023) to perform pairwise comparisons and generate contrasts between infected and uninfected plants, resulting in an effect size of the influence of virus infection on each trait (**Figure 3.2**). Additionally, to generate effect sizes for individual taxa for each trait, we ran separate generalized linear models with the interaction of species and infection status as a fixed effect. We then used 'emmeans' to generate pairwise comparisons between infected and uninfected plants in each taxon (**Figure 3.3**).

We started by analyzing data from 2019 and 2021 separately and noticed a stronger effect of infection in the magnitude and number of traits in 2021, therefore, we report results from each year separately. Additionally, for traits that were recorded in both years we combined datasets and ran GLS models with the interaction of infection status and year as fixed effects. When running analyses, we performed model diagnostics as above and log or log(x+1) transformed response variables in cases where it improved model assumptions.

3.2.5 Fruit and Seed Traits

We estimated fruit number, fruit weight, defective and non-defective seed number, and seed germination rate for each treatment to assess plant reproductive fitness. Fruit number and fruit weight were measured at the end of the season by counting the number of mature fruit and weighing in the field using an ElectroSamson hanging scale, respectively (Brecknell, Fairmont, MN, USA). We used the average fruit weight per plant for all analyses. To quantify seed traits, we removed a subset of fruits from the field (at least from 5 plants per species per treatment) and harvested and dried seeds, taking enough fruit from each plant to obtain at least 200 seeds. After drying, we first weighed 50 seeds per plant, including seeds that appeared defective – because they either likely lacked viable endosperm or were deformed. We then divided this number by 50 to calculate individual seed weight per plant. We then weighed all seeds from that plant and divided this by the estimated weight of an individual seed to estimate the total number of seeds per plant. We adjusted this estimate multiplying by the proportion of defective seeds out of 50, producing total seed counts that were adjusted for the number of defective seeds. We then divided this by the number of fruits per plant to ultimately obtain the average number of viable seeds per fruit per plant which we used for analyses along with the number of defective seeds per 50 seeds.

Additionally, we performed germination tests with seemingly viable seeds from each plant. We placed 50 seeds onto wet paper towels inside plastic petri dishes with tight-fitting lids. We then placed containers in a growth chamber with an 8 hr:16 hr, 30°C: 20°C, and light:dark cycle. We checked the dishes periodically and moistened the towels if they appeared dry. After 7 days of incubation, we recorded the percentage of germinated seeds for each plant. We conducted these germination trials three separate times for a total of 150 seeds and used the average of all three trials as the germination rate for statistical analyses.

3.3 Results

3.3.1 Hosts Differ in Symptom Severity but not Viral Load

We found that both ZYMV and SqMV efficiently accumulated in all taxa after 60 days, but we did not detect differences in the average relative level between taxa for ZYMV (X^2 = 4.49; df = 5; p = 0.48), SqMV (X^2 = 2.83, df = 5, p = 0.73), or the ratio of ZYMV:SqMV (X^2 = 9.14, df = 5, p = 0.1) (**Table S3.3**). However, we found significant differences in symptom severity between taxa (F = 5.95, df = 5, p < 0.001) with *C. pepo ovifera* var. *texana* and *C. pepo ovifera* var. *ovifera* having the most severe symptoms (**Figure 3.1**; **Table S3.3**). When analyzing the relationship between viral load and symptom severity, we found no significant associations between severity and levels of ZYMV (F = 2.6, df = 1, p = 0.1), SqMV (F = 0.18; df = 1, p = 0.67), or the ratio of ZYMV:SqMV (F = 1.3, df = 1, p = 0.25) (**Table S3.4**). We also found that domestication status was not significantly associated with symptom severity (F = 0.61; df = 1, p = 0.4) or viral load (ZYMV: [X^2 = 1.75; df = 1, p = 0.19]; SqMV [X^2 = 1.36; df = 1, p = 0.24]; ZYMV:SqMV [X^2 = 2.97; df = 1, p = 0.08]) (**Table S3.4**; **Table S3.5**).

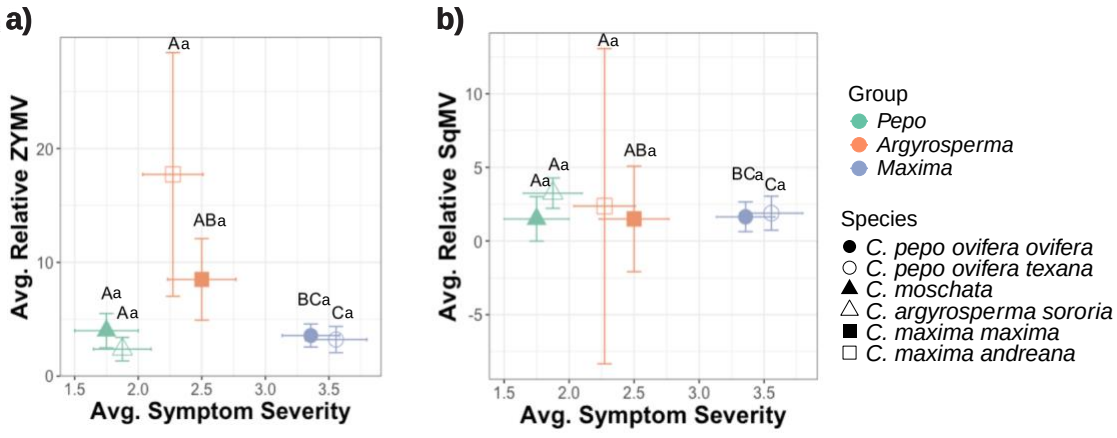

Figure 3.1: Average relative viral load of (**A**) ZYMV or (**B**) SqMV and average foliar symptom severity for different taxa measured in the field ~60 days post inoculation with bars indicating +/-

one standard error. Results of linear mixed models indicate that individual taxa differ in symptom severity but have similar viral loads quantified by RT-qPCR. Different letters indicate significantly different groups at alpha=0.05. Uppercase letters are used for symptom severity and lower-case letters are used for viral load. Taxa and phylogenetic groups are indicated by shape and color, respectively. Open shapes represent wild taxa and filled shapes represent domesticated taxa.

3.3.2 Virus Infection Impacts Diverse Life History Traits

In both years, we found that virus infection impacted traits related to plant reproduction and plant-pollinator interactions (**Figure 3.2**; **Table S3.6**). We found no evidence of association between domestication and infection outcomes for any trait in either year (**Table 3.1**). Thus, for subsequent analyses we report results of models using virus infection as the only fixed effect.

In 2019, we detected reductions in specialist pollinator (squash bee) visitation for resource collection (X^2 = 5.09, df = 1, $p < 0.05$) and marginal reductions in female flower number (X^2 = 3.74, df = 1, $p = 0.053$) (**Figure 3.2**). In 2021, we found that virus infection impacted all categories of traits with significant reductions in male flowers (X^2 = 4.81, df = 1, $p < 0.05$), female flowers (X^2 = 5.26, df = 1, $p < 0.05$), fruit number (X^2 = 38.05, df = 1, $p < 0.001$), and fruit weight (X^2 = 23.59, df = 1, $p < 0.001$). We also found significant reductions in germination rates of seeds from virus-infected plants (X^2 = 5.49, df = 1, $p < 0.05$). For plant-pollinator interactions, we found an increase in landing rates of the specialist pollinator (squash bees), in contrast to results from 2019 (X^2 = 20.74, df = 1, $p < 0.001$). Lastly, we observed changes in the quality of floral rewards in virus-infected plants with significant reductions in pollen dry weight (X^2 = 4.46, df = 1, $p < 0.05$) and nectar sucrose concentration (X^2 = 6.76, df = 1, $p < 0.01$). Our results show that models with phylogenetic corrections outperformed those without for all traits impacted by infection in both years, except for male flower number and nectar sucrose concentration recorded in 2021 (**Table S3.7**).

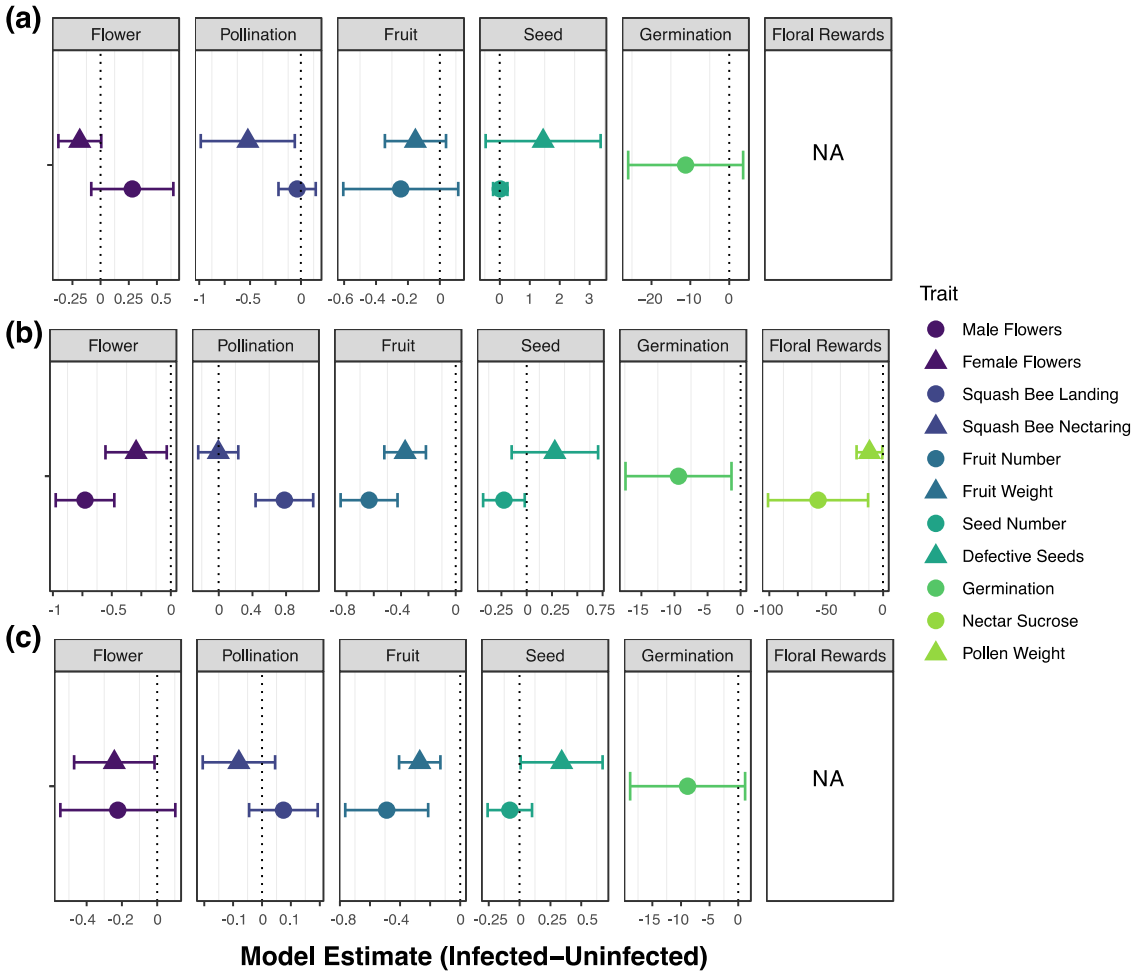

Figure 3.2: Contrasts of estimated marginal means from generalized least squares models evaluating the impact of virus infection on plant traits in (**A**) 2019, (**B**) 2021, and (**C**) both years combined. Circles represent model estimates and bars are +/- two standard errors. Negative model estimates indicate that virus infection reduced trait values in inoculated compared to uninoculated plants. Error bars that do not cross zero indicate results significant at $p < 0.05$. All traits were log transformed except for germination rate, pollen weight, and nectar sucrose concentration.

Table 3.1: Results of ANOVA showing the interaction of virus infection and domestication status. Domestication status did not influence infection outcomes as the interaction between domestication status and infection was not significant for any traits ($p > 0.05$).

Category	Year	Trait	Fixed Effect	Chisq	DF	p-value	Transformation	Lambda	Alpha
Flower	2019	Female flower	Infection* Status	0.57	1	0.452	log(x+1)	0.88	
Flower	2019	Male flower	Infection* Status	1.58	1	0.208	log	0.12	

Pollination	2019	Bumble bee landing	Infection* Status	0.26	1	0.612	log(x+1)	0.12	
Pollination	2019	Bumble bee nectaring	Infection* Status	0	1	0.969	log(x+1)		49.4
Pollination	2019	Squash bee landing	Infection* Status	0.01	1	0.913	log(x+1)	0.15	
Pollination	2019	Squash bee nectaring	Infection* Status	0.59	1	0.444	log	0.37	
Fruit	2019	Fruit number	Infection* Status	0.27	1	0.607	log	0.95	
Fruit	2019	Fruit weight	Infection* Status	0.57	1	0.45	log	0.99	
Seed	2019	Defective seeds	Infection* Status	0.19	1	0.664	log(x+1)	0.73	
Seed	2019	Seed number	Infection* Status	3.1	1	0.078	none	0.55	
Germination	2019	Germination	Infection* Status	0.45	1	0.5	none	0.77	
Flower	2021	Female flower	Infection* Status	2.06	1	0.152	log(x+1)	0.72	
Flower	2021	Male flower	Infection* Status	0.24	1	0.628	log	0.48	
Pollination	2021	Bumble bee landing	Infection* Status	0.06	1	0.81	log(x+1)		
Pollination	2021	Bumble bee nectaring	Infection* Status	1.54	1	0.215	log(x+1)		14.7
Pollination	2021	Squash bee landing	Infection* Status	1.1	1	0.294	log	0.33	
Pollination	2021	Squash bee nectaring	Infection* Status	0.07	1	0.798	log	0.79	
Fruit	2021	Fruit number	Infection* Status	0.14	1	0.712	log	0.98	
Fruit	2021	Fruit weight	Infection* Status	0.27	1	0.607	log	0.99	
Seed	2021	Defective seeds	Infection* Status	0.14	1	0.706	log(x+1)	0.59	
Seed	2021	Seed number	Infection* Status	0.08	1	0.78	log	0.95	
Germination	2021	Germination	Infection* Status	0.38	1	0.537	none	0.82	
Floral Rewards	2021	Nectar sucrose	Infection* Status	1.13	1	0.289	none	0.24	
Floral Rewards	2021	Nectar volume	Infection* Status	0.09	1	0.762	log		
Floral Rewards	2021	Pollen protein	Infection* Status	0.45	1	0.503	none	0.87	
Floral Rewards	2021	Pollen weight	Infection* Status	2.03	1	0.154	none	0.72	

Additionally, we saw differences between taxa in the number of traits and their effect sizes (**Figure 3.3**; **Table S3.8**). In 2019, some traits were significantly impacted by virus infection within specific taxa but were not detected as significantly different between virus-inoculated and mock-inoculated plants when analyzing all taxa together. Specifically, we detected differences in female flowers (*C. pepo* ssp. *ovifera* var. *ovifera, C. pepo* ssp. *ovifera* var. *texana*), fruit weight (*C. pepo* ssp. *ovifera* var. *texana*, *C. argyrosperma* ssp. *sororia*, *C. maxima* spp. *andreana*), and seed number (*C. pepo* ssp. *ovifera* var. *texana*) for several taxa, but not when combining all taxa. This is likely due to unique impacts per taxon that were not strong enough to be detected when analyzing all host taxa together. Conversely, we also found that seed number in 2021 was detected as significantly decreased in inoculated plants when looking at overall impacts, but we did not find significant differences in any particular taxa. This may be due to the penalization from running multiple tests when looking at individual taxa, or a relatively small overall effect of virus on seed number (X^2 = 4.81, df = 1, *p*-value = 0.028).

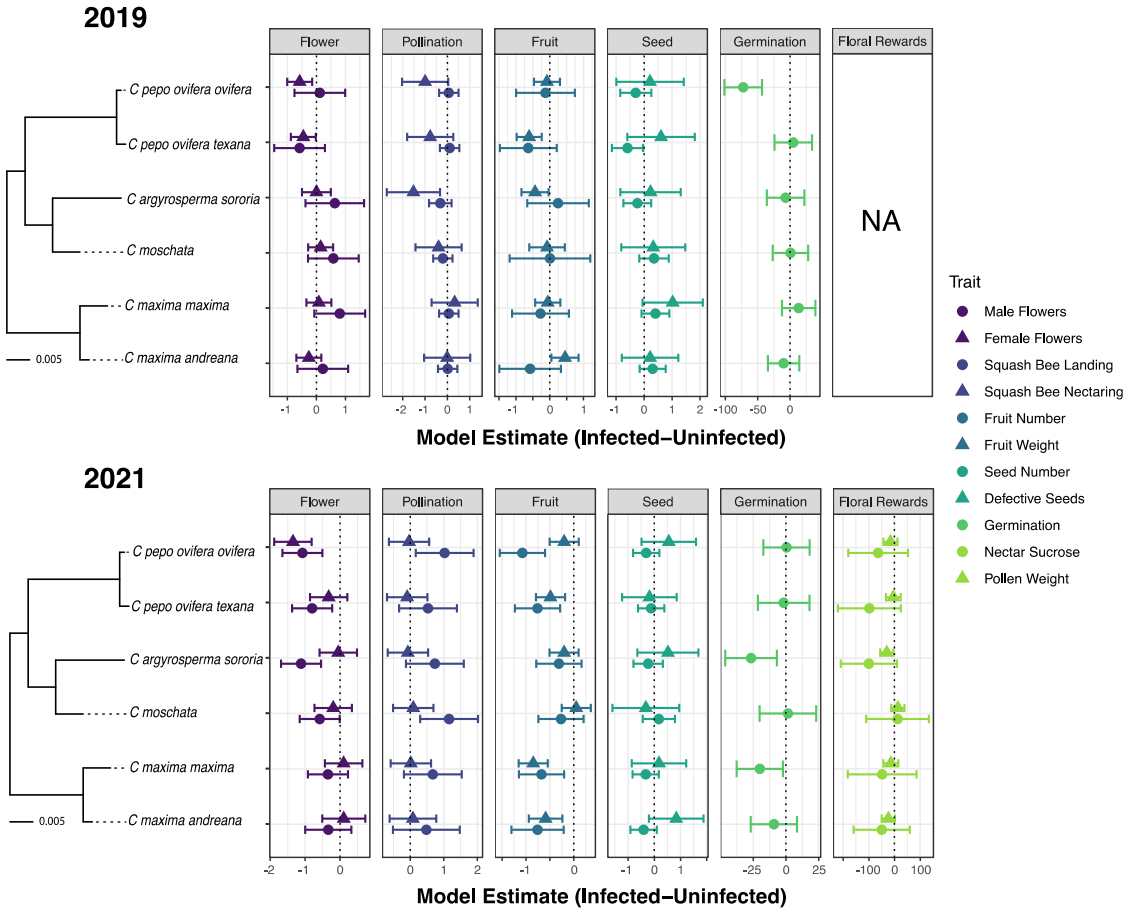

Figure 3.3: Contrasts of model estimates comparing virus-inoculated to uninoculated plants for different life history traits in 2019 and 2021. Circles represent model estimates and bars are +/- two standard errors. Negative estimates indicate reductions in trait values. Error bars that do not cross 0 are significant at $p < 0.05$. Estimates for individual plant taxa were obtained via post-hoc analyses of generalized least squares models. All traits underwent log transformation except for germination rate, pollen weight, and nectar sucrose concentration. Phylogeny was adapted from Kates et al. 2017, with scale bar representing 0.005 nucleotide substitutions per site.

The strength of virus impacts on host fitness differed between years, and thus combined analysis of 2019 and 2021 data provided insights on which traits were similar in their strength and directionality between years. The strongest effects of virus infections were on the reduction of

fruit number ($X^2 = 20.54$, $p < 0.001$) and fruit weight ($X^2 = 15.92$, $p < 0.001$). Additionally, we found a significant increase in defective seed number based on infection ($X^2 = 4.02$, $p < 0.05$) when combining the data from both years. Interestingly, we also found significant interactions between infection and year for male flower number ($X^2 = 20.23$, $p < 0.001$) and squash bee landing events ($X^2 = 4.38$, $p < 0.05$), indicating the direction of these traits was different between years (**Figure 3.2**).

