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MICROBE–HERBIVORE–NATURAL ENEMY MULTITROPHIC INTERACTIONS IN AGROECOSYSTEMS

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by

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Abstract

As primary producers, plants form the basis of terrestrial food webs and are associated with many organisms that collectively form the phytobiome. Plants can detect and respond to cues from these organisms, inducing plant defenses through phytohormone-regulated signalling pathways. These defenses not only thwart pathogens and herbivores directly, but attract and sustain natural enemies that regulate herbivore populations. In Chapter 1, I synthesize research demonstrating that natural enemies can also alter plant defenses, both directly and indirectly through modifying their herbivore hosts. I discuss the effects of natural enemy modulation of plant defenses on ecological communities, identify hypotheses for the evolution of NE–plant interactions, suggest applications for biological control, and propose future research directions.

The phytobiome includes phylloplane bacteria that colonize aboveground plant tissues. These bacteria are ingested by herbivorous insects and influence herbivore gut microbiomes. I used high-throughput sequencing to examine gut bacterial communities in field-collected lepidopteran larvae. *Spodoptera frugiperda* midgut bacterial communities differed from those of *Helicoverpa zea* collected from the same host plant species at the same site. In laboratory experiments with *S. frugiperda*, I found that host plant (*Zea mays* or *Glycine max*) had a greater influence on bacterial communities than egg source. I also observed differences between regurgitant (foregut) and midgut bacterial communities of *S. frugiperda*, suggesting differential colonization of bacteria between gut regions. These results indicate that host plant, insect species, and gut region can influence lepidopteran gut bacterial communities. Given the importance of *S. frugiperda* bacterial isolates in modulating or enhancing plant defenses, the composition of lepidopteran gut bacterial communities is likely to be an important dynamic of multitrophic interactions.

Entomopathogenic microbes are also important components of phytobiomes. In addition to acting as natural enemies of herbivorous insects, they can modulate plant defenses. Baculoviruses are entomopathogenic viruses that are transmitted in the environment as occlusion bodies (OBs). Caterpillars ingest OBs on plant surfaces, and the virus multiplies within the insect, eventually causing host tissues to rupture and liquify. I used *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and host *Trichoplusia ni* caterpillars to study the effects of virus-killed cadavers on tomato (*Solanum lycopersicum*) defenses and *T. ni* behavior.

Because both OBs and cadaver-associated bacteria could play a role in these interactions, I characterized the bacterial communities of virus-killed cadavers and uninfected (freeze-killed cadavers). I found no overall differences in the composition of bacterial communities of virus-killed or freeze-killed cadavers. However, bacterial communities of virus-killed cadavers from two separate experiments were significantly different, suggesting that host plant could play a greater role in shaping bacterial communities than virus infection status. I found that virus-killed cadavers suppressed plant defenses induced by mechanical wounding, providing the first evidence that baculoviruses can influence plant defenses through host cadavers. However, cadavers did not influence defenses induced by herbivory from healthy or AcMNPV-infected larvae. When applied to intact plants, virus-killed or freeze-killed cadavers did not influence *T. ni* oviposition or larval choice, indicating that *T. ni* could not discriminate cadaver cues. This research provides initial insight on the possible effects of virus-killed cadavers on plant defenses and insect behavior and points to a need for more studies on cadaver ecology.

Plant–insect interactions can also be affected by external factors such as pesticide applications. Although it is clear that insecticides have toxic effects on pests and non-target organisms, less is known about the indirect, plant-mediated effects of insecticidal applications. I used cotton (*Gossypium hirsutum*) to investigate the impacts of neonicotinoid seed treatments on extrafloral nectar (EFN), an important food resource for natural enemies. I found that neonicotinoids were translocated to the EFN of clothianidin and imidacloprid-treated, greenhouse-grown cotton plants. There was no difference in the quantity or metabolite composition of EFN in neonicotinoid-treated plants compared to untreated plants. In bioassays, female *Cotesia marginiventris* parasitoid wasps that fed on EFN from untreated, clothianidin-treated, or imidacloprid-treated plants demonstrated no difference in mortality or parasitization success. Using neonicotinoid-spiked honey to conduct acute toxicity assays, I established LC_{50} values for male and female wasps. Although LC_{50} values were substantially higher than neonicotinoid concentrations detected in EFN, caution should be used when translating these results to the field where other stressors could alter the effects of neonicotinoids.