3.4 Discussion

Here, we found that some host taxa are greatly impacted by virus infection with reductions in multiple traits while others have minimal costs of infection. We detected significant impacts of virus infection on traits directly related to fecundity, including production of fruits, viable seeds, and seed germination rate. Overall, virus infection significantly impacted most of the life history traits measured. Phylogenetic relatedness contributed to differences in infection outcomes for several traits, but domestication status was not a significant indicator of susceptibility for any of the measured traits. Additionally, we show that accumulation of both ZYMV and SqMV was roughly equivalent in all taxa, indicating that differences in susceptibility between taxa can be attributed to tolerance and not resistance. We took a novel approach by quantifying traits directly or indirectly relevant for agronomy (foliar symptoms, fruits), plant reproduction (flowers, seeds), and plant-pollinator interactions (bee visitation and floral rewards) over multiple years. We found significant impacts of virus infection on 11 out of 15 life history traits in at least one year. Except for the landing activity of squash bees and the number of defective seeds, all traits were decreased in virus-inoculated plants including floral resource collection by squash bees.

Historically, many studies have focused on plants exhibiting foliar symptoms when looking for impacts of viral diseases (Bock et al., 2022). However, it is becoming increasingly apparent that both wild and cultivated plants can harbor viral infections without showing visually apparent symptoms (Prendeville et al., 2012; Roossinck et al., 2015). All the taxa in our study exhibited visible foliar symptoms, but we show that fitness effects differ drastically between taxa, even for those with similar foliar symptoms (**Figure 3.1**). Foliar symptoms were also not indicative of viral burden, as we detected no significant relationship between viral load and symptom severity for either virus or the ratio of ZYMV:SqMV. This is unsurprising, given that plant virus infections do not always have a direct relationship between viral burden and symptom severity or host plant fitness (Thurston et al., 2001; Pallett et al., 2002; Pagán et al., 2007). Based on our results, we encourage future studies characterizing disease susceptibility to focus on diverse life history traits to provide more complete descriptions of virus impacts.

Although many crop plants are dependent on insect pollinators, the effects of plant viruses on pollinator behavior are largely unknown (Prendeville and Pilson, 2009; Groen et al., 2016; Murphy et al., 2023). Here we did not find differences in landing or resource collection of bumble bees, which are generalist pollinators perceiving diverse floral cues that may not be modified by virus infection in this system (Katzenberger et al., 2013). Although they frequently collect nectar and are efficient pollinators of cucurbits, bumble bees actively avoid collecting *Cucurbtia* pollen as it contains toxic chemical and physical defenses (Brochu et al., 2020). In contrast, we found decreased resource acquisition and increased landing of squash bees in infected plants in 2019 and 2021, respectively. Squash bees source all their pollen from cucurbits and most of their nectar resources as well. Taken together, this suggests that squash bees may be initially more attracted to virus-infected flowers but prefer to acquire resources from healthy plants. This differs from previous research which found that squash bee visitation was not influenced by ZYMV infection in *Cucurbita pepo*, but the authors mentioned the plants were

inoculated much later and were not severely symptomatic during pollinator observations (Prendeville and Pilson, 2009). Floral visitation by bees is known to be influenced by the quality of floral rewards, including pollen availability and nectar sugar concentration (Vaudo et al., 2016; Mallinger and Prasifka, 2017). Sufficient nutritional resources are also important for larval development and immunocompetence in bees (Brunner et al., 2014; Bryś et al., 2021). Therefore, plant pathogen outbreaks that decrease floral resources could present added nutritional stress to pollinators in agricultural landscapes and potentially reduce pollination services. Research has started to consider the importance of high-quality floral resources for bees (Pamminger et al., 2019), but the downstream impacts of plant pathogens on pollinator health and nutrition deserve further investigation. Volatile organic compounds (VOCs) are also important for plant-pollinator interactions (Burkle and Runyon, 2019) and may contribute to the differences we observed in squash bee behavior on infected plants. Zucchini yellow mosaic virus can alter VOC profiles of cucurbits attracting its aphid vectors and repelling cucumber beetles which frequently transmit a bacterial pathogen responsible for a fatal wilt disease (Shapiro et al., 2012b; Salvaudon et al., 2013). Future studies could measure if pollinators spend more time on flowers or exhibit preference for VOCs of virus-infected plants to provide further insight on how plant viruses may be altering pollinator behavior in this system.

Studying variations in both symptom severity and associated virus accumulation facilitates a better understanding of the underlying mechanisms of plant defenses to virus infections and is required for characterizations of tolerance (Paudel and Sanfaçon, 2018). We found differing levels of virus tolerance in hosts, as we did not find differences in virus abundance between taxa but observed that some taxa had severe foliar symptoms and reductions in several traits while others did not. In 2019, the most affected host (*C. pepo ovifera* var. *texana*) had reductions in female flower number, fruit weight, and seed number, while the least affected host did not experience any reductions (**Figure 3.3**). In 2021, the most affected taxon was *C. pepo*

ovifera var. *ovifera*, with reductions in pistillate flowers, staminate flowers, fruit number, and an increase in squash bee landing (without resource acquisition). The least affected taxon in 2021 was *C. moschata* which similarly experienced reductions in staminate flower number and an increase in squash bee landing. Since we observed no other impacts of virus infection in *C. moschata* in either year, we conclude that *C. moschata* is the most tolerant taxon. Mechanisms of tolerance are mostly unknown but other studies have shown that for some virus infections there is a shift in resource allocation from growth to reproduction (Hily et al., 2016), or conversely a delay in reproduction and a longer reproductive phase (Montes et al., 2020). It is possible there are differences in resource allocation between taxa but here we quantified reproductive, not vegetative traits. We did see that the most tolerant taxon exhibited no decreases in progeny during infection (production of fruit, seed, or seed viability), thus maintaining its fitness, while other taxa exhibited reductions in reproductive output.

The variations we observed in virus impacts have implications for both fitness of wild plants and crop productivity which can be described by different biological traits. Both ZYMV and SqMV have large distributions, are frequently found in the same fields, and have varying effects on plant fitness (Köklü and Yilmaz, 2006; Jossey and Babadoost, 2007; Ali et al., 2012a, 2012b; Khanal et al., 2021). These viruses are known to have devastating impacts on crops, due to reductions in yield and marketable fruits (Fuchs and Gonsalves, 1995; da Silva et al., 2016) with fitness impacts differing between host genotypes (Prendeville et al., 2012, 2014b). We found that virus infection overall reduced many fitness related traits, but the impacts on traits relevant for crops were more variable, as some plants did not exhibit significant yield reductions.

Additionally, our results show that virus infection may reduce seed viability without reducing fruit production in the same host, indicating that reductions in plant fitness do not always produce reductions in agronomic yield. In addition to large differences in infection impacts between hosts, we found differences between years, indicating that outcomes of virus

infection may be environmentally dependent. Indeed, abiotic factors are known to influence plant-virus interactions and increased temperature can enhance symptom expression of plant viral diseases (Roden and Ingle, 2009; Chung et al., 2015; Velásquez et al., 2018; Tsai et al., 2022). Interestingly, we found disease severity was higher during the 2021 field season, which also had a higher average daily temperature and daily precipitation over the months of June-August (2019: 20.6°C, 2.7mm; 2021: 21.4°C, 4.5mm) (https://newa.cornell.edu/). Another possible reason we saw minor fitness impacts for some hosts, could be that both ZYMV and SqMV are vertically transmitted in addition to horizontally transmitted via vectors. Transmission modes can influence the evolution of virulence (May and Anderson, 1983) and the virulence-tradeoff hypothesis posits that vertical transmission modes will favor reduced virulence and the preservation of host fitness to maintain pathogen fitness (Bull et al., 1991; Read, 1994; Alizon et al., 2009). We find support for the ability of plants to maintain fitness during coinfection with ZYMV and SqMV, but show that the relative fitness impacts are dependent on the host in question.

Although all plants in our study belong to the same genus (*Cucurbita*), we saw strong variation in susceptibility to virus infection between hosts which was influenced by phylogenetic distance. Phylogenetic signal has been observed for plant-pathogen interactions, in both host range and severity of infection, indicating that host relatedness can be an important predictor of infection outcomes for specialist and generalist pathogens (Gilbert and Webb, 2007; Gilbert et al., 2015; Lynch et al., 2020). Research suggests that this is a byproduct of shared evolutionary history, and thus constraints in host defense mechanisms, that result in host competence and disease severity exhibiting phylogenetic signal (Gougherty and Davies, 2021). While dominant resistance genes have been thoroughly investigated to explain variation in plant pathogen susceptibility between hosts (Maule et al., 2007), host responses are also influenced by underlying genetic architecture at multiple loci (Wilfert and Schmid-Hempel, 2008). Cucurbits have fewer dominant plant pathogen resistance genes compared to other plant families (Lin et al.,

2013). Yet, the ability of viruses to efficiently accumulate in all hosts we used for this study indicates that dominant resistance genes are likely not responsible for the differences we observed in virus susceptibility between taxa. Our results suggest that there are evolutionary constraints between hosts resulting in closely related taxa having similar defense responses due to conservation of genetically encoded defense mechanisms. With this gradient of susceptibility between closely related hosts and reference genomes available for several taxa (Sun et al., 2017; Montero-Pau et al., 2018), future studies could leverage this pathosystem to provide valuable insight into the molecular mechanisms of virus tolerance.

The domestication process, and its impact on host evolution, does not appear to have impacted host responses to virus infection in this system, as we observed no differences in infection outcomes between domesticated and wild plants. Artificial selection during domestication has led to changes in the genomic architecture and genetic diversity of plants (Meyer and Purugganan, 2013). Resulting loss in genetic diversity is hypothesized to increase pathogen susceptibility in domesticated plants through reductions in pathogen resistance genes and secondary metabolites involved in host defenses (Flint-Garcia, 2013; Gaillard et al., 2018). However, studies investigating decreased susceptibility to viruses in crop wild relatives are almost exclusively concerned with qualitative or complete resistance, in which hosts prevent or greatly reduce virus replication (Maule et al. 2007, but see Nygren et al. 2015; Ferrero et al. 2019). Specifically for ZYMV, resistance genes have been identified in wild relatives, but have been difficult to introgress into other cultivars of interest due to sparing cross-fertility (Pachner et al., 2011). Overall, the evidence on whether domesticated plants have reduced pathogen defenses is mixed, is biased towards qualitative resistance, and likely depends on a range of factors, including the specific pathogen, host, and environmental conditions. Using domesticated crops and wild counterparts, which are all competent hosts, our results indicate that reductions in plant fitness are not predicted by domestication status in this pathosystem.

In conclusion, we demonstrate that susceptibility greatly varies across closely related species, and that host phylogenetic relatedness is one of the most important predictors to explain susceptibility to viral disease. Additionally, virus tolerance in plants is likely more prevalent than previously thought and is a defense mechanism of growing interest based on its potential for sustainable disease management (Pagán and García-Arenal, 2018; Paudel and Sanfaçon, 2018). Our results highlight the feasibility of deploying plants with virus tolerance in a field setting. We observed differences in tolerance between taxa but observed minimal impacts on plant fitness for the most tolerant hosts. Further studies are needed to address the underlying mechanisms and epidemiological or ecological consequences of virus tolerance. However, we provide relevant information with implications for the sustainable management of viral diseases. Our results also show that plant viruses can negatively impact floral rewards and change how pollinators interact with their host plants. We encourage future efforts to document the understudied effects of plant microbes on the perception and nutrition of non-vector insects which could present yet another threat to insect biodiversity.

3.5 References

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3.6 Supplemental Material

Supplemental Tables

Table S3.1: Sources for seed of each host taxon.

Host Scientific Name	Common Name	Supplier
Cucurbita pepo ssp. *ovifera* var. *ovifera*	"Spinning gourd"	www.seedsavers.org Cat# 0867
Cucurbita pepo ssp. *ovifera* var. *texana*	"Wild gourd"	Collected and maintained from wild populations (Harth et al. 2018: https://doi.org/10.3389/fpls.2018.00792)

Cucurbita argyrosperma ssp. *sororia*	"Halawe chipu"	Courtesy of donation by www.nativeseeds.org
Cucurbita moschata	"Waltham butternut"	www.johnnyseeds.com Cat# 671
Cucurbita maxima ssp. *maxima*	"Golden hubbard"	www.seedsavers.org Cat# 0410
Cucurbita maxima ssp. *andreana*	"Cucurbita andreana"	www.bobby-seeds.com Cat# WKCA644

Table S3.2: Primer and probes sequences used for quantification of zucchini yellow mosaic virus (ZYMV) and squash mosaic virus (SqMV).

Target	F Primer (5'-3')	R Primer (5'-3')	Probe (5'-3')	Source
Eukaryotic 18s rRNA Endogenous Control	Proprietary	Proprietary	Proprietary (VIC™/MGB)	ThermoFisher Cat #: 4319413E
ZYMV	ACTGATCCAGCAGCATTACC	CTGAGCTGATAGTGTCGAAGTG	FAM-TGCCGAGGA-ZEN-AATGATGAAGCGGAA-IBFQ	This study
SqMV	TCTGGGTTTTGTTGTGGCT	GCATCAAAGAAAACAAGCTCTT	FAM-TCTTTCCCAGTTCGCTTTAGAAGCCTCT-TAMRA	Liu et al. 2016

Table S3.3: Results of emmeans pairwise comparisons investigating differences in viral load or symptom severity between taxa. Taxa with different letters are significant at $p < 0.05$.

Response	Species	emmean	SE	df	lower.CL	upper.CL	Group
ZYMV	*C_maxima_andreana*	-0.199	0.537	11.2	-1.91	1.51	A
	C_pepo_ovifera_texana	0.671	0.486	13.9	-0.815	2.16	A
	C_pepo_ovifera_ovifera	0.735	0.457	12.3	-0.695	2.17	A
	C_argyrosperma_sororia	0.827	0.627	16	-1.054	2.71	A
	C_moschata	0.949	0.492	14.2	-0.553	2.45	A
	C_maxima_maxima	1.01	0.531	17	-0.57	2.59	A

SqMV	*C_moschata*	0.0128	0.361	17.6	-1.056	1.08	A
	C_maxima_maxima	0.1747	0.408	18.6	-1.025	1.37	A
	C_pepo_ovifera_ovifera	0.2269	0.328	16.9	-0.748	1.2	A
	C_pepo_ovifera_texana	0.2913	0.339	20.1	-0.697	1.28	A
	C_maxima_andreana	0.386	0.369	11.2	-0.787	1.56	A
	C_argyrosperma_sororia	0.9308	0.494	22.7	-0.492	2.35	A
ZYMV:SqMV Ratio	*C_maxima_andreana*	1.12	1.8	11.2	-4.63	6.87	A
	C_argyrosperma_sororia	1.64	2.36	22	-5.18	8.46	A
	C_pepo_ovifera_texana	2.09	1.63	17.6	-2.75	6.92	A
	C_pepo_ovifera_ovifera	2.28	1.55	15.2	-2.41	6.97	A
	C_maxima_maxima	3.01	1.87	18.9	-2.5	8.51	A
	C_moschata	6.71	1.69	16.7	1.66	11.76	A
Symptom Severity	*C_argyrosperma_sororia*	-2.042	0.896	Inf	-4.399	0.316	A
	C_moschata	-1.786	0.926	Inf	-4.222	0.65	A
	C_maxima_andreana	-0.758	0.779	Inf	-2.808	1.291	A
	C_maxima_maxima	0.264	0.777	Inf	-1.78	2.307	AB
	C_pepo_ovifera_ovifera	2.612	0.836	Inf	0.413	4.812	BC
	C_pepo_ovifera_texana	3.176	0.935	Inf	0.717	5.635	C

Table S3.4: Results of ANOVA on cumulative link linear mixed models testing the impact of plant traits on symptom severity.

Response	Fixed effect	Random effect	*F*.ratio	*p*-value
Severity	Species	Block	5.946	<.0001
Severity	Domestication status	Block	0.613	0.4338
Severity	Viral load (ZYMV)	Block	2.556	0.1099
Severity	Viral load (SqMV)	Block	0.183	0.669
Severity	ZYMV:SqMV Ratio	Block	1.348	0.2457

Table S3.5: Results of ANOVA on generalized linear mixed models evaluating the impact of plant host species or domestication status on viral load.

Response	Fixed effect	Random effect	Chisq	DF	*p*-value
ZYMV	Species	Block	4.49	5	0.4812
SqMV	Species	Block	2.8318	5	0.7259
ZYMV:SqMV Ratio	Species	Block	9.1426	5	0.1035
ZYMV	Domestication Status	Block	1.7545	1	0.1853
SqMV	Domestication Status	Block	1.3632	1	0.243
ZYMV:SqMV Ratio	Domestication Status	Block	2.9746	1	0.08458

Table S3.6: Results of emmeans pairwise comparisons run on generalized least squares (GLS) models evaluating the impact of virus infection on life history traits. Traits significantly impacted by infection at *p*-value <0.05 are in bold.

Trait	Year	Predictors	Estimates	SE	DF	t-ratio	*p*-value
Male Flowers	2019	Infected-Uninfected	0.282	0.182	40.9	1.545	0.1302
Female Flowers	2019	Infected-Uninfected	-0.184	0.0952	38.1	-1.935	0.0605
Fruit Number	2019	Infected-Uninfected	-0.245	0.18	34.6	-1.36	0.1827
Fruit Weight	2019	Infected-Uninfected	-0.153	0.0956	34.9	-1.597	0.1193
Bumble Bee Landing	2019	Infected-Uninfected	-0.0509	0.042	40.6	-1.212	0.2325
Bumble Bee Nectaring	2019	Infected-Uninfected	-0.0611	0.0393	43.9	-1.554	0.1273

Squash Bee Landing	2019	Infected-Uninfected	-0.0371	0.0918	39.8	-0.404	0.6883
Squash Bee Nectaring	2019	Infected-Uninfected	-0.522	0.231	30.8	-2.257	**0.0313**
Seed Number	2019	Infected-Uninfected	0.0177	0.119	27.3	0.149	0.8828
Defective Seeds	2019	Infected-Uninfected	1.45	0.96	25.5	1.515	0.1421
Germination	2019	Infected-Uninfected	-11.2	7.39	23.4	-1.511	0.1442
Male Flowers	2021	Infected-Uninfected	-0.727	0.124	37.8	-5.885	**<.0001**
Female Flowers	2021	Infected-Uninfected	-0.294	0.13	38.3	-2.264	**0.0293**
Fruit Number	2021	Infected-Uninfected	-0.634	0.104	37.9	-6.082	**<.0001**
Fruit Weight	2021	Infected-Uninfected	-0.37	0.0761	38.9	-4.857	**<.0001**
Bumble Bee Landing	2021	Infected-Uninfected	-0.00179	0.00689	42	-0.259	0.7967
Bumble Bee Nectaring	2021	Infected-Uninfected	0.0178	0.00982	43	1.815	0.0765
Squash Bee Landing	2021	Infected-Uninfected	0.78	0.171	39.9	4.554	**<.0001**
Squash Bee Nectaring	2021	Infected-Uninfected	-0.00456	0.119	39.2	-0.038	0.9695
Seed Number	2021	Infected-Uninfected	-0.225	0.103	25.5	-2.194	**0.0375**
Defective Seeds	2021	Infected-Uninfected	0.279	0.214	26.6	1.306	0.2028
Germination	2021	Infected-Uninfected	-9.38	4	25.5	-2.343	0.0272
Nectar Volume	2021	Infected-Uninfected	-0.217	0.182	30	-1.189	0.2438
Nectar Sucrose	2021	Infected-Uninfected	-57.1	22	27.6	-2.599	**0.0148**
Pollen Weight	2021	Infected-Uninfected	-11.9	5.65	27.7	-2.111	**0.0439**
Pollen Protein	2021	Infected-Uninfected	-10.6	14.5	25	-0.729	0.4727
Male Flowers	Both	Infected-Uninfected	-0.223	0.163	85.5	-1.368	0.1747
Female Flowers	Both	Infected-Uninfected	-0.243	0.114	83.6	-2.126	**0.0364**
Fruit Number	Both	Infected-Uninfected	-0.489	0.138	80.7	-3.533	**0.0007**
Fruit Weight	Both	Infected-Uninfected	-0.269	0.0688	81	-3.911	**0.0002**
Bumble Bee Landing	Both	Infected-Uninfected	-0.0263	0.0237	87	-1.11	0.2699
Bumble Bee Nectaring	Both	Infected-Uninfected	-0.0435	0.0693	84.2	-0.627	0.5324
Squash Bee Landing	Both	Infected-Uninfected	0.0742	0.0594	85.2	1.25	0.2148
Squash Bee Nectaring	Both	Infected-Uninfected	-0.08	0.0626	82	-1.278	0.205
Seed Number	Both	Infected-Uninfected	-0.079	0.0899	60.6	-0.879	0.3831
Defective Seeds	Both	Infected-Uninfected	0.341	0.166	58.9	2.047	**0.0451**
Germination	Both	Infected-Uninfected	-8.84	5.02	55.1	-1.761	0.0838

Table S3.7: Results of model comparisons between GLS models without phylogenetic corrections and those with either Pagel's lambda or Ornstein-Uhlenbeck phylogenetic correlation structure for each trait. Model performance was evaluated based on delta > 4.

Trait	Year	Models	DF	logLik	AICc	Delta	Weight	Transf
Fruit Number	2019	Pagel's lambda	6	-46.887	108.2	0	0.969	log
Fruit Number	2019	Ornstein-Uhlenbeck	6	-50.327	115.1	6.88	0.031	log
Fruit Number	2019	GLS	5	-74.456	160.6	52.4	0	log
Fruit Weight	2019	Pagel's lambda	6	-25.76	65.9	0	1	log

Fruit Weight	2019	Ornstein-Uhlenbeck	6	-45.119	104.6	38.72	0	log
Fruit Weight	2019	GLS	5	-75.081	161.8	95.91	0	log
Female Flower	2019	Pagel's lambda	6	-24.095	62.3	0	1	log(x+1)
Female Flower	2019	Ornstein-Uhlenbeck	4	-38.818	86.6	24.27	0	log(x+1)
Female Flower	2019	GLS	5	-44.52	100.5	38.2	0	log(x+1)
Male Flower	2019	GLS	5	-46.075	103.6	0	0.664	log
Male Flower	2019	Pagel's lambda	6	-45.43	105	1.36	0.336	log
Male Flower	2019	Ornstein-Uhlenbeck	6	-52.014	118.2	14.53	0	log
Seed Number	2019	GLS	5	-187.6	387.3	0	0.68	none
Seed Number	2019	Pagel's lambda	6	-187.02	389	1.77	0.28	none
Seed Number	2019	Ornstein-Uhlenbeck	6	-188.97	392.9	5.68	0.04	none
Defective Seeds	2019	Pagel's lambda	6	-39.187	93.4	0	0.785	log(x+1)
Defective Seeds	2019	GLS	5	-41.949	96	2.59	0.215	log(x+1)
Germination Rate	2019	GLS	5	-144.72	301.6	0	0.533	none
Germination Rate	2019	Pagel's lambda	6	-143.37	301.9	0.27	0.466	none
Germination Rate	2019	Ornstein-Uhlenbeck	6	-149.21	313.5	11.94	0.001	none
Squash Bee Landing	2019	GLS	5	-16.716	44.9	0	0.709	log(x+1)
Squash Bee Landing	2019	Pagel's lambda	6	-16.282	46.7	1.79	0.291	log(x+1)
Squash Bee Landing	2019	Ornstein-Uhlenbeck	6	-24.815	63.8	18.85	0	log(x+1)
Squash Bee Nectaring	2019	Pagel's lambda	6	-55.571	125.3	0	0.98	log
Squash Bee Nectaring	2019	GLS	5	-60.83	133.2	7.86	0.019	log
Squash Bee Nectaring	2019	Ornstein-Uhlenbeck	6	-62.771	139.7	14.4	0.001	log
Bumble Bee Landing	2019	GLS	5	14.923	-18.3	0	0.627	log(x+1)
Bumble Bee Landing	2019	Pagel's lambda	6	15.447	-16.7	1.6	0.281	log(x+1)
Bumble Bee Landing	2019	Ornstein-Uhlenbeck	6	14.334	-14.5	3.83	0.092	log(x+1)
Bumble Bee Nectaring	2019	Ornstein-Uhlenbeck	6	-6.728	27.6	0	1	log(x+1)
Bumble Bee Nectaring	2019	GLS	5	-17.525	46.6	18.94	0	log(x+1)
Bumble Bee Nectaring	2019	Pagel's lambda	6	-16.796	47.7	20.14	0	log(x+1)
Defective Seeds	2021	Pagel's lambda	6	-35.574	86.4	0	0.93	log
Defective Seeds	2021	GLS	5	-39.994	92.2	5.83	0.05	log
Defective Seeds	2021	Ornstein-Uhlenbeck	6	-39.452	94.1	7.76	0.019	log
Germination Rate	2021	Pagel's lambda	6	-117.55	250.5	0	0.945	none
Germination Rate	2021	Ornstein-Uhlenbeck	6	-120.42	256.2	5.73	0.054	none
Germination Rate	2021	GLS	5	-125.59	263.5	13.02	0.001	none
Squash Bee Landing	2021	Pagel's lambda	6	-44.397	102.9	0	0.897	log
Squash Bee Landing	2021	GLS	5	-47.895	107.3	4.34	0.102	log
Squash Bee Landing	2021	Ornstein-Uhlenbeck	6	-52.319	118.8	15.84	0	log
Squash Bee Nectaring	2021	Ornstein-Uhlenbeck	6	-31.077	76.3	0	0.789	log

Squash Bee Nectaring	2021	Pagel's lambda	6	-32.395	78.9	2.64	0.211	log
Squash Bee Nectaring	2021	GLS	5	-41.002	93.5	17.2	0	log
Bumble Bee Landing	2021	GLS	5	93.308	-175.1	0	0.788	log(x+1)
Bumble Bee Landing	2021	Pagel's lambda	6	93.323	-172.5	2.62	0.212	log(x+1)
Bumble Bee Landing	2021	Ornstein-Uhlenbeck	6	84.994	-155.8	19.28	0	log(x+1)
Bumble Bee Nectaring	2021	Ornstein-Uhlenbeck	6	51.596	-89	0	0.984	log(x+1)
Bumble Bee Nectaring	2021	Pagel's lambda	5	46.167	-80.8	8.2	0.016	log(x+1)
Bumble Bee Nectaring	2021	GLS	5	40.172	-68.8	20.19	0	log(x+1)
Pollen Weight	2021	Pagel's lambda	6	-134.69	284.5	0	0.99	none
Pollen Weight	2021	Ornstein-Uhlenbeck	6	-139.49	294.1	9.59	0.008	none
Pollen Weight	2021	GLS	5	-142.68	297.5	13.02	0.001	none
Pollen Protein	2021	Pagel's lambda	6	-165.06	345.2	0	0.886	none
Pollen Protein	2021	Ornstein-Uhlenbeck	6	-167.14	349.4	4.16	0.111	none
Pollen Protein	2021	GLS	5	-172.18	356.5	11.28	0.003	none
Nectar Volume	2021	GLS	5	-16.923	46.2	0	0.755	log
Nectar Volume	2021	Pagel's lambda	6	-16.525	48.4	2.26	0.245	log
Nectar Volume	2021	Ornstein-Uhlenbeck	6	-25.417	66.2	20.04	0	log
Nectar Sucrose	2021	Pagel's lambda	6	-161.28	337.9	0	0.513	none
Nectar Sucrose	2021	GLS	5	-162.86	338	0.12	0.483	none
Nectar Sucrose	2021	Ornstein-Uhlenbeck	6	-166.1	347.6	9.64	0.004	none
Fruit Number	2021	Pagel's lambda	6	-31.364	76.9	0	1	log
Fruit Number	2021	Ornstein-Uhlenbeck	6	-48.225	110.6	33.72	0	log
Fruit Number	2021	GLS	5	-78.884	169.3	92.39	0	log
Fruit Weight	2021	Pagel's lambda	6	-21.118	56.4	0	1	log
Fruit Weight	2021	Ornstein-Uhlenbeck	6	-45.363	104.9	48.49	0	log
Fruit Weight	2021	GLS	5	-83.04	177.6	121.19	0	log
Seed Number	2021	Pagel's lambda	6	-18.736	52.7	0	1	log
Seed Number	2021	Ornstein-Uhlenbeck	6	-26.922	69.1	16.37	0	log
Seed Number	2021	GLS	5	-33.748	79.7	27.02	0	log
Fruit Number	2021	Pagel's lambda	6	-31.364	76.9	0	1	log
Fruit Number	2021	Ornstein-Uhlenbeck	6	-48.225	110.6	33.72	0	log
Fruit Number	2021	GLS	5	-78.884	169.3	92.39	0	log
Fruit Weight	2021	Pagel's lambda	6	-21.118	56.4	0	1	log
Fruit Weight	2021	Ornstein-Uhlenbeck	6	-45.363	104.9	48.49	0	log
Fruit Weight	2021	GLS	5	-83.04	177.6	121.19	0	log
Seed Number	2021	Pagel's lambda	6	-169.61	354.4	0	0.999	log
Seed Number	2021	GLS	5	-178.77	369.8	15.32	0	log
Seed Number	2021	Ornstein-Uhlenbeck	6	-177.66	370.5	16.1	0	log

Fruit Weight	Both	Pagel's lambda	4	-44.434	97.3	0	1	log
Fruit Weight	Both	Ornstein-Uhlenbeck	4	-80.997	170.5	73.13	0	log
Fruit Weight	Both	GLS	3	-161.54	329.4	232.02	0	log
Fruit Number	Both	Pagel's lambda	4	-99.664	207.8	0	1	log
Fruit Number	Both	GLS	3	-159.32	324.9	117.12	0	log
Male Flower	Both	Pagel's lambda	4	-111.82	232.1	0	0.741	log
Male Flower	Both	GLS	3	-113.96	234.2	2.1	0.259	log
Male Flower	Both	Ornstein-Uhlenbeck	4	-151.08	310.6	78.52	0	log
Female Flower	Both	Pagel's lambda	4	-84.604	177.7	0	1	log(x+1)
Female Flower	Both	GLS	3	-110.68	227.6	49.96	0	log(x+1)
Female Flower	Both	Ornstein-Uhlenbeck	4	-122.39	253.2	75.57	0	log(x+1)
Bumble Bee Landing	Both	GLS	3	63.216	-120.2	0	0.704	log(x+1)
Bumble Bee Landing	Both	Pagel's lambda	4	63.445	-118.4	1.73	0.296	log(x+1)
Bumble Bee Landing	Both	Ornstein-Uhlenbeck	4	26.982	-45.5	74.65	0	log(x+1)
Bumble Bee Nectaring	Both	Pagel's lambda	4	-36.776	82	0	0.858	log(x+1)
Bumble Bee Nectaring	Both	GLS	3	-39.666	85.6	3.59	0.142	log(x+1)
Bumble Bee Nectaring	Both	Ornstein-Uhlenbeck	4	-81.481	171.4	89.41	0	log(x+1)
Squash Bee Nectaring	Both	Pagel's lambda	4	-101.63	211.7	0	1	log
Squash Bee Nectaring	Both	GLS	3	-110.9	228.1	16.34	0	log
Squash Bee Nectaring	Both	Ornstein-Uhlenbeck	4	-132.58	273.6	61.89	0	log
Squash Bee Landing	Both	Pagel's lambda	4	-20.51	49.5	0	0.516	log(x+1)
Squash Bee Landing	Both	GLS	3	-21.668	49.6	0.13	0.484	log(x+1)
Squash Bee Landing	Both	Ornstein-Uhlenbeck	4	-56.518	121.5	72.02	0	log(x+1)
Seed Number	Both	Pagel's lambda	4	-390.03	788.7	0	0.998	none
Seed Number	Both	GLS	3	-397.54	801.5	12.76	0.002	none
Defective Seeds	Both	Pagel's lambda	4	-76.999	162.6	0	0.995	log(x+1)
Defective Seeds	Both	GLS	3	-83.357	173.1	10.46	0.005	log(x+1)
Germination Rate	Both	GLS	3	-296.96	600.3	0	0.509	none
Germination Rate	Both	Pagel's lambda	4	-295.87	600.4	0.07	0.491	none
Germination Rate	Both	Ornstein-Uhlenbeck	4	-315.07	638.8	38.47	0	none

Table S3.8: Results of pairwise comparisons to evaluate the impact of virus infection on trait values for individual taxa. Traits significantly impacted by infection at *p*-value <0.05 are in bold.

Trait	Year	Species	Trt	Estimate	SE	DF	t.ratio	transf	*p*-value
Fruit Number	2019	C_argyrosperma_sororia	Inf-Uninf	0.24	0.46	30	0.537	log	0.595
Fruit Number	2019	C_maxima_andreana	Inf-Uninf	-0.58	0.46	30	-1.278	log	0.211
Fruit Number	2019	C_maxima_maxima	Inf-Uninf	-0.27	0.42	30	-0.652	log	0.520

Fruit Number	2019	C_moschata	Inf-Uninf	0.01	0.60	30	0.010	log	0.992
Fruit Number	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-0.13	0.44	30	-0.290	log	0.774
Fruit Number	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.64	0.42	30	-1.511	log	0.141
Fruit Weight	2019	C_argyrosperma_sororia	Inf-Uninf	-0.44	0.20	30	-2.167	log	0.038
Fruit Weight	2019	C_maxima_andreana	Inf-Uninf	0.45	0.20	30	2.246	log	0.032
Fruit Weight	2019	C_maxima_maxima	Inf-Uninf	-0.06	0.19	30	-0.317	log	0.753
Fruit Weight	2019	C_moschata	Inf-Uninf	-0.08	0.26	30	-0.308	log	0.760
Fruit Weight	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-0.08	0.19	30	-0.430	log	0.670
Fruit Weight	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.61	0.19	30	-3.280	log	**0.003**
Female Flowers	2019	C_argyrosperma_sororia	Inf-Uninf	0.00	0.25	34	0.000	log(x+1)	1.000
Female Flowers	2019	C_maxima_andreana	Inf-Uninf	-0.26	0.22	34	-1.218	log(x+1)	0.232
Female Flowers	2019	C_maxima_maxima	Inf-Uninf	0.09	0.22	34	0.400	log(x+1)	0.692
Female Flowers	2019	C_moschata	Inf-Uninf	0.15	0.22	34	0.677	log(x+1)	0.503
Female Flowers	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-0.58	0.22	34	-2.687	log(x+1)	**0.011**
Female Flowers	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.45	0.22	34	-2.099	log(x+1)	**0.043**
Male Flowers	2019	C_argyrosperma_sororia	Inf-Uninf	0.64	0.51	34	1.257	log	0.217
Male Flowers	2019	C_maxima_andreana	Inf-Uninf	0.22	0.44	34	0.505	log	0.617
Male Flowers	2019	C_maxima_maxima	Inf-Uninf	0.80	0.44	34	1.838	log	0.075
Male Flowers	2019	C_moschata	Inf-Uninf	0.58	0.44	34	1.333	log	0.192
Male Flowers	2019	C_pepo_ovifera_ovifera	Inf-Uninf	0.12	0.44	34	0.265	log	0.793
Male Flowers	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.58	0.44	34	-1.327	log	0.193
Seed Number	2019	C_argyrosperma_sororia	Inf-Uninf	-0.24	0.25	23	-0.980	log	0.337
Seed Number	2019	C_maxima_andreana	Inf-Uninf	0.30	0.23	23	1.306	log	0.204
Seed Number	2019	C_maxima_maxima	Inf-Uninf	0.40	0.25	23	1.613	log	0.120
Seed Number	2019	C_moschata	Inf-Uninf	0.35	0.26	23	1.335	log	0.195
Seed Number	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-0.30	0.28	23	-1.087	log	0.288
Seed Number	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.60	0.28	23	-2.141	log	**0.043**
Defective Seeds	2019	C_argyrosperma_sororia	Inf-Uninf	0.23	0.54	23	0.423	log(x+1)	0.676
Defective Seeds	2019	C_maxima_andreana	Inf-Uninf	0.21	0.50	23	0.420	log(x+1)	0.679

Defective Seeds	2019	C_maxima_maxima	Inf-Uninf	1.02	0.54	23	1.887	log(x+1)	0.072
Defective Seeds	2019	C_moschata	Inf-Uninf	0.33	0.57	23	0.569	log(x+1)	0.575
Defective Seeds	2019	C_pepo_ovifera_ovifera	Inf-Uninf	0.21	0.60	23	0.348	log(x+1)	0.731
Defective Seeds	2019	C_pepo_ovifera_texana	Inf-Uninf	0.60	0.60	23	1.001	log(x+1)	0.327
Germination	2019	C_argyrosperma_sororia	Inf-Uninf	-6.97	14.50	22	0.423	none	0.676
Germination	2019	C_maxima_andreana	Inf-Uninf	-10.07	12.10	22	0.420	none	0.679
Germination	2019	C_maxima_maxima	Inf-Uninf	13.28	12.90	22	1.887	none	0.072
Germination	2019	C_moschata	Inf-Uninf	0.57	13.70	22	0.569	none	0.575
Germination	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-72.42	14.50	22	0.348	none	0.731
Germination	2019	C_pepo_ovifera_texana	Inf-Uninf	4.81	14.50	22	1.001	none	0.327
Squash Bee Landing	2019	C_argyrosperma_sororia	Inf-Uninf	-0.32	0.25	34	-1.271	log(x+1)	0.212
Squash Bee Landing	2019	C_maxima_andreana	Inf-Uninf	0.01	0.22	34	0.047	log(x+1)	0.963
Squash Bee Landing	2019	C_maxima_maxima	Inf-Uninf	0.06	0.22	34	0.286	log(x+1)	0.776
Squash Bee Landing	2019	C_moschata	Inf-Uninf	-0.21	0.22	34	-0.945	log(x+1)	0.352
Squash Bee Landing	2019	C_pepo_ovifera_ovifera	Inf-Uninf	0.06	0.22	34	0.277	log(x+1)	0.783
Squash Bee Landing	2019	C_pepo_ovifera_texana	Inf-Uninf	0.10	0.22	34	0.453	log(x+1)	0.654
Squash Bee Nectaring	2019	C_argyrosperma_sororia	Inf-Uninf	-1.52	0.60	34	-2.556	log	**0.015**
Squash Bee Nectaring	2019	C_maxima_andreana	Inf-Uninf	-0.01	0.52	34	-0.027	log	0.979
Squash Bee Nectaring	2019	C_maxima_maxima	Inf-Uninf	0.32	0.52	34	0.622	log	0.538
Squash Bee Nectaring	2019	C_moschata	Inf-Uninf	-0.40	0.52	34	-0.771	log	0.446
Squash Bee Nectaring	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-1.00	0.52	34	-1.937	log	0.061
Squash Bee Nectaring	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.77	0.52	34	-1.495	log	0.144
Bumble Bee Landing	2019	C_argyrosperma_sororia	Inf-Uninf	-0.11	0.12	34	-0.865	log(x+1)	0.393
Bumble Bee Landing	2019	C_maxima_andreana	Inf-Uninf	0.00	0.11	34	-0.026	log(x+1)	0.979
Bumble Bee Landing	2019	C_maxima_maxima	Inf-Uninf	-0.19	0.11	34	-1.765	log(x+1)	0.087
Bumble Bee Landing	2019	C_moschata	Inf-Uninf	-0.03	0.11	34	-0.288	log(x+1)	0.775
Bumble Bee Landing	2019	C_pepo_ovifera_ovifera	Inf-Uninf	0.00	0.11	34	0.014	log(x+1)	0.989
Bumble Bee Landing	2019	C_pepo_ovifera_texana	Inf-Uninf	0.00	0.11	34	0.038	log(x+1)	0.970
Bumble Bee Nectaring	2019	C_argyrosperma_sororia	Inf-Uninf	-0.05	0.24	34	-0.211	log(x+1)	0.834