These studies collectively enhance our understanding of herbivore, natural enemy, and microbe interactions with crop plants and the role that insecticide applications can have in these interactions. This information could have important applications for integration of biological control, host plant resistance, and pesticide components of biological control programs.

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Preface

Interactions between plants and herbivores are key to understanding ecological communities and ecosystem functioning because they link primary production to wider food webs. In terrestrial ecosystems, insects are particularly important herbivores; coevolution between plants and herbivorous insects is thought to have driven the vast diversity of defenses expressed by plants. In addition to continually produced constitutive defenses, plants upregulate defenses in response to herbivore attack. These induced defenses include defensive proteins and secondary metabolites that directly impair herbivore growth and survival and indirect defenses such as herbivore-induced plant volatiles and extrafloral nectar that attract and sustain natural enemies. Natural enemies (predators, entomopathogens, parasitoids) not only influence plants through regulation of herbivore populations, but can directly and indirectly modulate plant defenses, with cascading effects on ecosystems (Chapter 1). Microbes act on all components of plant–herbivore–natural enemy tripartite systems—as natural enemies of herbivores, as plant and insect pathogens, and as symbionts. Better understanding of how plants interact with other trophic levels is important for influencing ecological theory, shaping our view of the evolution of multitrophic interactions, and informing host plant resistance and biological control components of integrated pest management (IPM). Because IPM strategies may also include the judicious use of pesticides, it is also important to consider how chemical applications affect plant defenses and natural enemies. In Chapter 2, I show that host plant species and other factors shape bacterial communities of two important pest lepidopteran larvae species. In Chapter 3, I used another pest lepidopteran system to investigate how baculovirus infection altered bacterial communities of cadavers. I also examined how virus-killed cadavers influenced plant defenses and insect behavior. In Chapter 4, I measured the influences of neonicotinoid seed treatments on cotton extrafloral nectar and implications for parasitoid survival. Collectively, these studies provide insight into novel interactions among organisms associated with crop plants, and further our understanding of complex multitrophic interactions in crop systems.

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Chapter 1

Mutualism, manipulation, or mistaken identity: natural enemy modulation of plant defenses

Abstract

Ecological theory of interactions among plants, insect herbivores, and natural enemies (NEs) has traditionally been understood through studying how herbivore populations are affected by "bottom-up" plant defenses and "top-down" influences of predation and parasitism. Yet, interactions among organisms in different trophic levels are much more complex than these theories imply. Over the last several decades accumulating research has demonstrated that bottom-up traits also influence top-down control of herbivores by affecting NE populations. Plant volatiles and extrafloral nectar attract and sustain NEs, and defensive compounds can negatively or positively affect NEs through modification of host or prey quality. Here, I discuss how NEs also modulate plant defenses, highlighting how top-down effects can influence bottom-up defenses. Predators, parasitoids, microbial pathogens, and entomopathogenic nematodes produce a variety of cues that are directly recognized by plants (direct cues), or they can regulate host cues to indirectly alter plant traits (indirect cues). By influencing plant production of defensive compounds and volatile emissions, NEs have cascading effects on herbivores, plant pathogens, and plant fitness. Currently little is known about whether plant detection of NEs evolved as mutualisms between plants and NEs, manipulation of plants by NEs, or "mistaken identity" where plants recognize these organisms based on overlapping cues with pests. Expanding our view of NE interactions with plants will likely improve understanding of food web ecology and presents new opportunities to integrate biological control and host plant resistance components of integrated pest management programs.

Introduction: Plant–herbivore–natural enemy interactions

The relative importance of "bottom-up" and "top-down" effects in regulating herbivore populations has been discussed for decades to explain "why the world is green" (Hairston et al., 1960; Power, 1992; Slobodkin et al., 1967; Wilkinson and Sherratt, 2016). Top-down theory predicts that herbivores are limited predominantly by natural enemies (NEs) in the third trophic level (Hairston et al., 1960; Figure 1.1: 2). NEs, such as parasitoids, predators, microbial

pathogens, and entomopathogenic nematodes (EPNs), can protect plants through consumption and removal of herbivores (De Bach and Rosen, 1991; Hajek et al., 2007; Letourneau et al., 2009). The bottom-up view posits that herbivore populations are primarily regulated by plant resources, in terms of productivity (Lindeman, 1942), nutritional quality (White, 1978), or chemical defenses that deter, poison, or inhibit digestion of herbivores (Fraenkel, 1959; Figure 1.1: 1). In general, it is likely that bottom-up and top-down effects both contribute and interact to regulate herbivore populations and shape ecological communities (Hunter et al., 1997; Mooney et al., 2012; Vidal and Murphy, 2018; Walker and Jones, 2001; Wilkinson and Sherratt, 2016).

Over the last several decades it has also become clear that plant–herbivore–NE tritrophic interactions do not follow a simple linear food web structure (Figure 1.1). A growing body of research has unravelled the remarkable complexity of ecological interactions among trophic levels (Figure 1.1). An important driver of this complexity appears to be plant defenses. Plants possess continually expressed constitutive defense traits (Wittstock and Gershenzon, 2002) and a second line of inducible defenses that are upregulated in response to pathogen or herbivore attack (Karban and Baldwin, 1997). However, plant defenses affect not only herbivores and pathogens, but can also directly and indirectly affect NEs (Price et al., 1980; Figure 1.1: 3a and 3b). Herbivore-induced traits such as plant volatiles and production of extrafloral nectar (EFN) (Heil, 2008, 2015) are often called "indirect plant defenses" because they can recruit and sustain predators, parasitoids, and EPNs (Ali et al., 2010; Dicke and Baldwin, 2010; McCormick et al., 2012; Turlings et al., 2012). Plant defensive compounds can also indirectly affect parasitoid and predator development, entomopathogen efficacy, and EPN infectivity by altering host quality (Cory and Hoover, 2006; Hazir et al., 2016; Ode, 2006; Shikano, 2017). By modifying NE behavior, physiology, survival, and efficacy, bottom-up plant defenses can affect top-down regulation of herbivores (Figure 1.1: 3a and 3b).