Bumble Bee Nectaring	2019	C_maxima_andreana	Inf-Uninf	0.01	0.21	34	0.056	log(x+1)	0.956
Bumble Bee Nectaring	2019	C_maxima_maxima	Inf-Uninf	-0.36	0.21	34	-1.748	log(x+1)	0.090
Bumble Bee Nectaring	2019	C_moschata	Inf-Uninf	-0.05	0.21	34	-0.220	log(x+1)	0.827
Bumble Bee Nectaring	2019	C_pepo_ovifera_ovifera	Inf-Uninf	-0.03	0.21	34	-0.164	log(x+1)	0.871
Bumble Bee Nectaring	2019	C_pepo_ovifera_texana	Inf-Uninf	-0.12	0.21	34	-0.579	log(x+1)	0.567
Fruit Number	2021	C_argyrosperma_sororia	Inf-Uninf	-0.31	0.24	34	-1.306	log	0.200
Fruit Number	2021	C_maxima_andreana	Inf-Uninf	-0.76	0.27	34	-2.768	log	**0.009**
Fruit Number	2021	C_maxima_maxima	Inf-Uninf	-0.68	0.24	34	-2.846	log	**0.007**
Fruit Number	2021	C_moschata	Inf-Uninf	-0.27	0.24	34	-1.128	log	0.267
Fruit Number	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-1.08	0.24	34	-4.531	log	**<.0001**
Fruit Number	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.76	0.24	34	-3.202	log	**0.003**
Seed Number	2021	C_argyrosperma_sororia	Inf-Uninf	-0.24	0.28	21	-0.839	log	0.411
Seed Number	2021	C_maxima_andreana	Inf-Uninf	-0.41	0.25	21	-1.623	log	0.120
Seed Number	2021	C_maxima_maxima	Inf-Uninf	-0.33	0.25	21	-1.302	log	0.207
Seed Number	2021	C_moschata	Inf-Uninf	0.17	0.31	21	0.566	log	0.577
Seed Number	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-0.31	0.25	21	-1.234	log	0.231
Seed Number	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.12	0.25	21	-0.478	log	0.638
Fruit Weight	2021	C_argyrosperma_sororia	Inf-Uninf	-0.20	0.15	34	-1.331	log	0.192
Fruit Weight	2021	C_maxima_andreana	Inf-Uninf	-0.59	0.18	34	-3.357	log	**0.002**
Fruit Weight	2021	C_maxima_maxima	Inf-Uninf	-0.85	0.15	34	-5.588	log	**<.0001**
Fruit Weight	2021	C_moschata	Inf-Uninf	0.05	0.15	34	0.347	log	0.730
Fruit Weight	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-0.20	0.15	34	-1.316	log	0.197
Fruit Weight	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.49	0.15	34	-3.206	log	**0.003**
Female Flowers	2021	C_argyrosperma_sororia	Inf-Uninf	-0.05	0.27	34	-0.191	log(x+1)	0.850
Female Flowers	2021	C_maxima_andreana	Inf-Uninf	0.11	0.31	34	0.343	log(x+1)	0.733
Female Flowers	2021	C_maxima_maxima	Inf-Uninf	0.10	0.27	34	0.385	log(x+1)	0.703
Female Flowers	2021	C_moschata	Inf-Uninf	-0.20	0.27	34	-0.731	log(x+1)	0.470
Female Flowers	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-1.35	0.27	34	-5.039	log(x+1)	**<.0001**
Female Flowers	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.33	0.27	34	-1.214	log(x+1)	0.233

Male Flowers	2021	C_argyrosperma_sororia	Inf-Uninf	-1.12	0.29	34	-3.902	log	**<.0001**
Male Flowers	2021	C_maxima_andreana	Inf-Uninf	-0.34	0.33	34	-1.018	log	0.316
Male Flowers	2021	C_maxima_maxima	Inf-Uninf	-0.35	0.29	34	-1.210	log	0.235
Male Flowers	2021	C_moschata	Inf-Uninf	-0.58	0.29	34	-2.036	log	0.050
Male Flowers	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-1.08	0.29	34	-3.767	log	**0.001**
Male Flowers	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.80	0.29	34	-2.794	log	**0.009**
Seed Weight	2021	C_argyrosperma_sororia	Inf-Uninf	-0.02	0.26	21	-0.094	log	0.926
Seed Weight	2021	C_maxima_andreana	Inf-Uninf	-0.58	0.23	21	-2.527	log	**0.020**
Seed Weight	2021	C_maxima_maxima	Inf-Uninf	-0.27	0.23	21	-1.176	log	0.253
Seed Weight	2021	C_moschata	Inf-Uninf	-0.09	0.28	21	-0.336	log	0.740
Seed Weight	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-0.20	0.23	21	-0.887	log	0.385
Seed Weight	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.14	0.23	21	-0.622	log	0.541
Defective Seeds	2021	C_argyrosperma_sororia	Inf-Uninf	0.52	0.58	21	0.893	log(x+1)	0.382
Defective Seeds	2021	C_maxima_andreana	Inf-Uninf	0.84	0.52	21	1.608	log(x+1)	0.123
Defective Seeds	2021	C_maxima_maxima	Inf-Uninf	0.18	0.52	21	0.345	log(x+1)	0.733
Defective Seeds	2021	C_moschata	Inf-Uninf	-0.32	0.64	21	-0.504	log(x+1)	0.620
Defective Seeds	2021	C_pepo_ovifera_ovifera	Inf-Uninf	0.55	0.52	21	1.061	log(x+1)	0.301
Defective Seeds	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.19	0.52	21	-0.358	log(x+1)	0.724
Germination	2021	C_argyrosperma_sororia	Inf-Uninf	-26.66	9.87	20	-2.702	none	**0.014**
Germination	2021	C_maxima_andreana	Inf-Uninf	-9.19	8.83	20	-1.041	none	0.310
Germination	2021	C_maxima_maxima	Inf-Uninf	-19.96	8.83	20	-2.262	none	**0.035**
Germination	2021	C_moschata	Inf-Uninf	1.50	10.81	20	0.139	none	0.891
Germination	2021	C_pepo_ovifera_ovifera	Inf-Uninf	0.44	8.83	20	0.050	none	0.960
Germination	2021	C_pepo_ovifera_texana	Inf-Uninf	-1.78	9.87	20	-0.180	none	0.859
Squash Bee Landing	2021	C_argyrosperma_sororia	Inf-Uninf	0.74	0.43	34	1.707	log	0.097
Squash Bee Landing	2021	C_maxima_andreana	Inf-Uninf	0.48	0.50	34	0.967	log	0.341
Squash Bee Landing	2021	C_maxima_maxima	Inf-Uninf	0.67	0.43	34	1.563	log	0.127
Squash Bee Landing	2021	C_moschata	Inf-Uninf	1.16	0.43	34	2.688	log	**0.011**
Squash Bee Landing	2021	C_pepo_ovifera_ovifera	Inf-Uninf	1.03	0.43	34	2.378	log	**0.023**

Squash Bee Landing	2021	C_pepo_ovifera_texana	Inf-Uninf	0.53	0.43	34	1.229	log	0.227
Squash Bee Nectaring	2021	C_argyrosperma_sororia	Inf-Uninf	-0.07	0.30	34	-0.232	log	0.818
Squash Bee Nectaring	2021	C_maxima_andreana	Inf-Uninf	0.08	0.35	34	0.227	log	0.822
Squash Bee Nectaring	2021	C_maxima_maxima	Inf-Uninf	0.02	0.30	34	0.058	log	0.954
Squash Bee Nectaring	2021	C_moschata	Inf-Uninf	0.09	0.30	34	0.304	log	0.763
Squash Bee Nectaring	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-0.03	0.30	34	-0.116	log	0.909
Squash Bee Nectaring	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.09	0.30	34	-0.298	log	0.768
Bumble Bee Landing	2021	C_argyrosperma_sororia	Inf-Uninf	-0.03	0.02	34	-1.682	log	0.102
Bumble Bee Landing	2021	C_maxima_andreana	Inf-Uninf	0.00	0.02	34	-0.111	log	0.912
Bumble Bee Landing	2021	C_maxima_maxima	Inf-Uninf	0.00	0.02	34	0.062	log	0.951
Bumble Bee Landing	2021	C_moschata	Inf-Uninf	-0.02	0.02	34	-1.213	log	0.234
Bumble Bee Landing	2021	C_pepo_ovifera_ovifera	Inf-Uninf	0.02	0.02	34	1.127	log	0.268
Bumble Bee Landing	2021	C_pepo_ovifera_texana	Inf-Uninf	0.02	0.02	34	1.192	log	0.242
Bumble Bee Nectaring	2021	C_argyrosperma_sororia	Inf-Uninf	0.06	0.04	34	1.567	log	0.126
Bumble Bee Nectaring	2021	C_maxima_andreana	Inf-Uninf	-0.01	0.05	34	-0.194	log	0.848
Bumble Bee Nectaring	2021	C_maxima_maxima	Inf-Uninf	0.03	0.04	34	0.789	log	0.436
Bumble Bee Nectaring	2021	C_moschata	Inf-Uninf	-0.02	0.04	34	-0.401	log	0.691
Bumble Bee Nectaring	2021	C_pepo_ovifera_ovifera	Inf-Uninf	0.00	0.04	34	-0.093	log	0.927
Bumble Bee Nectaring	2021	C_pepo_ovifera_texana	Inf-Uninf	0.02	0.04	34	0.383	log	0.704
Pollen Weight	2021	C_argyrosperma_sororia	Inf-Uninf	-29.79	12.90	22	-2.313	none	**0.031**
Pollen Weight	2021	C_maxima_andreana	Inf-Uninf	-23.75	12.90	22	-1.844	none	0.079
Pollen Weight	2021	C_maxima_maxima	Inf-Uninf	-14.05	14.40	22	-0.976	none	0.340
Pollen Weight	2021	C_moschata	Inf-Uninf	13.25	12.90	22	1.029	none	0.315
Pollen Weight	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-15.54	13.70	22	-1.137	none	0.268
Pollen Weight	2021	C_pepo_ovifera_texana	Inf-Uninf	-4.29	14.40	22	-0.298	none	0.768
Pollen Protein	2021	C_argyrosperma_sororia	Inf-Uninf	-9.42	35.60	22	-0.265	none	0.794
Pollen Protein	2021	C_maxima_andreana	Inf-Uninf	13.98	35.60	22	0.393	none	0.698
Pollen Protein	2021	C_maxima_maxima	Inf-Uninf	7.15	39.70	22	0.180	none	0.859
Pollen Protein	2021	C_moschata	Inf-Uninf	-7.56	35.60	22	-0.213	none	0.834

Pollen Protein	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-51.81	37.70	22	-1.374	none	0.183
Pollen Protein	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.48	39.70	22	-0.012	none	0.991
Nectar Volume	2021	C_argyrosperma_sororia	Inf-Uninf	-0.38	0.34	20	-1.105	log	0.282
Nectar Volume	2021	C_maxima_andreana	Inf-Uninf	-0.26	0.34	20	-0.755	log	0.459
Nectar Volume	2021	C_maxima_maxima	Inf-Uninf	0.32	0.42	20	0.764	log	0.454
Nectar Volume	2021	C_moschata	Inf-Uninf	-0.19	0.38	20	-0.501	log	0.622
Nectar Volume	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-0.54	0.36	20	-1.499	log	0.149
Nectar Volume	2021	C_pepo_ovifera_texana	Inf-Uninf	-0.25	0.38	20	-0.659	log	0.517
Nectar Sucrose	2021	C_argyrosperma_sororia	Inf-Uninf	-99.50	54.70	20	-1.819	none	0.084
Nectar Sucrose	2021	C_maxima_andreana	Inf-Uninf	-49.60	54.70	20	-0.907	none	0.375
Nectar Sucrose	2021	C_maxima_maxima	Inf-Uninf	-47.90	67.00	20	-0.715	none	0.483
Nectar Sucrose	2021	C_moschata	Inf-Uninf	11.90	61.10	20	0.195	none	0.847
Nectar Sucrose	2021	C_pepo_ovifera_ovifera	Inf-Uninf	-63.50	58.00	20	-1.095	none	0.287
Nectar Sucrose	2021	C_pepo_ovifera_texana	Inf-Uninf	-97.60	61.10	20	-1.597	none	0.126

Supplemental Figures

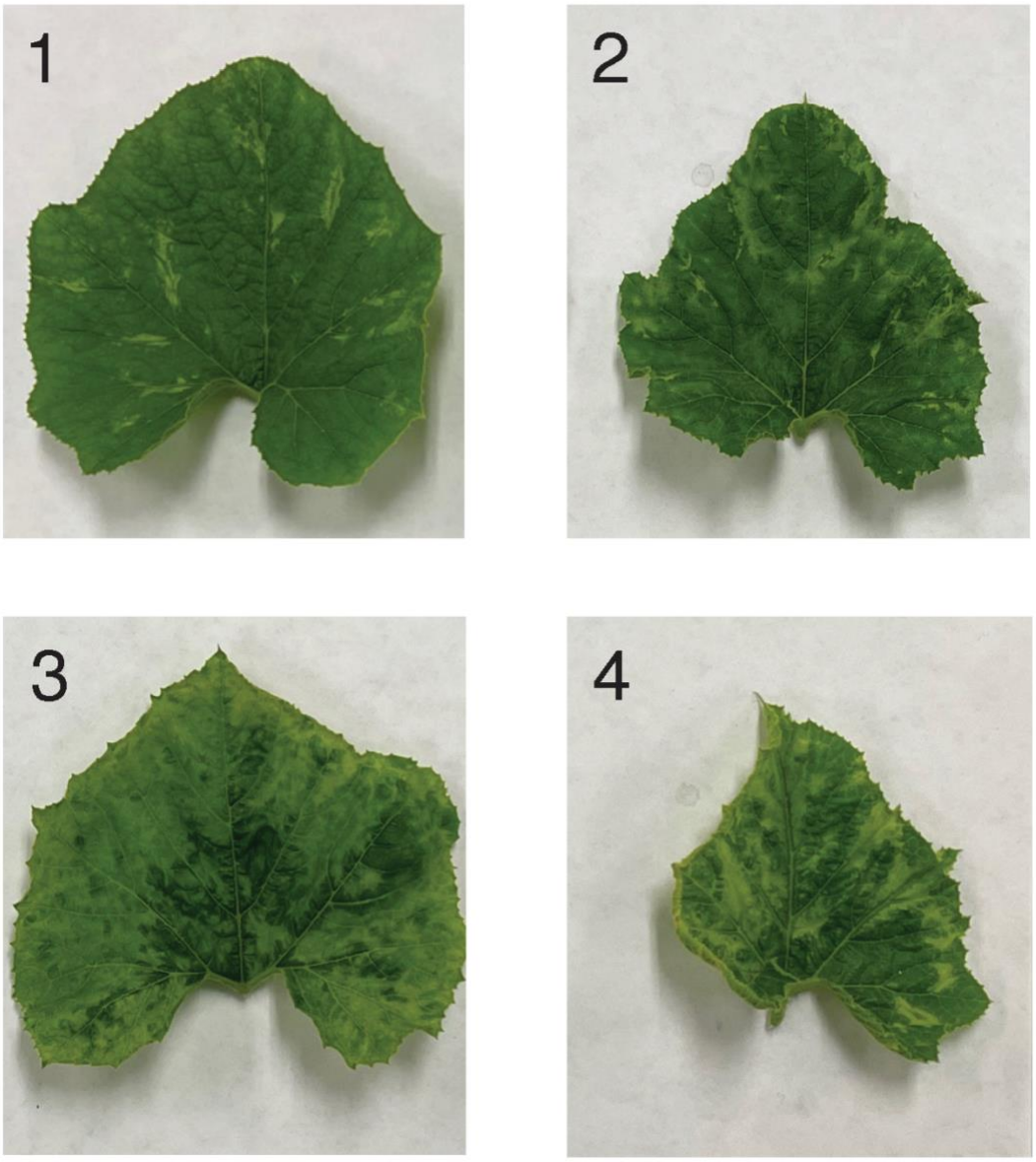

Figure S3.1: Example of the scale used for rating virus-induced foliar symptoms, modified from Kone et al. 2017. 1 = mild mottling on ≤ 10% of leaf area; 2 = mottling on ≤ 50% of leaf area/light downward cupping; 3 = pronounced downward or up cupping of leaf/chlorosis/75–100% leaf mottling; 4 = severe mottling/severe distortion of leaf/crinkled leaf/stunting of entire plant/leaf bunching.

Chapter 4

Investigating Transcriptional Responses to Virus Infection in Three *Cucurbita* spp. via RNA-sequencing

Plant viruses can cause devastating disease to agricultural crops. Resistance is the most extensively studied defense mechanism in plants and qualitative host resistance refers to a high capacity for reducing pathogen replication, or complete inhibition. While some plant hosts can reduce disease without completely blocking virus replication, the molecular mechanisms behind reduced susceptibility in the absence of qualitative resistance are largely unknown. Here, we examined transcriptional responses to mixed infection of zucchini yellow mosaic virus (ZYMV) and squash mosaic virus (SqMV) at 2-, 4-, and 8-days post-inoculation (dpi) in hosts lacking sources of qualitative resistance. We used three *Cucurbita* species with high (*C. pepo ovifera* var. *ovifera*), medium (*C. maxima* ssp. *maxima*), or low (*C. moschata*) susceptibility to disease based on previous characterizations of plant fitness in the field. In the present study, both viruses efficiently accumulated in all hosts, but *C. moschata* had reduced levels of ZYMV and symptom severity compared to other hosts. Results of RNA sequencing show that hosts with low susceptibility had fewer differentially expressed genes (DEGs) compared to highly susceptible hosts. This trend was also observed when comparing mock-inoculated to uninoculated plants, suggesting similar responses to various stressors. At early time points in locally inoculated leaves, virus infection triggered responses related to photosynthesis in the more susceptible hosts, but not in the least susceptible host. We also detected significant enrichment of pathways related to plant-pathogen interaction in the most susceptible host in systemically infected leaves at 8 dpi. With the strongest defense responses detected in the most susceptible host, our results suggest that in this system lower virus susceptibility is marked by reduced but efficient responses that limit virus

accumulation and contribute to reduced foliar symptoms and disease severity in the long term. This dataset provides a valuable resource for further investigating the genetic mechanisms associated with reduced susceptibility to viral disease beyond qualitative resistance.

4.1 Introduction

Due to decreases in host fitness caused by pathogen infections, hosts have evolved responses to limit the severity of damage, or pathogen virulence. While resistance is the most studied defense mechanism against plant viruses because it limits the replication of pathogens, the observation that many plants are infected with viruses but show limited damage suggests that other defenses may be more widespread than previously thought (Prendeville et al., 2012; Roossinck et al., 2015).

Qualitative or complete resistance that strongly inhibits the replication or spread of plant viruses within a host is often conferred by individual or a few R-genes (Kang et al., 2005). Theory and experimental evidence indicate that qualitative resistance places strong selection pressures on pathogens to evolve and avoid recognition or to interfere with the defense pathways and can lead to resistance-breaking strains of plant viruses (Pink et al., 1992; Salomon, 1999; Crescenzi et al., 2013; Heo et al., 2020; Lahre et al., 2023). Thus, there is a need for increased understanding of how plants can limit the negative effects of virus infection in the absence of qualitative resistance.

The genetic basis of host antiviral defenses beyond qualitative resistance is largely unknown. In many cases, reduced susceptibility is associated with multiple loci with major or minor effects and is associated with the presence of host susceptibility factors (del Blanco et al., 2014; Foresman et al., 2016; Jones et al., 2018). Some studies have been able to identify individual genes associated with incomplete or partial resistance, but these can be dominant,

semi-dominant, or recessive, further contributing to the complexity of investigating the genetics of antiviral defenses (Zamir et al., 1994; Pachner et al., 2011; Riedel et al., 2011; Senjam et al., 2018). Disease manifestation is ultimately a result of either damage from uncontrolled and non-specific host responses or direct toxicity of viral proteins (Mandadi and Scholthof, 2013; Paudel and Sanfaçon, 2018). Therefore, the ability to survive viral infections comes from controlling either of these two mechanisms (Mandadi and Scholthof, 2013; Paudel and Sanfaçon, 2018). Investigating genome-wide transcriptional responses to virus infection can provide insight on the diversity of genes and processes associated with disease susceptibility.

Cucurbits (*Cucurbita* spp., “squash, pumpkins, gourds") are grown around the world and are currently known to be susceptible to at least 96 viruses (Khanal et al., 2021).Two of the most prevalent viruses infecting cucurbits in the US are zucchini yellow mosaic virus (ZYMV; *Potyvirus*) and squash mosaic virus (SqMV; *Comovirus*), transmitted by aphids and beetles, respectively (Desbiez and Lecoq, 1997; Tolin et al., 2016). Both viruses are also transmitted vertically through the seeds of infected plants and are commonly found co-infecting the same fields and plants (Alvarez, 1978; Simmons et al., 2013). Although these viruses rarely kill host plants, they contribute to significant yield losses through distortions of leaves and malformation of fruits (Blua and Perring, 1989; Lecoq and Desbiez, 2012).

There have been considerable efforts to breed for virus resistance in cucurbits either by introgressing sources of resistance from wild species (Pachner et al., 2011, 2015), or expressing viral coat proteins to confer resistance in transgenic plants (Tricoli et al., 1995; Provvidenti and Tricoli, 2002). Several dominant and recessive resistance genes have been identified against ZYMV (Paris et al., 1988; Paris and Cohen, 2000; Pachner et al., 2011; Nacar Ç et al., 2012). Recessive *eIF4E* genes have been identified with mutations that inhibit interactions with potyvirus VPg proteins, preventing viral translation and ultimately replication of several potyviruses (Ling et al., 2009; Chandrasekaran et al., 2016). Additionally, when various

resistance genes are introgressed into susceptible *C. pepo* cultivars, there is still symptom expression, and viral replication after inoculation with ZYMV and hosts can be considered tolerant (Pachner et al., 2015). Previous studies have taken transcriptomic or proteomic approaches to identify important factors conferring tolerance to ZYMV (Nováková et al., 2015; Amoroso et al., 2022). However, these studies have focused on a single species, *C. pepo*, with resistance genes introgressed from one of the several resistant genotypes of *C. moschata* (Paris et al., 1988; Pachner et al., 2011; Nacar et al., 2012).

Here, we used three *Cucurbita* spp. genotypes that are not known to carry any major or minor resistance genes to ZYMV, SqMV, or other viruses. Previously, we characterized the susceptibility of these hosts to mixed infection with ZYMV and SqMV over multiple growing seasons (Hinshaw et al., *unpublished*). We found that impacts of virus infection on symptom severity and life history traits varied greatly between *C. pepo*, *C. maxima*, and *C. moschata*, and fitness costs for hosts were phylogenetically structured. Yet, when examining plants collected in the field approximately 60 dpi, all three hosts exhibited similarly high levels of SqMV and ZYMV. Additionally, responses to foliar damage may be linked with responses to incoming pathogen infection, as both damage-associated molecular patterns and pathogen-associated molecular patterns can activate similar responses to aid in plant recovery (Savatin et al., 2014). Thus, the ability to mount appropriate responses to various stresses (wounding or infection) may be indicative of general "immunocompetence". As there are likely multiple genes and unknown interactors that contribute to reductions in disease, here we investigated transcriptional responses to virus infection and wounding via RNA sequencing in three closely related hosts with different levels of susceptibility.

Based on previous models of virus defense mechanisms, we hypothesized that reduced susceptibility is characterized by generally reduced transcriptional reprogramming in response to virus infection (Bengyella et al., 2015). We found support for this, as the least susceptible host

had reduced responses to both wounding and virus infection compared to other hosts. We also found that differentially expressed genes were largely unique to each host, and identified specific defense related genes that were uniquely expressed during early responses in the least susceptible host. Responses were also more similar in the two most closely related hosts regarding timing and overlap in gene expression. By identifying genome-wide expression involved in effective plant defenses we can start to uncover the molecular basis behind defense strategies beyond qualitative resistance.

4.2 Materials and Methods

4.2.1 Virus Inoculation and Growth Conditions

We placed seeds of *C. pepo ovifera* var. *ovifera, C. moschata*, and *C. maxima* ssp. *maxima* (hereafter, *C. pepo*, *C. moschata*, and *C. maxima*) in plastic dishes with moistened paper towels to initiate germination. After gemination we transplanted seedlings into 4-inch pots containing Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA) amended with Osmocote slow release fertilizer (14-14-14) (Osmocote, Bloomington, IN). Seed sources and the variety name of each host species are provided in **Table S4.1**. The phylogenetic relationships between the three hosts are depicted in **Figure S4.1**. After transplanting, plants were grown in a controlled growth room at approximately 25-27°C, 30% relative humidity, with a light:dark cycle of 16:8 hrs. Plants were inoculated with a combination of ZYMV and SqMV at the three true leaf stage using coinfected leaves from symptomatic *C. pepo* ssp. *ovifera* var *texana* collected from a natural field epidemic (Harth et al., 2018). Leaves used for inoculum had ZYMV and SqMV infection confirmed by ELISA based Immunostrips® (Agdia Inc., Elkhart, IN) tests. Symptomatic leaves were homogenized in phosphate buffer (0.1 M Na2HPO4·2H2O + 0.1 M

KH2PO4) using a mortar and pestle containing activated carbon and carborundum. The pestle was then dipped in the inoculum and rubbed on the adaxial leaf surface of each plant. The rest of the plants were either mock-inoculated using the same buffer but without leaf tissue or kept undamaged to serve as controls. Plants were arranged in a randomized block design. To investigate initial responses to infection, we sampled plants at two time points before either SqMV or ZYMV was detected systemically (2 dpi, 4 dpi on inoculated leaves) and one time point after virus accumulation and symptom expression (8 dpi on youngest leaves). At each time point we used approximately 20 plants per species per treatment. After sampling, virus-inoculated plants were monitored for symptom development until 12 dpi, and only plants which developed symptomatic infections were chosen for sequencing.

4.2.2 Symptom Severity Assessment

Symptom severity in virus-inoculated plants was rated using a modified scale from (Kone et al., 2017b). Plants were assigned values based on the following: 0 = no disease symptoms; 1 = mild mottling on ≤ 10% of leaf area; 2 = mottling on ≤ 50% of leaf area/light downward cupping; 3 = pronounced downward or up cupping of leaf/chlorosis/75–100% leaf mottling; 4 = severe mottling/severe distortion of leaf/crinkled leaf/stunting of entire plant/leaf bunching. Using severity ratings from all plants at 12 dpi, we then ran cumulative link mixed-effect models to determine differences in symptom severity between host species using the ‘ordinal’ package in R version 4.2.2 (R Core Team, 2019; Christensen, 2022). We used severity as the response, host species as a fixed effect, and time point as a random effect. We performed pairwise comparisons using the ‘emmeans’ package with a Tukey adjustment for multiple comparisons (Lenth, 2020).

4.2.3 Sampling, RNA Extraction, and Library Preparation

Three biological replicates from each treatment, taxon, and time point combination were sampled for a total of 81 samples used for RNA-sequencing. Leaf tissue was collected by taking three, 1cm hole punches of inoculated leaves for plants sampled at 2 dpi and 4 dpi, and the youngest systemic leaf >~1in^2 for plants sampled at 8 dpi. Thus, our sampling represented early/local (2 dpi), late/local (4 dpi), and late/systemic responses (8 dpi). Immediately after sampling, leaf tissue from each plant was combined and flash frozen using liquid nitrogen and stored at -80°C until RNA extraction could be performed. RNA was extracted from samples using Zymo Resarch quick-RNA plant miniprep kit, according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). We then sent samples to Novogene Inc. for RNA quality control and paired-end sequencing (2 x 150bp) of total mRNA after poly(A) enrichment. Sequencing was performed on an Illumina NovaSeq 6000 system. After sequencing, quality control was performed by trimming adapters and removing low quality reads with >10% undetermined bases, Q-score $\leq$ 5.

4.2.4 RNA-Sequencing Analyses

The cleaned reads from each sample were mapped to their respective host reference genomes (**Table 4.1**) using HISAT2 with default parameters (Kim et al., 2019). We then used featureCounts v 2.0.1, to assign alignments to specific genes (Liao et al., 2014). After alignment, we used DESeq2 for analysis of differential gene expression between mock-inoculated and virus-inoculated plants or between uninoculated and mock-inoculated plants (Love et al., 2014). We calculated the total number of expressed genes using a threshold of baseMean > 100 and the number of differentially expressed genes between treatments based on *p*-adjusted < 0.05 and $\log_2$

fold change > 1. To make direct comparisons of DEGs between the three host taxa, we identified reciprocal best hits as a proxy for orthologs with MMseqs2 using Genome Coverage Format (GCF) files from each host as input (Steinegger and Söding, 2017). We then used DAVID v2021 to identify gene ontology terms associated with DEGs in each treatment and perform gene enrichment analysis (Sherman et al., 2022).

Table 4.1: Reference genomes used for analysis. Relative level of susceptibility for each species is defined based on previous characterizations of fitness costs during virus infection in the field.

Host	Reference Genome	Level of Susceptibility
C. pepo pepo var *ovifera*	GCF_002806865.1	High
C. maxima maxima	GCF_002738345.1	Medium
C. moschata	GCF_002738365.1	Low

4.2.3 Analysis of Virus Sequences

To analyze viral reads, we first used HISAT2 to map reads from each sample to ZYMV (MT882336.1) or SqMV (concatenation of RNA1 NC_003799.1 and RNA2 NC_003800.1) reference genomes, separately. For samples collected at 8 dpi, we used the percentage of all viral reads in a sample to determine differences in relative virus abundance between hosts. We used generalized linear models with a fixed effect of host species percentage of virus-aligned reads as the response. We then used the 'emmeans' package to perform pairwise comparisons between species, with a Tukey adjustment for multiple comparisons (Lenth, 2020). All analyses were conducted in R version 4.2.2 (R Core Team, 2019).

We then performed variant calling using BCFTools and performed visualizations in the Integrative Genomics Viewer to detect single nucleotide variants (SNVs) that were present in virus genomes in each biological replicate of the three host species (Li, 2011; Robinson et al., 2011). We also used the resulting multi-sequence alignments to generate a consensus sequence for our isolates of ZYMV or SqMV. We then used the consensus sequence from each virus to generate phylogenies to determine the relatedness of our isolates with other isolates. We performed phylogenetic analysis separately for the complete sequence of ZYMV and SqMV RNA 1. We downloaded complete viral genomes from NCBI Genbank (http://www.ncbi.nlm.nih.gov) in FASTA format and performed a multiple sequence alignment in MEGA11 software with ClustalW with a gap opening penalty of 15 and a gap extension penalty of 6.7 (Tamura et al., 2021). Then we generated a maximum-likelihood phylogeny in MEGA11 using the Kimura-2 substitution model, with 1,000 bootstrap replicates, collapsing any nodes with less than 60% bootstrap support (**Figure S4.2**; **Figure S4.3**).

4.3 Results

4.3.1 Quantification of Host Symptom Severity and Viral Load

Symptom expression was consistently observed in all virus-inoculated hosts at 12 dpi but not at 2 or 4 dpi time points, and never on the inoculated leaves. We found that foliar symptoms at 12 dpi were significantly different between hosts (F = 13.1, df = 2, $p < 0.001$). The severity of symptoms was similar between *C. pepo* and *C. maxima* but was significantly lower in *C. moschata* (**Figure 4.1B**). Additionally, within each host the three biological replicates used for RNA-sequencing exhibited similar levels of symptom severity.

We aligned transcripts from each sample to the genome of either SqMV or ZYMV. We found that at 2 dpi less than 0.14 % reads were identified as belonging to SqMV or ZYMV. At 4 dpi, the number of reads corresponding to either virus dropped to < 0.02 % for all samples (and < 0.01% for most samples), indicating that viral reads from 2 dpi were likely from the inoculated viruses and not a result of within-host virus replication.

At 8 dpi, we found that a large percentage of all reads were assigned to ZYMV, and a smaller amount were identified as SqMV (**Figure 4.1A**; **Table S4.2**). Additionally, we found a significant effect of host on the percentage of reads identified as ZYMV (X^2= 16.03, df = 2, p = $3.3x10^{-4}$). On average, *C. moschata* had significantly fewer ZYMV transcripts than *C. pepo* (2.7x less) or *C. maxima* (2.9x less), while no significant differences were detected between *C. pepo* and *C. maxima*. No differences in the amount of SqMV were detected between hosts (X^2= 1.29, df = 2, p = 0.52).

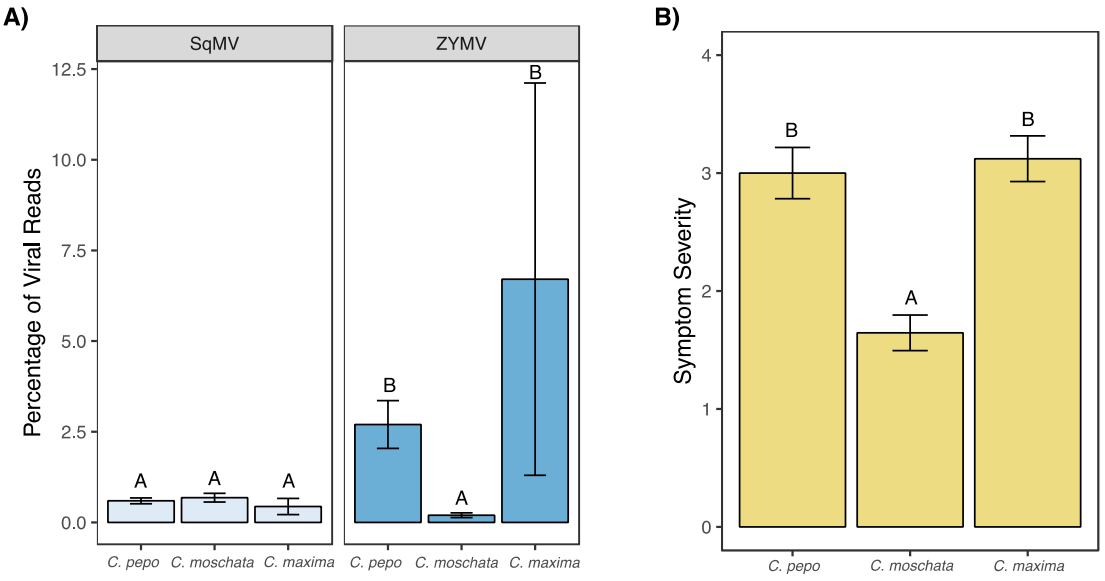

Figure 4.1: Quantification of virus accumulation and symptom severity in three plant hosts. (**A**) Percentage of reads corresponding to ZYMV or SqMV detected in each host at 8 dpi based on three biological replicates. (**B**) Average foliar symptom severity based on all symptomatic infections observed in this experiment at 12 dpi (*C. pepo*, n= 31; *C. maxima*, n = 33; *C. moschata*, n = 31). Bars represent standard error of the mean. Different letters indicate significant differences between groups at alpha=0.05.

Multi-sequence alignments of ZYMV from all nine virus-inoculated samples collected at 8 dpi show that several single nucleotide variants (SNVs) were unique to the ZYMV populations infecting *C. moschata*. Specifically, we detected three nucleotide mutations that were present in all three samples from *C. moschata* but not from the other hosts. These mutations were found in regions encoding the P3 protein (position 3105), NiB protein (position 7815), and the coat protein (position 9030). However, all three were synonymous mutations and did not result in amino acid changes. Analysis of SqMV sequences from each biological sample revealed three mutations that were present in all three samples from *C. pepo* but no other samples. These mutations were found

in the large coat protein encoding region at positions 2648, 2651, and 2654. All mutations were synonymous.

Phylogenetic analyses using the consensus sequence of ZYMV from all samples at 8 dpi show that our isolate was most closely related to other isolates from Pennsylvania, USA and shares the most similarity with a previous isolate from the same fields (ref: JQ716413.1) from which this isolate was propagated (Simmons et al., 2008) (**Figure S4.2**). Isolates from Pennsylvania were also more closely related to each other than to other isolates. Phylogenetic analyses of SqMV show that our isolate forms its own clade but shares a common ancestor with two isolates from Japan (**Figure S4.3**). However, there are relatively few complete sequences of SqMV RNA 1 available in NCBI GenBank and thus descriptions of phylogenetic relationships remain incomplete.

4.3.2 Gene Expression Trends in Local Leaves and Conserved Genes Between Hosts

We obtained an average of 59M raw reads per sample with a GC content of 44.5%. After cleaning, over 98% of all reads remained for each sample. We achieved an average alignment rate of 85% for all samples using HISAT2. Overall, approximately half of all gene features in each host were expressed at each time point (**Table S4.3**). The number of DEGs in local responses to wounding or virus infection was markedly different between hosts and time points (**Figure 4.2**).

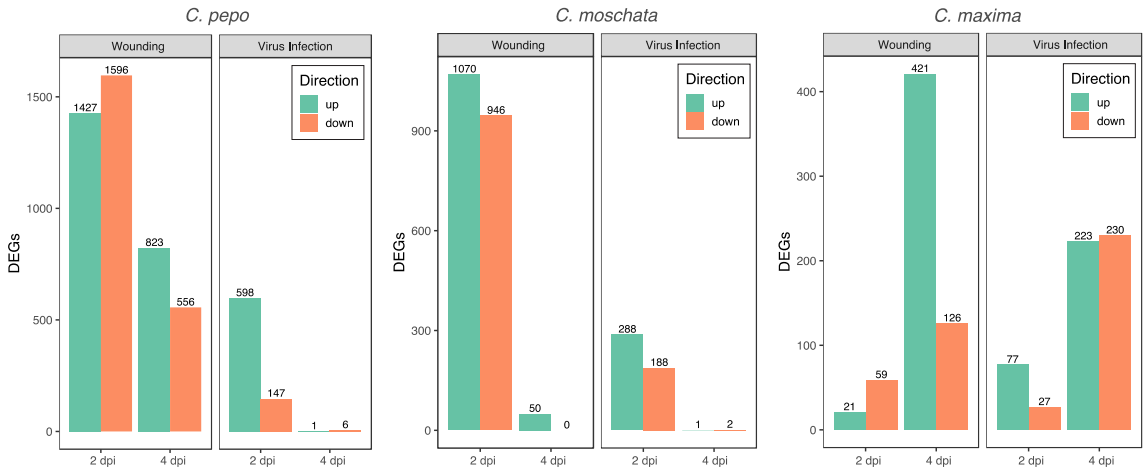

Figure 4.2: Total number of differentially expressed genes (DEGs) during local responses to wounding (mock-inoculation vs. undamaged) or virus infection (virus-inoculation vs. mock-inoculation) in each host at two or four days post inoculation (dpi). Note: y-axes are on different scales.

To determine if the three host species responded differently to wounding during the inoculation process, we calculated the number of genes that were differentially expressed in mock-inoculated compared to undamaged plants. At 2 dpi in response to wounding, *C. pepo* had the highest number of DEGs (3023), followed by *C. moschata* (2016), and *C. maxima* (80) (**Figure 4.2**). We found that wounding responses were decreased at 4 dpi compared to 2 dpi in *C. pepo* and *C. moschata* with reductions of 54.3% and 97.5%, respectively, but we detected the strongest responses to wounding in *C. maxima* at 4 dpi. At 4 dpi, *C. pepo* had the highest number of DEGs (1379), followed by *C. maxima* (547), and *C. moschata* (50) (**Figure 4.2**).