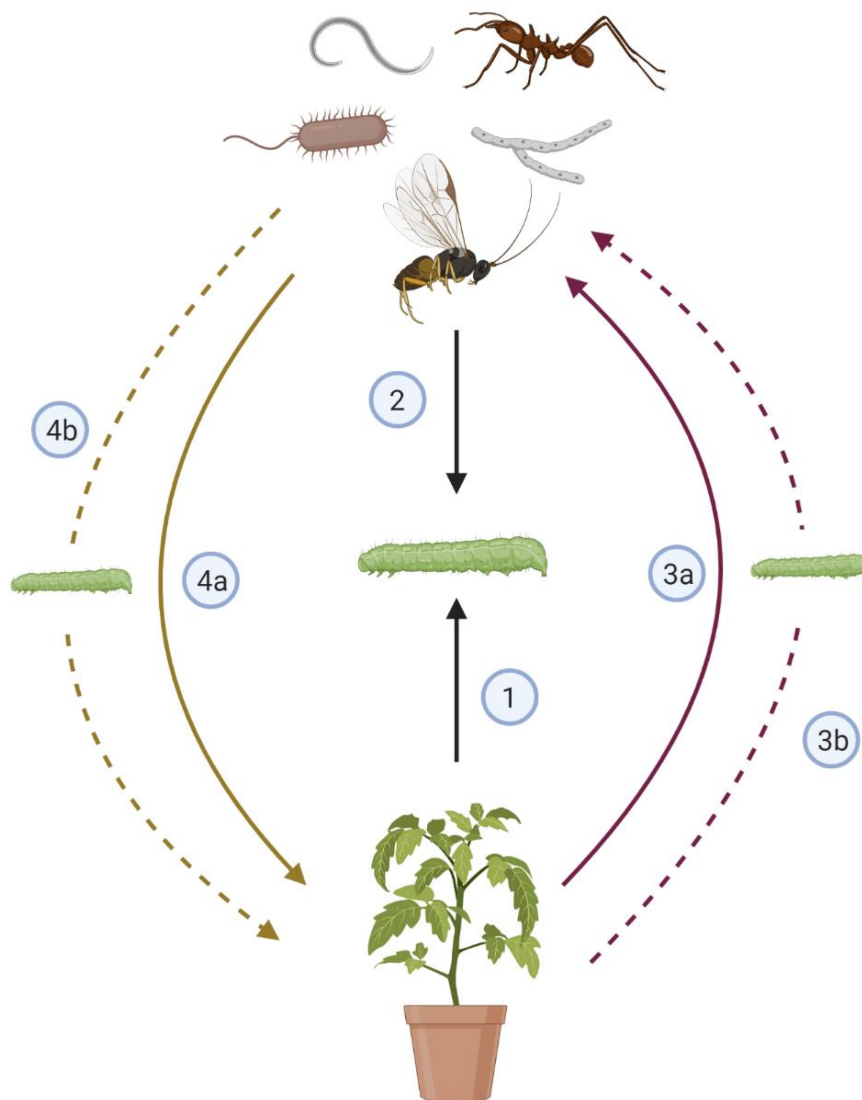

Figure 1.1: The complexity of tritrophic plant–herbivore–natural enemy (NE) interactions 1) Bottom-up effects of plant traits on herbivore populations, 2) Top-down impacts of NEs on herbivore populations, 3a) Bottom-up effects of plant traits on NEs (direct effects), 3b) Bottom-up effects of plant traits on NEs via changes to host/prey quality (indirect effects) , 4a) Top-down effects of NEs on plant traits (direct effects), 4b) Top-down effects of NEs on plants via changes to host/prey physiology or behavior (indirect effects). Direct and indirect effects are depicted with solid and dotted lines, respectively.

Reciprocally, the effects of NEs in ecosystems may be more complex than previously appreciated. Much research has focused on benefits NEs provide to plants through consumption and removal of herbivores, particularly in a biological control context (De Bach and Rosen, 1991; Hajek et al., 2007; Hawkins et al., 1997, Hawkins et al., 1999; Letourneau et al., 2009). However, emerging research broadens our understanding of the complexity of NE interactions in ecosystems. NEs can influence plant defenses, both directly when they use plant resources (Wäckers et al., 2005) and indirectly through modification of herbivore phenotypes during infection and parasitization (Kaplan, 2016). These findings show that NEs can influence bottom-up traits (Figure 1.1: 4a and 4b), with cascading effects on plants, herbivores, and NEs themselves. Although the prevalence and evolutionary context of NE impacts on plant defenses has only just begun to be explored, these interactions could be important components of complex multitrophic networks and aid our understanding of terrestrial food webs.

In this review, I synthesize research on NE modulation of plant chemical defenses and seek to expand the current paradigm of NE interactions in ecosystems. I discuss types of NE cues and plant responses, implications for NEs, plants, and herbivores, and the evolutionary context for such interactions. Finally, I examine how this knowledge could apply to biological control and propose avenues for future research.

Section 1: Plant detection of NEs

Plant defenses

To effectively mount defense responses to pathogen and herbivore attack, plants must detect and identify their attackers. Plant perception of pathogens involves recognition of microbial elicitors or microbial-associated molecular patterns (MAMPs) (Dodds and Rathjen, 2010; Teixeira et al., 2019). MAMPs are usually evolutionarily conserved molecules that are common to many microbes. Recognition of MAMPs by receptors on the cell surface triggers a general defense response known as microbial-triggered immunity (MTI) (Dodds and Rathjen, 2010; Teixeira et al., 2019). Pathogenic microbes can also possess effectors, virulence molecules that suppress MTI and cause disease (Boller and He, 2009). In turn, plants have a second response called effector-triggered immunity (ETI) that is induced by intracellular receptor recognition of effectors (Boller and He, 2009).

Plant detection of herbivorous insects is thought to follow a similar model to that described for pathogens. Insects produce herbivore-associated molecular patterns (HAMPs) that act as elicitors or effectors (suppressors) of plant defense responses (Maffei et al., 2012). HAMPs include mechanical damage from feeding (Heil, 2009), compounds present in saliva and other secretions (Acevedo et al., 2015), insect hormones (Helms et al., 2013), and even microbial symbionts (Mason et al., 2018). Although numerous herbivore elicitors have been described (Alborn et al., 1997; Bonaventure et al., 2011), the receptors that recognize these HAMPs are poorly understood (Basu et al., 2017; Heil, 2009; Schmelz, 2015; but see: Steinbrenner et al., 2019; Truitt et al., 2004).

After plant recognition of an invader, signalling cascades lead to changes in intracellular Ca^{2+}, production of reactive oxygen species, activation of protein kinases, and accumulation of phytohormones (Fraire-Velázquez et al., 2011). Jasmonic acid (JA) and ethylene (ET) are thought to mainly control defenses against necrotrophic pathogens and chewing herbivores, whereas salicylic acid (SA) and ET coordinate defenses against biotrophic pathogens and piercing-sucking insects (Erb et al., 2012; Li et al., 2019). The SA and JA pathways are often mutually antagonistic (Li et al., 2019; Thaler et al., 2012), meaning that induction of plant resistance to one class of attacker could make plants more vulnerable to other biotic threats. Crosstalk between signalling pathways and influence of other plant hormones may also allow plants to “fine-tune” their response to attackers (Beckers and Spoel, 2006; Erb et al., 2012; Fraire-Velázquez et al., 2011).