In comparison to wounding at 2 dpi, we detected approximately 75% fewer DEGs in response to virus infection at the same time point in *C. pepo* and *C. moschata*, but 30% more DEGs in *C. maxima*. Regarding virus-specific responses at 2 dpi, *C. pepo* had the highest total number of DEGs (745) followed by *C. moschata* (476) and *C. maxima* (104). The total number of DEGs then decreased drastically at 4 dpi in *C. pepo* (7) and *C. moschata* (3). In contrast, *C. maxima* had more DEGs at 4 dpi (453) compared to 2 dpi (104). When comparing across time

points, *C. pepo* and *C. moschata* exhibited similar trends in local responses to wounding and to virus infection, with more DEGs at 2 dpi than 4 dpi, while *C. maxima* had the highest differential expression at 4 dpi for both wounding and virus infection.

When evaluating overlap in wounding responses between hosts, we found that the two more closely related hosts (*C. pepo* and *C. moschata*) shared the most genes based on sequence similarity. The comparisons with the most overlap were between *C. pepo* and *C. moschata* at 8 dpi (518 orthologous DEGs), *C. pepo* at 2 dpi and 4 dpi (503 orthologous DEGs), and *C. pepo* at 4 dpi and *C. moschata* at 2 dpi (226 orthologous DEGs) (**Figure 4.3A**).

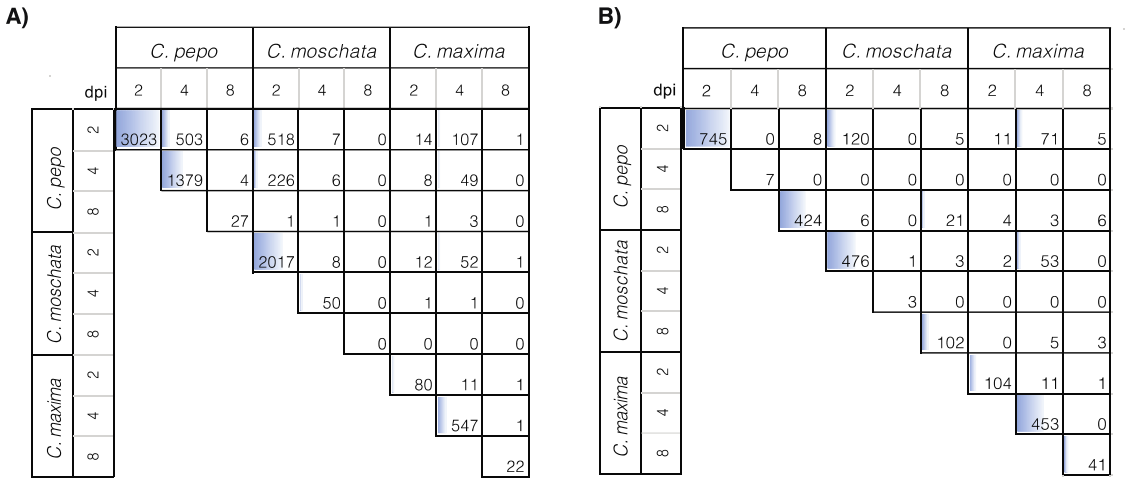

Figure 4.3: Number of overlapping differentially expressed genes between hosts and time points in response to (**A**) wounding (mock-inoculation vs. undamaged) or (**B**) virus infection (virus-inoculation vs. mock-inoculation). Comparisons between different hosts represent reciprocal best hits as proxies for orthologs.

We also found a similar overlap between hosts and time points in response to virus infection as to wounding. There was considerable overlap between *C. pepo* and *C. moschata* at 2 dpi, with 120 orthologous genes between the two hosts (**Figure 4.3B**). At this time point, there

were also 11 genes shared between *C. maxima* and *C. pepo* and two genes shared between *C. maxima* and *C. moschata*. We found only one orthologous gene that was differentially expressed in all hosts at 2 dpi. This was "photosystem II 5 kDa protein, chloroplastic", which encodes photosystem II 5kDa subunit PSII-T. This gene had the highest log_2 fold change in *C. pepo* (2.07) followed by *C. maxima* (1.90) and *C. moschata* (1.75).

Interestingly, several of the genes expressed by *C. maxima* in response to virus infection at 4 dpi were orthologous to those expressed by *C. pepo* (71) and *C. moschata* (53) at 2 dpi, suggesting a delay in responses to virus infection in *C. maxima*. At 8 dpi, several genes were differentially expressed during virus infection in both *C. pepo* and *C. moschata* (21 orthologous DEGs), *C. pepo* and *C. maxima* (6 orthologous DEGs) and *C. maxima* and *C. moschata* (3 orthologous DEGs) (**Figure 4.3B**). Generally, there was minimal overlap of genes in all comparisons, indicating responses are largely unique to hosts and time points.

4.3.3 Gene Expression in Systemic Leaves

Systemic responses to wounding at 8 dpi in uninoculated leaves were highly decreased compared to virus infection at the same time point (**Figure 4.4**). At 8 dpi, we detected no DEGs in *C. moschata* and found 27 and 22 DEGs in *C. pepo* and *C. maxima*, respectively. When comparing the two treatments at 8 dpi, we found fewer genes in response to wounding than to virus infection, with reductions of 93.6%, 100%, and 46.3% for *C. pepo*, *C. moschata*, and *C. maxima*, respectively. For the late, systemic responses to virus infection at 8 dpi we detected the highest number of DEGs in *C. pepo* (424) followed by *C. moschata* (102) and *C. maxima* (41) (**Figure 4.4**).

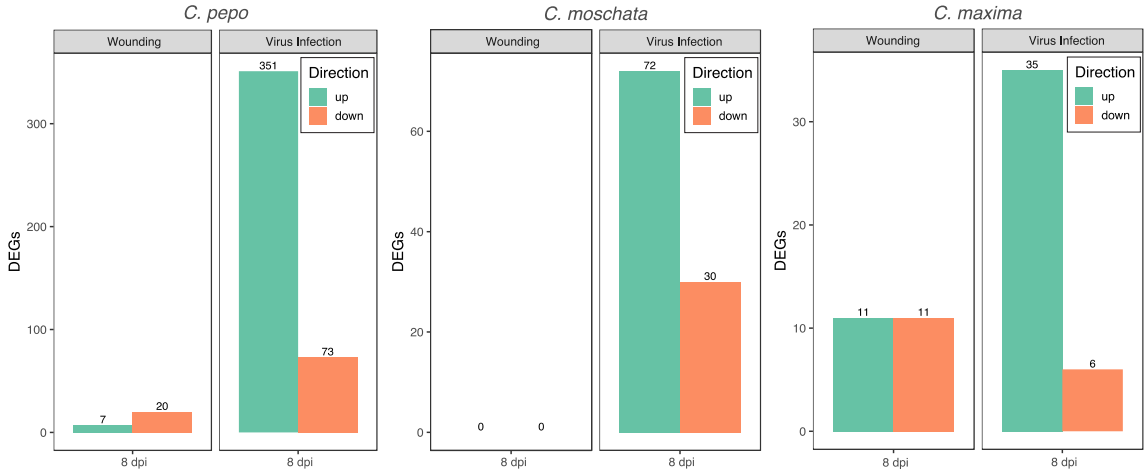

Figure 4.4: Number of differentially expressed genes (DEGs) during systemic responses to wounding (mock-inoculation vs. undamaged) or virus infection (virus-inoculation vs. mock-inoculation) in each host at eight days post inoculation (dpi). Note: y-axes are on different scales.

4.3.4 Local Responses to Virus Infection

In all hosts, we detected fewer than 1,000 total DEGs in local responses to virus infection. To determine if responses were concentrated on certain processes, we first investigated the GO terms associated with DEGs. In general, we found that GO terms varied widely within hosts, as few GO terms were significantly enriched for any time point or host combination using a false discovery rate (FDR) < 0.05 (**Table S4.4**; **Table S4.5**). Thus, we extracted all the identified GO terms and focused our reporting on those with known functions in defense responses or other associations of interest.

Regarding early local responses at 2 dpi, we found enrichment of 10 GO terms in *C. pepo* including those related to photosynthesis, translation, cell wall organization, chlorophyll biosynthesis, and fucose metabolic processes (**Table S4.4**). In *C. maxima* we also found multiple genes associated with photosynthesis and chlorophyll biosynthesis GO terms as well as terpenoid biosynthesis. Surprisingly, in *C. moschata*, photosynthesis processes were not represented by

multiple genes. Indeed, we only found two out of 476 DEGs directly associated with either photosynthesis or photoreceptor activity. We found multiple genes associated with GO terms such as cell wall organization, methylation, and methionine biosynthesis.

Examining DEGs with known relations to plant responses to biotic stresses, we found several GO terms related to various functions involved in pathogen responses including pathogenesis-related proteins, leucine-rich repeat receptor-like protein kinases, and salicylic acid signaling molecules (**Table 4.2**). When combining early and late local responses, *C. pepo* had the highest number of defense related genes for several categories.

Table 4.2: Functions of DEGs in local responses to virus infection.

Gene Function/ Process	*C. pepo* 2 dpi	*C. pepo* 4 dpi	*C. moschata* 2 dpi	*C. moschata* 4 dpi	*C. maxima* 2 dpi	*C. maxima* 4 dpi
Pathogenesis related (PR) protein	4	0	0	0	0	0
WRKY transcription factor	0	0	0	1	1	0
Leucine-rich repeat	8	0	5	0	0	3
Protein kinase activity	25	0	22	0	3	18
Salicylic acid signaling	1	0	1	0	2	3
Abscisic acid signaling	4	0	1	0	1	1
Defense response	7	0	3	0	0	0
Response to hormone	2	0	1	0	0	3

Examination of KEGG pathways in *C. pepo* at 2 dpi showed overrepresentation of photosynthesis, metabolic pathways, biosynthesis of secondary metabolites, carbon metabolism, porphyrin metabolism, and ribosome based on FDR < 0.05 (**Table S4.6**). In *C. maxima*, we detected overrepresentation of KEGGs corresponding to photosynthesis at 2 dpi, and metabolic pathways and secondary metabolite biosynthesis at 4 dpi (**Table S4.7**). We then focused on genes in specific KEGG pathways (**Table 4.3**). In the plant-pathogen interaction pathway, we found 4, 5, and 2 genes in *C. pepo*, *C. moschata*, and *C. maxima*, respectively when combing early and late local responses. For the plant hormone signal transduction pathway, we found the highest number of genes in *C. maxima* (9) followed by *C. pepo* (6), and *C. moschata* (5). Also, the mRNA surveillance pathway was represented by a small number of genes (4 in *C. pepo*, 1 in *C. moschata*, and 2 in *C. maxima*).

Table 4.3: Categories of KEGG pathways in local responses to virus infection.

Gene Function	*C. pepo* 2 dpi	*C. pepo* 4 dpi	*C. moschata* 2 dpi	*C. moschata* 4 dpi	*C. maxima* 2 dpi	*C. maxima* 4 dpi
Plant-pathogen interaction	4	0	5	0	0	2
Plant hormone signal transduction	6	0	5	0	2	7
mRNA surveillance	4	0	1	0	1	1

4.3.5 Systemic Responses to Virus Infection

Systemic responses in all hosts were characterized by fewer DEGs than local responses. GO enrichment analysis showed that at 8 dpi *C. pepo* had overrepresentation of molecular functions: transcription factor activity, sequence-specific DNA binding, and calcium ion binding, based on FDR < 0.05 (**Table S4.8**). In all hosts, we found genes corresponding to pathogenesis-

related proteins, WRKY transcription factors, and protein kinases, but the number differed between hosts. Interestingly, *C. pepo* had the highest number of DEGs in all categories related to defense responses (**Table 4.4**).

Table 4.4: Functions of DEGs in systemic responses to virus infection.

Gene Function/ Process	*C. pepo* 8 dpi	*C. moschata* 8 dpi	*C. maxima* 8 dpi
Pathogenesis related (PR) protein	3	2	1
WRKY transcription factor	15	3	2
Leucine-rich repeat	6	3	0
Protein kinase activity	29	10	2
Salicylic acid signaling	2	0	0
Abscisic acid signaling	1	0	0
Defense response	8	3	2
Response to hormone	0	0	0

We found enrichment of KEGG pathways for plant-pathogen interaction and MAPK signaling pathway in *C. pepo* at 8 dpi with 21 and 14 DEGs, respectively (**Table S4.9**). We identified fewer genes related to plant-pathogen interactions in the other hosts with three in *C. moschata* and one in *C. maxima* (**Table 4.5**). Also, *C. moschata* had enrichment of sulfur metabolism (4 DEGs), and *C. maxima* was not enriched for any pathways. We also found genes involved in plant hormone signal transduction (*C. pepo* = 8, *C. moschata* = 2, *C. maxima* = 1). In total, we found more genes involved in plant-pathogen interactions at 8 dpi compared to the earlier time points. All genes assigned to the plant-pathogen interaction pathway were upregulated in the three host species, apart from one gene encoding putative disease resistance protein RGA3 (LOC111779949) in *C. pepo*. We did not find orthologs for this gene in *C. moschata*, *C. maxima*, or *A. thaliana*.

Table 4.5: Categories of KEGG pathways in systemic responses to virus infection.

Gene Function	*C. pepo* 8 dpi	*C. moschata* 8 dpi	*C. maxima* 8 dpi

Plant-pathogen interaction	21	3	1
Plant hormone signal transduction	8	2	1
mRNA surveillance	1	1	1

Further investigation of specific genes showed that several genes related to pathogen resistance were upregulated at 8 dpi. Specifically, we found that a gene for tobacco mosaic virus (TMV) resistance protein N (LOC111791155) was upregulated in *C. pepo* (log_2 fold change = 3.5) and had orthologs in the other two hosts. Similarly, a gene encoding TMV resistance protein N-like (LOC111450603) was also upregulated in *C. moschata* (log_2 fold change = 3.0), but no orthologs were identified for this gene. We also found two genes corresponding to NDR1/HIN1-like protein 10 were upregulated exclusively in *C. pepo* at 8 dpi. Interestingly, one of these genes was also found to be upregulated in *C. moschata* at 2 dpi (LOC111452658) and a similar gene was uniquely expressed in *C. moschata* at 2 dpi (LOC111447589). Also uniquely expressed in *C. pepo* at 8 dpi were DEGs encoding two Leaf rust 10 disease-resistance protein-kinases (LOC111794096, LOC111808520), rust resistance kinase Lr10-like (LOC111797553), and nematode resistance protein-like HSPRO2 (LOC111809400). Lastly, we found upregulation of a gene encoding PYRICULARIA ORYZAE RESISTANCE 21-like (LOC111477939) in *C. maxima* and no orthologs were identified for this gene.

4.4 Discussion

Our results provide insight into the dynamic nature of defense responses against two economically important viruses in closely related hosts. We show that the highest number of DEGs in response to virus infection occurs at 2 or 4 dpi before viral load increases or before

symptoms occur. This suggests that early responses are important for long term infection outcomes and that there are key players in early responses that deserve further investigation. We also found that the most susceptible host (*C. pepo*) exhibited the highest elicitation of responses to both wounding and virus infection and the greatest number of defense related genes DEGs at nearly all time points. Our results indicate that decreased disease susceptibility is associated with a lower magnitude or delayed activation of responses, potentially decreasing the availability of factors needed for pathogen replication (Bengyella et al., 2015).

We found that while levels of SqMV accumulation were similar in all three plant hosts, *C. moschata* had significantly lower levels of ZYMV and reduced symptoms compared to the other hosts. This suggests some level of resistance is responsible for reductions in disease in *C. moschata*. However, appreciable levels of both viruses are still detected in this host indicating that any resistance is not complete, and viruses can still effectively replicate. Additionally, previous field experiments with the same host genotypes and inoculum showed that *C. pepo* had the greatest fitness costs during virus infection, *C. maxima* had moderate reductions in fitness, and *C. moschata* had minimal fitness costs. However, all hosts had similar viral loads ~60 dpi and thus, differences in susceptibility were attributed to host tolerance. We expand on our previous descriptions of host susceptibility by showing that *C. moschata* has a lower initial accumulation of ZYMV, and this is associated with substantial local responses followed by reduced systemic responses, contributing to lasting reductions in disease phenotypes. Although the moderately susceptible *C. maxima* has reduced fitness costs in the field compared to the highly susceptible *C. pepo*, we did not observe reduced symptom severity or viral load at early stages of infection. Yet, responses are generally delayed in *C. maxima* compared to the other two hosts. Viral load or the expression of antiviral defenses may fluctuate over the course of an infection and can be dependent on environmental factors such as temperature (Szittya et al., 2003; Qu et al., 2005; Bergès et al., 2018; Paudel et al., 2018). This may contribute to differences in the results from our

field and grow room studies, but the data from early responses described in the present experiments offers an increased understanding of how hosts mitigate the negative impacts of virus infection.

We also detected host-specific nucleotide mutations in both viruses, suggesting host adaptation of the viruses. For ZYMV, there were 3 SNVs in regions encoding for the coat protein, NiB, or P3 that were found in all 3 biological replicates of *C. moschata* plants used for RNA-Sequencing at 8 dpi, but not in other host taxa at this time point. For SqMV, we detected unique mutations in the large capsid protein region of the genome that were in all virus-inoculated samples from *C. pepo* at 8 dpi. While we only assessed three plants per host species, and virus sequence comparison was only performed on samples from 8 dpi due to low number of reads at other time points, the repeated occurrence of these mutations unique to host taxa indicates there may be selection pressures for these mutations to occur. Single amino acid mutations can alter the biology of potyviruses (González-Jara et al., 2005; Wallis et al., 2007), and although we did not detect any mutations at the amino acid level it is possible that the nucleotide substitutions observed in *C. moschata* or *C. pepo* are having an influence on virus biology and host phenotypes. However, additional experiments are needed to confirm this. Furthermore, phylogenetic analyses revealed that our isolate of ZYMV was most closely related to sequenced isolates from Pennsylvania, USA, which all came from *C. pepo* or *C. pepo* ssp. *texana*. Phylogenetic analyses of SqMV RNA 1 show that our isolate shares a common ancestor with isolates from Japan, but these relationships should be interpreted with caution due to the limited number and geographic distribution of sequences available for SqMV.

Regarding local responses to virus infection, the least susceptible host, *C. moschata*, had reduced responses compared to the most susceptible host, *C. pepo*, in terms of total DEGs and defense related genes. The moderately susceptible host, *C. maxima*, had delayed local responses but overall expressed an intermediate number of genes compared to the other two hosts. The most

susceptible host, *C. pepo*, was characterized by the highest number of total DEGs at 2 dpi, and the highest number of defense related genes in nearly all categories. Previous studies of transcriptional reprogramming associated with virus tolerance have suggested models where early host responses include a reduction in the number of DEGs in attempts to limit resources for viral replication and we found support for this hypothesis in our data (Allie et al., 2014; Bengyella et al., 2015; Liu et al., 2019; Amoroso et al., 2022).

Systemic responses to virus infection were characterized by a similar trend with the highest number of DEGs detected in *C. pepo*, followed by *C. moschata*, and *C. maxima*. Interestingly, *C. moschata* had more DEGs than the moderately tolerant, *C. maxima*, at 2 and 8 dpi. We also detected an intermediate number of differentially expressed defense related genes in several categories in *C. moschata* compared to the other two hosts. This suggests that reduced susceptibility in *C. moschata* is not simply a lack of differential gene expression, but limited and efficient early transcriptional responses, combined with a conserved systemic response that is effective at reducing viral load and ultimately, limiting virulence.