Activation of plant hormone signalling pathways ultimately leads to upregulation of defense-related genes that confer resistance to pathogens and herbivores (Erb et al., 2012). In response to pathogens, MTI and ETI involve local responses such as reinforcement of cell walls and programmed cell death (hypersensitive response) that restrict pathogen spread (Kombrink and Schmelzer, 2001). Plants also synthesize pathogenesis-related (PR) proteins such as chitinases and glucanases (van Loon and van Strien, 1999). PR proteins not only provide local resistance against pathogens, but accumulation of these proteins in distal tissues is important for generating systemic acquired resistance (SAR), a long-lasting and broad-spectrum state of resistance across the whole plant (Ding and Ding, 2020; Durrant and Dong, 2004).

In response to herbivory, plants produce secondary metabolites (e.g. phenolics, terpenoids) and defensive proteins (e.g. protease inhibitors, polyphenol oxidases, peroxidases) that affect herbivores directly by decreasing nutrient availability, acting as toxins, or making plants unpalatable (Baldwin and Preston, 1999; Chen, 2008; Zhu-Salzman et al., 2008). In addition to these direct defenses, plants may also upregulate the production of indirect defenses such as herbivore-induced plant volatiles (HIPVs) and EFN which attract and sustain NEs that indirectly protect plants through consumption of herbivores (Aljbory and Chen, 2016; Arimura et al., 2005; Dicke, 1999; Elliot et al., 2000; Mumm and Dicke, 2010).

NE cues may elicit similar plant responses to those induced by pathogens or herbivores, involving modulation of plant hormone signalling pathways, induction of defensive compounds, and emission of volatiles. NEs are both phylogenetically and ecologically diverse, comprising arthropod predators, parasitoids, EPNs, and microbial entomopathogens (De Bach and Rosen, 1991; Gonzalez et al., 2016; Hajek et al., 2007), and thus it is likely that NEs produce an equally diverse array of cues that are detectable by plants. These cues can be produced by NEs themselves (direct cues) during life phases where NEs use plants as habitat and food (Table 1.1). Or plant detection of NEs can be mediated through indirect modulation of host cues (indirect cues; Table 1.1). Although some NEs may possess unique cues, it is possible that they also share effectors and elicitor compounds with phylogenetically related pathogens and herbivores.

NE direct cues

Many predominantly predaceous insects occasionally feed on plants to obtain water and supplemental nutrients or for sustenance when prey are scarce (Dumont et al., 2018; Torres and Boyd, 2009). This omnivorous behavior—known as zoophytophagy—has been documented in plant bugs (Hemiptera) (Torres and Boyd, 2009), coccinellid beetles (Moser and Obrycki, 2009; Moser et al., 2008), and thrips (Spence et al., 2007); most research on plant defense induction by predators has focused on mirid (Bouagga et al., 2017; Moayeri et al., 2007; Pappas et al., 2015, 2016; Pérez-Hedo et al., 2015a; Zhang et al., 2018) and anthocorid (Bouagga et al., 2018) plant bugs. During plant feeding, omnivorous predators can inflict mechanical damage and introduce salivary enzymes into wounds (Castañé et al., 2011), both of which could provide cues for plant detection of these NEs (Table 1.1). Variation in tomato plant defense responses to feeding by three species of mirid bugs (Pérez-Hedo et al., 2015b) suggests that plants could differentiate between even closely related species on the basis of these cues.

Like feeding, oviposition by NEs can wound plant tissue and introduce oviposition secretions and glues. Predaceous bugs often use plants as substrates for oviposition and may prefer some plants over others to lay their eggs (Lundgren and Fergen, 2006; Steidle et al., 2008). Although plant perception of herbivorous insect eggs has been well-documented (Bertea et al., 2020; Hilker and Meiners, 2006), currently only one study that I am aware of has reported plant defense induction in response to predator oviposition (De Puysseleyr et al., 2011). *Orius laevigatus* (Hemiptera: Miridae) induced similar defense responses in tomato through both oviposition and ovipositor puncture without addition of eggs, suggesting that the plant responded to mechanical damage, rather than egg elicitors (De Puysseleyr et al., 2011) (Table 1.1).