Additionally, we found similar trends in local transcriptional responses between wounding and virus infection within plants hosts. Most plant viruses require wounded plant tissue or insect vectors to successfully infect plant hosts (Jones and Naidu, 2019). Thus, it is perhaps unsurprising that responses to wounding can overlap considerably with inducible defenses against pathogens and can be both localized and systemic (Savatin et al., 2014). However, it is improbable that the transcriptional profiles would still be similar when performing inoculations with insect vectors, since many insects including aphids inject effectors in plants that change plant responses (Pitino and Hogenhout, 2013). Our results show that the strength of local responses to wounding are typically broader than virus-specific responses, with more DEGs detected in response to wounding than in response to virus infection. The most susceptible host was characterized by strong local responses to both wounding and infection, and systemic

responses consisting of the highest number of DEGs out of all hosts. *C. moschata* had high induction of local responses to both stresses at 2 dpi, but by 4 dpi the responses to wounding were considerably lower (97.5% reduction) and virus-specific responses were essentially absent at this time point (only 3 DEGs). In contrast, *C. maxima* had the lowest number of DEGs in early, local responses to wounding and infection at 2 dpi but a large increase in gene expression at 4 dpi, indicating a general delay in defense responses compared to other hosts. This drastic difference in local responses of *C. maxima* compared to the hosts could be a result of the evolutionary history of these species, as *C. pepo* and *C. moschata* are more closely related to each other than to *C. maxima* (**Fig. S4.1**). Our previous field experiments also showed that the impact of virus infection on many plant traits are informed by host phylogenetic relationships (Hinshaw et al., *unpublished*). Indeed, interactions between plants and pathogens are known to exhibit phylogenetic signal, likely due to evolutionary constraints on host defenses (Gilbert et al., 2015; Gilbert and Parker, 2016). In addition, defense genes are often evolutionarily conserved in plant lineages which could be contributing to the similar transcriptional responses observed between the two more closely related hosts (Lin et al., 2013; Zheng et al., 2016).

Systemic response to virus infection and wounding were more similar between hosts. Although systemic responses to wounding at 8 dpi in *C. pepo* and *C. maxima* consisted of fewer than 30 DEGs, we did not detect any differentially expressed genes in *C. moschata* at this time point, suggesting systemic responses in this host were not initiated or were already dissipated. Similar transcriptional profiles to wounding and virus infection provide further support for decreased susceptibility being characterized by a general reduction and limited investment in defense responses. This reduced induction could help limit self-induced damage from activation of toxic defenses or reduce the availability of host factors required for pathogen replication (Hanley-Bowdoin et al., 2013; Sirikantaramas et al., 2014; Ghoshal and Sanfaçon, 2015).

Photosynthesis is commonly impacted during plant virus infection and can have a strong impact on plant physiology (Souza et al., 2019). In the two more susceptible hosts, expression of genes in photosynthesis pathways was significantly enriched during early responses to infection. In contrast, we found that the least susceptible host did not have over-representation of photosynthesis related genes at 2 or 4 dpi, indicating this process was not significantly impacted by virus infection. A previous study investigated transcriptional responses to ZYMV infection in two *C. pepo* genotypes that were classified as either susceptible or tolerant based on a symptom recovery (Amoroso et al., 2022). They found that photosynthesis related genes were upregulated in the tolerant genotype at 12 dpi during the symptom recovery phase and suggested the ability to upregulate photosynthesis may aid in symptom recovery. Another study profiling protein expression of susceptible and partially resistant *C. pepo* showed that accumulation of photosynthesis related proteins was higher in the susceptible genotype at 6 dpi but higher in the resistant genotype at 15 dpi (Nováková et al., 2015). In agreement with Nováková et al., we also found susceptible hosts upregulated photosynthesis at early time points of 2 dpi and 4 dpi before the onset of symptoms, but the least susceptible host did not. Viruses can suppress photosynthesis related genes, which is one of the causes of the typical mosaic symptoms observed in many plant virus infections (Hanssen et al., 2011; Lu et al., 2012; Liu et al., 2014). During infection, potyviruses also recruit chloroplasts via virus-induced vesicles and form viral replication complexes within chloroplasts (Wei et al., 2010, 2013). As chloroplast resources are often required for virus replication, it is possible that limiting upregulation of photosynthesis genes during early infection stages reduces virus replication, while at later time points upregulating photosynthesis may limit disease symptoms (Budziszewska and Obrępalska-Stęplowska, 2018; Bwalya and Kim, 2023). Thus, the ability to maintain photosynthesis related gene expression at a consistent level in the least susceptible host may contribute to the lower virus replication and

symptom expression observed in this host. Surprisingly, at 8 dpi we found differential expression of only 1-2 photosynthesis related genes in all virus-inoculated hosts.

In addition to photosynthesis, other KEGG pathways represented in local responses to viruses included “metabolic pathways” and “biosynthesis of secondary metabolites” and were found in all taxa. The two more susceptible hosts, *C. pepo* and *C. maxima* had significant enrichment of these pathways at 2 and 4 dpi, respectively. Yet, *C. moschata* did not, suggesting transcriptional reprogramming during virus infection is enhanced in more susceptible hosts. Secondary metabolites are known to be involved in antiviral responses in plants and further characterization of these genes could provide additional insights into phenotypes of reduced susceptibility (Mishra et al., 2020).

During systemic infection, we also found differential expression of genes related to plant-pathogen interaction in all hosts based on KEGG pathway analysis and nearly all had positive $\log_2$ fold changes. Several of these genes encoded for pathogenesis-related (PR) proteins which are expressed locally and systemically during pathogen infections and aid in limiting virus replication or spread, and promote the development of systemic acquired resistance (SAR) (Musidlak et al., 2017). We detected differential expression of PR-like proteins in *C. pepo* (3 genes), *C. moschata* (2 genes), and *C. maxima* (1 gene). All three PR protein 1-like genes in *C. pepo* had orthologous genes detected in *C. moschata*, with higher expression in *C. moschata* for (LOC111811369) and vice versa for (LOC111811949) and (LOC111811953). We also found that the PR protein P2-like gene differentially expressed in *C. maxima* at 8 dpi was orthologous to a gene in *C. pepo* at 2 dpi, again suggesting a generally delayed reaction in *C. maxima*. The only other genes associated with plant-pathogen interactions in *C. maxima* and *C. moschata* at 8 dpi encoded for calmodulin-like proteins (CML) or calcium-binding protein, respectively. Both were uniquely expressed and did not overlap with other hosts or time points. *C. pepo* also had differential expression of CMLs or calcium-binding proteins and we detected four calcium

binding proteins, one calmodulin protein, and two CMLs at 8 dpi. These proteins are involved in the regulation of plant immunity and one CaM (rgs-CaM) in *Nicotiana benthamiana* has been directly linked to the RNA interference (RNAi) pathway, counteracting suppression of RNAi by virus infection (Nakahara et al., 2012; Cheval et al., 2013).

Although *C. pepo* had the strongest responses to wounding and virus infection regarding total number of DEGs and the number of defense related DEGs in nearly all categories, this host is highly susceptible to infection with high virus accumulation, severe foliar symptoms, and reductions of fitness metrics in the field. We previously categorized *C. maxima* as moderately tolerant based on similarly high viral loads and symptom severity, but reduced fitness costs in the field during virus infection compared to *C. pepo*. In *C. maxima*, we observed what can be considered moderate local transcriptional defense responses to physical damage and virus infection, but generally delayed responses compared to the other hosts. Additionally, this host had the lowest number of defense related DEGs for all categories during systemic infection. It is possible the delay in responses in combination with reduced systemic defenses contributes to increased susceptibility compared to hosts with lower pathogen burden or symptom expression. Similar to previous studies of virus infection in cucurbits, we found that the least susceptible host, *C. moschata*, exhibited reduced early responses to infection compared to more susceptible hosts (Nováková et al., 2015; Amoroso et al., 2022). However, we also found that systemic responses to virus infection in *C. moschata* were intermediate between the other hosts. In their transcriptome analysis, Amoroso et al. 2022 found that the more susceptible cultivar had a higher number of protein kinases expressed during virus infection and differential expression of response to oxidative stress and salicylic acid. Here, we also show that the most susceptible host had more protein kinase activity at both early and late time points than less susceptible hosts. While we also detected differential expression of genes related to oxidative stress and salicylic acid signaling in *C. pepo*, these pathways were not enriched, possibly because our study focused on responses at

earlier timepoints. Lastly, we observed fewer defense related genes expressed in *C. moschata* at early time points, but these responses were effective at reducing viral load and symptoms in the early stages of plant infection.

Our dataset constitutes a valuable resource for further investigating the molecular mechanisms involved in resilience to multiple virus infections, especially during the early stages of infection. Future studies could aim to characterize the functions of specific defense related genes and their roles in plant-virus interactions.

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4.6 Supplemental Material

Supplemental Tables

Table S4.1: Seed sources for each host.

Host Scientific Name	Variety Name	Supplier
Cucurbita pepo ssp. *ovifera* var *ovifera*	"Spinning gourd"	www.seedsavers.org Cat# 0867
Cucurbita maxima ssp. *maxima*	"Golden hubbard"	www.seedsavers.org Cat# 0410
Cucurbita moschata	"Waltham butternut"	www.johnnyseeds.com Cat# 671

Table S4.2: Results of mapping reads to genomes of either ZYMV or SqMV. The alignment column represents the percentage of all reads in a sample that were mapped successfully to reference genome of either virus.

Sample	Virus	Species	Treatment	dpi	Total paired reads	Alignment (%)
XZ1_8	ZYMV	C_maxima	Inoc	8	29715748	1.41
XZ2_8	ZYMV	C_maxima	Inoc	8	32008114	1.19
XZ3_8	ZYMV	C_maxima	Inoc	8	30958893	17.52
OZ1_8	ZYMV	C_pepo	Inoc	8	27441970	3.46
OZ2_8	ZYMV	C_pepo	Inoc	8	28090538	3.25
OZ3_8	ZYMV	C_pepo	Inoc	8	28297801	1.39
MZ1_8	ZYMV	C_moschata	Inoc	8	29288083	0.33
MZ2_8	ZYMV	C_moschata	Inoc	8	27855564	0.13
MZ3_8	ZYMV	C_moschata	Inoc	8	28434278	0.14
XZ1_8	SqMV	C_maxima	Inoc	8	29715748	0.61
XZ2_8	SqMV	C_maxima	Inoc	8	32008114	0.71
XZ3_8	SqMV	C_maxima	Inoc	8	30958893	0
OZ1_8	SqMV	C_pepo	Inoc	8	27441970	0.45
OZ2_8	SqMV	C_pepo	Inoc	8	28090538	0.61
OZ3_8	SqMV	C_pepo	Inoc	8	28297801	0.73
MZ1_8	SqMV	C_moschata	Inoc	8	29288083	0.61
MZ2_8	SqMV	C_moschata	Inoc	8	27855564	0.52
MZ3_8	SqMV	C_moschata	Inoc	8	28434278	0.92
XZ1_2	ZYMV	C_maxima	Inoc	2	28631059	0.09
XZ2_2	ZYMV	C_maxima	Inoc	2	28469793	0.13

XZ3_2	ZYMV	C_maxima	Inoc	2	30744216	0.02
OZ1_2	ZYMV	C_pepo	Inoc	2	28234050	0
OZ2_2	ZYMV	C_pepo	Inoc	2	27907546	0.01
OZ3_2	ZYMV	C_pepo	Inoc	2	31343562	0.05
MZ1_2	ZYMV	C_moschata	Inoc	2	27043297	0
MZ2_2	ZYMV	C_moschata	Inoc	2	28631091	0.03
MZ3_2	ZYMV	C_moschata	Inoc	2	29249746	0.02
XZ1_2	SqMV	C_maxima	Inoc	2	28631059	0.01
XZ2_2	SqMV	C_maxima	Inoc	2	28469793	0.02
XZ3_2	SqMV	C_maxima	Inoc	2	30744216	0.02
OZ1_2	SqMV	C_pepo	Inoc	2	28234050	0
OZ2_2	SqMV	C_pepo	Inoc	2	27907546	0
OZ3_2	SqMV	C_pepo	Inoc	2	31343562	0
MZ1_2	SqMV	C_moschata	Inoc	2	27043297	0
MZ2_2	SqMV	C_moschata	Inoc	2	28631091	0
MZ3_2	SqMV	C_moschata	Inoc	2	29249746	0
XZ1_4	ZYMV	C_maxima	Inoc	4	27878053	0.01
XZ2_4	ZYMV	C_maxima	Inoc	4	33803823	0.02
XZ3_4	ZYMV	C_maxima	Inoc	4	29175576	0
OZ1_4	ZYMV	C_pepo	Inoc	4	28203977	0
OZ2_4	ZYMV	C_pepo	Inoc	4	28983622	0
OZ3_4	ZYMV	C_pepo	Inoc	4	56885623	0
MZ1_4	ZYMV	C_moschata	Inoc	4	28833106	0
MZ2_4	ZYMV	C_moschata	Inoc	4	27337211	0
MZ3_4	ZYMV	C_moschata	Inoc	4	27854822	0
XZ1_4	SqMV	C_maxima	Inoc	4	27878053	0
XZ2_4	SqMV	C_maxima	Inoc	4	33803823	0
XZ3_4	SqMV	C_maxima	Inoc	4	29175576	0
OZ1_4	SqMV	C_pepo	Inoc	4	28203977	0
OZ2_4	SqMV	C_pepo	Inoc	4	28983622	0
OZ3_4	SqMV	C_pepo	Inoc	4	56885623	0
MZ1_4	SqMV	C_moschata	Inoc	4	28833106	0
MZ2_4	SqMV	C_moschata	Inoc	4	27337211	0
MZ3_4	SqMV	C_moschata	Inoc	4	27854822	0

Table S4.3: The number of genes in the genome of each host, the combined number of expressed genes based on base mean > 100 in all samples at each time point, and the number of differentially expressed gene in virus-inoculated vs. mock-inoculated samples, and mock-inoculated vs. uninoculated samples.

Host	dpi	Total Genes	Expressed Genes	Inoc vs Mock	Mock vs Uninoc

C_maxima	2	35289	17520	104	78
C_maxima	4	35289	17851	453	547
C_maxima	8	35289	17721	41	22
C_pepo	2	35013	17047	745	3023
C_pepo	4	35289	16309	4	1420
C_pepo	8	35013	11963	424	27
C_moschata	2	35355	17840	476	2016
C_moschata	4	35355	16531	3	50
C_moschata	8	35355	16240	102	0

Table S4.4: List of the top 5 most enriched gene ontology (GO) terms based on false discovery rate (FDR) associated with biological processes detected in each host at 2 dpi.

Host	Tolerance	Term	Description	Count	%	FDR
C_pepo	Low	GO:0015979	photosynthesis	13	1.94	1.30E-03
C_pepo	Low	GO:0006412	translation	29	4.32	5.38E-03
C_pepo	Low	GO:0006004	fucose metabolic process	8	1.19	1.96E-02
C_pepo	Low	GO:0009664	plant-type cell wall organization	7	1.04	1.96E-02
C_pepo	Low	GO:0015995	chlorophyll biosynthetic process	6	0.89	2.08E-02
C_maxima	Medium	GO:0009765	photosynthesis, light harvesting	5	4.81	7.42E-05
C_maxima	Medium	GO:0015979	photosynthesis	6	5.77	1.56E-04
C_maxima	Medium	GO:0018298	protein-chromophore linkage	5	4.81	4.68E-04
C_maxima	Medium	GO:0016114	terpenoid biosynthetic process	2	1.92	2.73E-01
C_maxima	Medium	GO:0015995	chlorophyll biosynthetic process	2	1.92	4.86E-01
C_moschata	High	GO:0071555	cell wall organization	9	1.89	2.98E-01
C_moschata	High	GO:0032259	methylation	12	2.52	3.34E-01
C_moschata	High	GO:0080162	intracellular auxin transport	3	0.63	5.19E-01
C_moschata	High	GO:0030245	cellulose catabolic process	4	0.84	5.42E-01
C_moschata	High	GO:0009664	plant-type cell wall organization	4	0.84	6.01E-01

Table S4.5: List of the top 5 most enriched gene ontology (GO) terms based on false discovery rate (FDR) associated with biological processes detected in *C. maxima* at 4 dpi. Note: only 7 and 3 DEGs were detected in *C. pepo* and *C. moschata*, respectively at 4 dpi.

Host	Tolerance	Term	Description	Count	%	FDR
C_maxima	Medium	GO:0009725	response to hormone	3	0.7	6.47E-01
C_maxima	Medium	GO:0009800	cinnamic acid biosynthetic process	3	0.7	6.47E-01
C_maxima	Medium	GO:0032259	methylation	10	2.2	6.47E-01
C_maxima	Medium	GO:0071555	cell wall organization	7	1.5	6.47E-01
C_maxima	Medium	GO:0006559	L-phenylalanine catabolic process	3	0.7	6.73E-01

Table S4.6: List of most enriched KEGG pathways based on false discovery rate (FDR) in each host at 2 dpi. Note: only 4 KEGG pathways were enriched in *C. maxima*.

Host	Tolerance	Term	Process	Count	%	FDR
C_pepo	Low	cpep00195	Photosynthesis	14	1.9	1.01E-05
C_pepo	Low	cpep01100	Metabolic pathways	135	18	1.01E-05
C_pepo	Low	cpep01110	Biosynthesis of secondary metabolites	76	10	3.56E-03
C_pepo	Low	cpep01200	Carbon metabolism	25	3.4	1.38E-02
C_pepo	Low	cpep00860	Porphyrin metabolism	9	1.2	1.89E-02
C_maxima	Medium	cmax00196	Photosynthesis - antenna proteins	5	4.8	2.50E-04
C_maxima	Medium	cmax00195	Photosynthesis	5	4.8	2.95E-03
C_maxima	Medium	cmax01100	Metabolic pathways	20	19	1.18E-01
C_maxima	Medium	cmax00860	Porphyrin metabolism	3	2.9	2.52E-01
C_moschata	High	cmos01100	Metabolic pathways	83	18	1.19E-01
C_moschata	High	cmos01230	Biosynthesis of amino acids	16	3.4	1.19E-01
C_moschata	High	cmos01110	Biosynthesis of secondary metabolites	49	11	1.26E-01
C_moschata	High	cmos00062	Fatty acid elongation	5	1.1	1.50E-01
C_moschata	High	cmos00520	Amino sugar and nucleotide	10	2.2	2.00E-01

			sugar metabolism			

Table S4.7: List of most enriched KEGG pathways based on false discovery rate (FDR) in *C. maxima* at 4 dpi. Note: only 7 and 3 DEGs were detected in *C. pepo* and *C. moschata*, respectively at 4 dpi.

Host	Tolerance	Term	Process	Count	%	FDR
C_maxima	Medium	cmax01100	Metabolic pathways	71	16	9.50E-04
C_maxima	Medium	cmax01110	Biosynthesis of secondary metabolites	41	9	2.84E-02
C_maxima	Medium	cmax01240	Biosynthesis of cofactors	12	2.6	1.90E-01
C_maxima	Medium	cmax00941	Flavonoid biosynthesis	4	0.9	1.90E-01
C_maxima	Medium	cmax00130	Ubiquinone and other terpenoid-quinone biosynthesis	5	1.1	2.55E-01

Table S4.8: List of the top most enriched gene ontology (GO) terms based on false discovery rate (FDR) associated with biological processes detected in all hosts at 8 dpi. Note: only 2 GO terms were detected as enriched in *C. maxima* and *C. moschata*.

Host	Tolerance	Term	Description	Count	%	FDR
C_pepo	Low	GO:0006839	mitochondrial transport	3	0.8	7.33E-02
C_pepo	Low	GO:0034976	response to endoplasmic reticulum stress	3	0.8	7.33E-02
C_pepo	Low	GO:0007186	G-protein coupled receptor signaling pathway	3	0.8	3.94E-01
C_pepo	Low	GO:0007034	vacuolar transport	3	0.8	3.94E-01
C_pepo	Low	GO:0006979	response to oxidative stress	5	1.4	3.94E-01
C_maxima	Medium	GO:0031408	oxylipin biosynthetic process	2	4.9	6.08E-01
C_maxima	Medium	GO:0006749	glutathione metabolic process	2	4.9	8.55E-01
C_moschata	High	GO:0000103	sulfate assimilation	2	2	6.66E-01
C_moschata	High	GO:0019509	L-methionine salvage from methylthioadenosine	2	2	6.66E-01

Table S4.9: List of most enriched KEGG pathways based on false discovery rate (FDR) in each host at 8 dpi.

Host	Tolerance	Term	Process	Count	%	FDR
C_pepo	Low	cpep04626	Plant-pathogen interaction	21	5	5.44E-08
C_pepo	Low	cpep04016	MAPK signaling pathway - plant	15	3.5	4.02E-04

C_pepo	Low	cpep00940	Phenylpropanoid biosynthesis	7	1.7	1.00E+00
C_maxima	Medium	cmax00480	Glutathione metabolism	3	7.3	2.75E-01
C_maxima	Medium	cmax00591	Linoleic acid metabolism	2	4.9	3.25E-01
C_moschata	High	cmos00920	Sulfur metabolism	4	3.9	1.32E-02
C_moschata	High	cmos00261	Monobactam biosynthesis	2	2	6.28E-01
C_moschata	High	cmos00450	Selenocompound metabolism	2	2	6.71E-01
C_moschata	High	cmos01100	Metabolic pathways	15	15	6.71E-01

Supplemental Figures

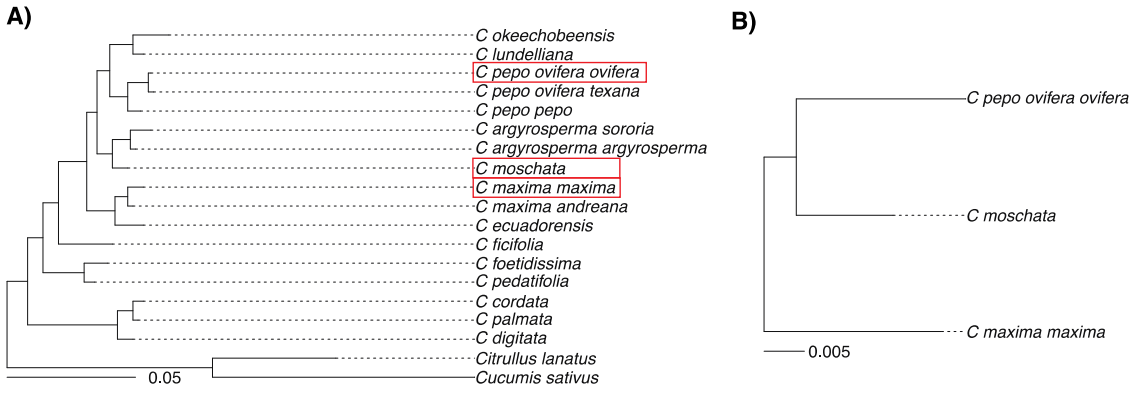

Figure S4.1: (**A**) Phylogeny of *Cucurbita* with *Citrullus lanatus* and *Cucumis sativus* as outgroups. Taxa used in this study are indicated by red boxes. (**B**) Phylogenetic relationships of host taxa used in this study. Phylogenies are adapted from Kates et al. 2017, with scale bar representing nucleotide substitutions per site.

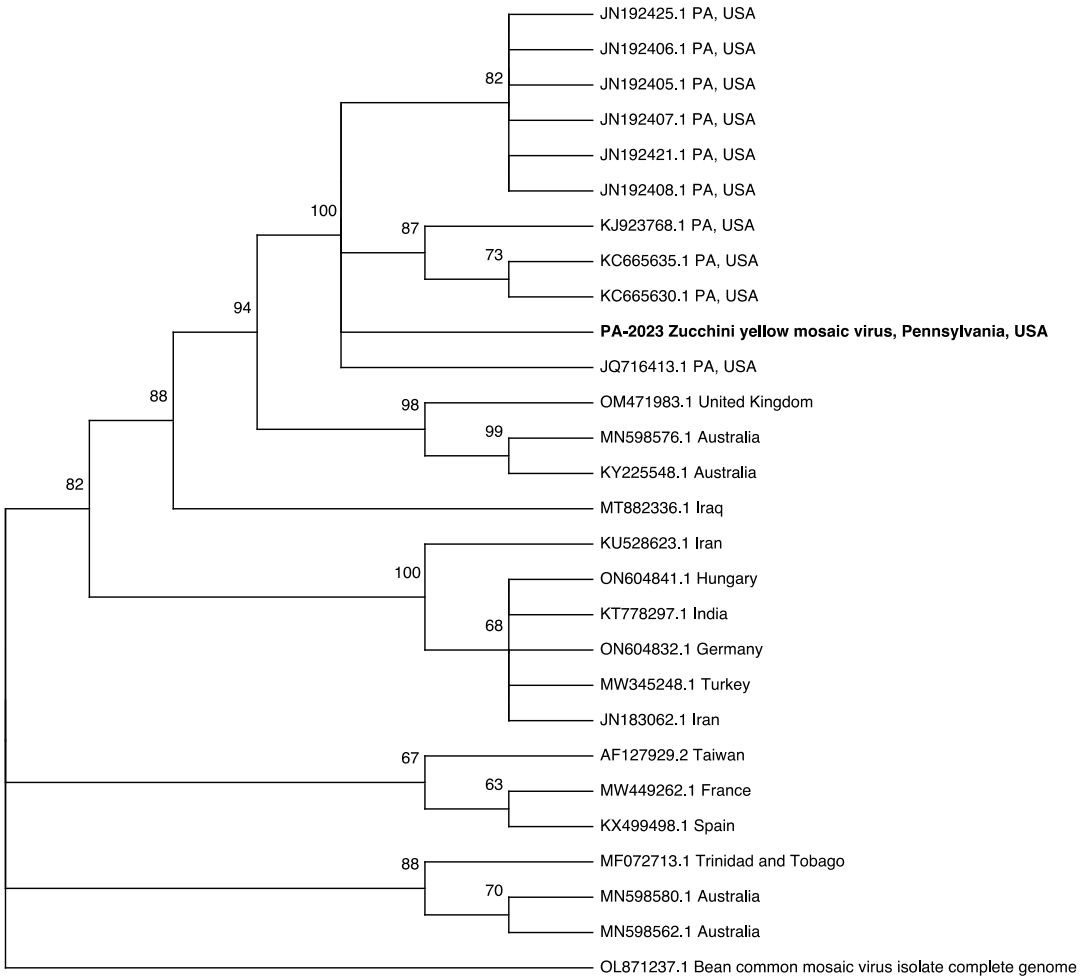

Figure S4.2: Phylogenetic analysis of ZYMV genome sequences showing that the isolate in this study (bold text) is most closely related to other isolates from PA, USA. Phylogenetic tree was generated using maximum-likelihood method and nodes with less than 60% bootstrap support are collapsed. The locations of all isolates are listed in the taxon name and values above each node are bootstrap values based on 1,000 replicates.

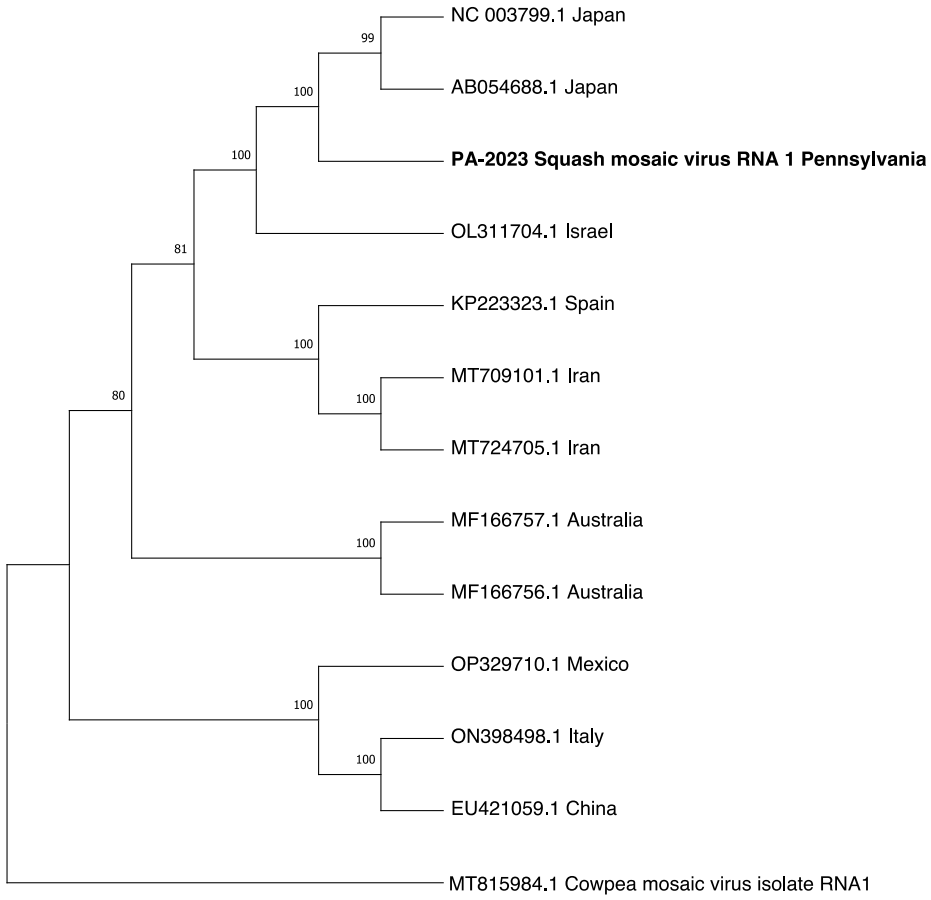

Figure S4.3: Phylogenetic analysis of SqMV RNA 1 genome sequences showing that the isolate in this study (bold text) is most closely related to isolates from Japan. Phylogenetic tree was generated using maximum-likelihood method and nodes with less than 60% bootstrap support are collapsed. The locations of all isolates are listed in the taxon name and values above each node are bootstrap values based on 1,000 replicates.

Chapter 5

Summary and Future Directions

Viruses can cause devastating diseases that negatively impact the health of host populations. Understanding how some hosts are able to accommodate virus replication while maintaining aspects of fitness can help guide efforts to control viral diseases. This research has identified hosts that can coexist with pathogenic viruses and investigated the mechanisms in which they do so. This dissertation had several aims: 1) Determine if feral honey bees are more tolerant to virus infection and if this is mediated by immunological mechanisms; 2a) Determine if wild *Cucurbita* spp. species have higher tolerance to virus infection; 2b) Determine the consequences of plant virus infection for plant-pollinator interactions; 3) Investigate molecular mechanisms of virus tolerance in *Cucurbita* spp.

In chapter 2, it was demonstrated that feral honey bees harbored greater amounts of deformed wing virus (DWV), one of the most notorious pathogens of honey bees, compared to managed colonies. Although DWV was positively associated with winter colony losses, feral and managed colonies had similar overwintering rates, suggesting feral colonies have some mechanism to tolerate high levels of DWV. Another main finding of this work was the positive associations between overwintering survival and the expression of two immune-related genes (*hymenoptaecin* and *vago*). This suggests that these genes could be used as biomarkers to select for colonies that have higher expression of these immune genes, and thus, higher likelihood of overwintering survival.

The genes *hymenoptaecin* and *vago* are part of complex immune pathways (IMD/Toll and RNAi, respectively). Therefore, future studies could further investigate the diverse players in these immune pathways to determine if there are genes that are more strongly associated with, or better able to predict, the ability of honey bees to mitigate the negative impacts of DWV

infection. Performing RNA-sequencing on managed and feral honey bees in response to DWV infection could provide more detailed descriptions of the role of immune-related genes in virus responses and how feral honey bees may have more efficient activation of immune mechanisms. Candidate genes from RNA-sequencing experiments or genes identified in chapter 2 could then be further characterized for their role in virus immunity by performing RNAi gene knockdown experiments. By knocking down the expression of individual genes (such as *hymenopateacin* and *vago*), followed by inoculating bees with DWV and measuring virus accumulation and disease phenotypes, it could be determined if these genes have direct effects on reducing viral loads or preventing disease. This type of validation would serve as a valuable confirmation that genes would be useful as biomarkers to evaluate immunocompetence in honey bee breeding programs.

One hypothesis for the increased tolerance to DWV observed in feral honey bees is that they are able to limit the damage caused by immune responses to virus infection. Immune activation may result in oxidative stress and inflammation that damages host tissues and increases mortality. Therefore, the ability to limit immune-related damage could contribute to the ability of feral colonies to survive the winter with high levels of DWV. Future experiments could directly test for differences in cellular and humoral immunity between feral and managed honey bees after an immune challenge. This could be done by measuring hemocyte concentration (cellular immunity), phenoloxidase activity, and lipid peroxidation, which are involved in oxidative stress response and wound healing, respectively. This would provide more detailed information beyond the expression of individual genes, to see if the physiological responses to stressors are different in feral honey bees. This could have broad implications for the ability of insects to tolerate pathogen infections and increase our understanding of the mechanisms involved in virus tolerance.

The results of chapter 3 demonstrated that responses to plant virus infection in *Cucurbita* spp. were associated with phylogenetic relationships between host plants but not domestication

status. Combining analysis of various life history traits with quantification of viral load in the field also provided information on which host taxa were the least impacted by virus infection, and thus exhibited virus tolerance. Some hosts were able to maintain nearly all aspects of fitness, indicating that host tolerance can effectively limit disease and may be valuable as a management strategy in agriculture for combatting yield losses from pathogens.

However, before being used widely as a disease management strategy in agriculture, it should be shown that virus tolerance is effective against various pathogen genotypes and is durable and does not break down over time, due to virus evolution. Specifically for ZYMV, there are a diversity of virus genotypes around the world which differ in their aggressiveness, determined by symptom severity and yield reductions. It is possible that the isolates of ZYMV and SqMV used in this research are less aggressive than others, and that greater reductions in host fitness would be seen if performing similar experiments with other strains. Therefore, future experiments could evaluate the effectiveness of host tolerance against multiple virus strains under controlled settings. A panel of virus strains could be inoculated onto hosts with different levels of virus tolerance and evaluated for symptom severity and fitness costs. This would determine if the tolerance observed to ZYMV and SqMV is effective against other virus genotypes. Additionally, to investigate the durability of virus tolerance, passaging experiments could be performed using hosts with different levels of tolerance and virus strains with different levels of aggressiveness. By measuring virus accumulation and symptom severity after serial passaging and comparing this to original virus strains, it would be determined if viruses evolve to become more aggressive on tolerant hosts and if this is associated with increased virus replication. These experiments would generate valuable information about the robustness of host tolerance against various virus genotypes and its durability over time, providing more evidence for its effectiveness as a disease management strategy in agricultural practices.

Another outcome of the research in chapter 3 was the confirmation that plant virus infection can impact plant-pollinator interactions. It was suggested that the decreased acquisition of floral resources observed by squash bees was possibly attributed to the avoidance of lower quality rewards, but this has yet to be tested. Future experiments could directly investigate the mechanisms responsible for these observed behavioral differences. Many plant viruses are known to alter the volatile organic compounds (VOCs) emitted from host plants, resulting in altered perception by insect vectors. Choice test experiments are commonly performed to measure the preference of insects for certain blends of VOCs or nutritional resources. Similar experiments could be performed to determine if altered behavior of squash bees is associated with differences in their perception of virus-infected compared to uninfected plants. Nectar and VOCs could be collected from virus-infected or healthy plants and then used in choice test assays to see if squash bees perceive differently nectar or VOCs when plants are infected with viruses. Identifying the mechanisms attributed to differences in pollinator behavior could help guide efforts to mitigate potential reductions in pollination caused by plant pathogens or decreased nutritional resources for pollinator populations. Specifically, breeding efforts could select for plants that still produce abundant, high quality floral resources and attractive VOC blends during virus infection, thus ensuring the health of both plants and pollinators.

The results of chapter 4 have provided novel insights into transcriptional profiles associated with virus tolerance in *Cucurbita* spp. In this system, virus tolerance was associated with a general reduction in differential gene expression compared to less tolerant host plants. The least susceptible host also had comparatively reduced accumulation of ZYMV and symptom severity at 8 days post-inoculation (dpi) and 12 dpi, respectively.

The number of defense-related genes that were differentially expressed also differed between hosts and for several categories the most tolerant host, *C. moschata*, did not have the lowest number of defense-related differentially expressed genes (DEGs). However, future

analyses are still required to determine how many of the genes in different categories are identical between hosts or unique to an individual host(s). Additionally, several genes found to be differentially expressed were not associated with any gene ontology (GO) terms and therefore, information is missing on the molecular function, biological process, and subcellular location of gene products associated with this dataset. Although the genomes of all *Cucurbita* spp. used in this host are relatively well-annotated, improvements in the functional annotations of cucurbit genomes will enhance the ability to associate differentially expressed genes with GO terms and KEGG pathways. This could provide more detailed biological interpretations of responses to virus infection and wounding. Generally, continued searches within the existing dataset will help identify candidate genes that may be involved in phenotypes of virus tolerance.

A logical next step is then to perform functional studies on candidate genes of interest to determine their roles in limiting virus replication or disease severity. First, genes that were differentially expressed between hosts and have putative roles in defense response could be analyzed for their phylogenetic similarity to other genes with known roles in pathogen defense. Genes with high levels of homology with known defense genes would be the strongest candidates for targeted functional analysis. Then, various candidate genes could be evaluated by performing RNAi knockdown or overexpression techniques to decrease or increase gene expression, respectively. Combining this with measurements of viral load and symptom severity would determine if genes are directly involved in infection outcomes previously observed. However, one of the main hypotheses in this dissertation is that virus tolerance is associated with expression of multiple genes. Therefore, it is possible that single genes with strong effects on virus replication or disease severity may not be identified.

The upregulation of genes involved in hormonal signaling at various timepoints suggests that hormone-mediated defenses could be involved in virus tolerant phenotypes. To further investigate this hypothesis, research could examine accumulation of plant hormones involved in

responses to biotic and abiotic stressors: salicylic acid, jasmonic acid, abscisic acid, and ethylene. All four of these hormonal pathways can be affected by plant virus infection and have roles in antiviral responses. Experiments could examine accumulation of hormones over time, the associations between hormone abundance and viral load or symptom severity, and possible antagonism or cross-talk between pathways. Describing hormone-mediated defenses would enhance our understanding of virus tolerance beyond the transcriptional level and allow for meaningful conclusions about the higher-level mechanisms associated with different responses to ZYMV and SqMV in *Cucurbita* hosts.

When considering the results of experiments in chapter 3 and chapter 4 together, it also appears that while the most tolerant host, *C. moschata*, reduces virus accumulation early on at 8 dpi, viral load becomes similar as plants age or are moved to field settings. Descriptions of virus accumulation at multiple timepoints over the lifespan of plants would allow for a better understanding of how tolerant plants can limit virus replication at early stages but then accommodate high viral loads later in the season. Thus, we could have more targeted approaches for which timepoints may be most informative for predicting plant disease outcomes and ultimately informing better breeding strategies.

VITA

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Publications

Chauncy R. Hinshaw, Evans KC, Rosa C and López-Uribe MM (2021) The Role of Pathogen Dynamics and Immune Gene Expression in the Survival of Feral Honey Bees. *Front. Ecol. Evol.* 8:594263. doi: 10.3389/fevo.2020.594263

Presentations

Chauncy R. Hinshaw, Margarita M. López-Uribe, Cristina Rosa. "The influence of domestication on host plant tolerance and consequences of plant virus infection for plant-pollinator interactions". International Advances in Plant Virology (2022).

Chauncy R. Hinshaw, Laine Hackenberg, Margarita M. López-Uribe, and Cristina Rosa. "Understanding Tolerance to Virus Infections and Consequences for Plant-Pollinator Interactions". American Phytopathological Society Annual Meeting, Virtual (2020).

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Chauncy R. Hinshaw, Kathleen C. Evans, Christina M. Grozinger, and Margarita M. López-Uribe. "Tracking Feral Bee Health in Pennsylvania". Entomological Society of America (ESA), Entomological Society of Canada (ESC) and Entomological Society of British Columbia (ESBC) Joint Annual Meeting, Vancouver, BC, Canada (2018).