NEs produce other mechanical cues that could be detectable by plants. During parasitization of eggs, parasitoid ovipositors may puncture plant tissue and cause mechanical damage (Li et al., 2020). Herbivore "footsteps" can damage leaf tissue and break trichomes, leading to induction of plant defenses (Hall et al., 2004; Peiffer et al., 2009; Tooker et al., 2010), and even mechanical stimulation that does not damage trichomes can influence systemic plant defenses (Markovic et al., 2016). Plants could similarly perceive mechanical cues from parasitoids and predators as they move across plant surfaces. Plants have also been shown to alter defenses in response to vibrations associated with herbivore feeding (Appel and Cocroft, 2014; Body et al., 2019), and evening primrose (*Oenothera drummondii*) flowers produced nectar with higher sugar content in response to pollinator wingbeat vibrations (Veits et al., 2019). Plants could similarly detect vibrations associated with predators or parasitoids, although this has not yet been investigated.

Fungal (e.g. *Beauveria, Metarhizium*), bacterial (e.g. *Bacillus, Serratia*) , and viral (e.g. Baculoviridae) entomopathogens interact with plants during endophytic (Bamisile et al., 2018; Monnerat et al., 2009; Praca et al., 2012), rhizosphere- (Bruck, 2010; Prischmann et al., 2008), and phylloplane-inhabiting (Cory and Myers, 2003; Smith and Couche, 1991) stages of their lifecycles. Like other microorganisms, entomopathogens possess MAMPs that can be detected by plant receptors (Hyakumachi et al., 2013) during these non-entomopathogenic phases. The diverse impacts that entomopathogenic fungi can have on plant resistance through these

Table 1.1: Types of cues natural enemies (NEs) use to modulate plant defenses, plant responses, and effects on NEs and other organisms.

Natural enemy	Plant	Type of cue[1]	Effects on plant[2]	Effects on NE[2]	Effects of plant defenses on herbivores, pathogens, and other trophic levels
Zoophytophagous predators					
Macrolophus pygmaeus (Hemiptera: Miridae)	Tomato (*Solanum lycopersicum*)	Direct, via feeding	•Induction of JA-related gene *PIN2*; no change in expression of ABA-related *ASR1* gene (Perez-Hedo et al., 2015b) •Enhanced protease inhibitor expression (Pappas et al., 2015)		•Spider mite (*Tetranychus urticae*; Acari: Trombidiformes: Tetranychidae) survival and number of eggs laid were reduced (Pappas et al., 2015) •Attraction of pest *Tuta absoluta* in Y-tube assays (Lepidoptera: Gelechiidae) (Perez-Hedo et al., 2015b) •Attraction of parasitoid *Encarsia formosa* in Y-tube assays (Hymenoptera: Aphelinidae) (Perez-Hedo et al., 2015b) •No difference in attraction of *B. tabaci* in Y-tube assays (Perez-Hedo et al., 2015b) •Whitefly (*Trialeurodes vaporariorum*; Hemiptera: Aleyrodidae) performance, attraction, and number of eggs not affected (Pappas et al., 2015)
Macrolophus pygmaeus (Hemiptera: Miridae)	Sweet pepper (*Capsicum annuum*)	Direct, via feeding	•Higher levels of phytohormones JA-Ile, JA OPDA, ABA, but not SA (Zhang et al., 2018; Bouagga et al., 2017) •Upregulation of emission of 12 volatile compounds (terpenoids, green leaf volatiles, methyl salicylate), plus induction of one compound (octyl acetate) not detected in undamaged or *N. tenuis*-damaged plants (Bouagga et al., 2017) •Induction of PIN2 and ASR1 genes, markers of JA and ABA phytohormone pathway, respectively. No difference in SA-regulated PR1 gene (Bouagga et al., 2017) •Fewer leaves and open flowers, similar numbers of fruits, shorter period between flowering and fruiting, 5x greater seed production per fruit (Zhang et al., 2019a)	•Reduced flower numbers on exposed plants correlated with reduced survival and reproduction of *M. pygmaeus* (Zhang et al., 2019)	•Reduced reproduction of *T. urticae* and western flower thrips (*Frankliniella occidentalis;* Thysanoptera: Thripidae) on both exposed leaves and non-exposed leaves (Zhang et al., 2018) •Reduced attraction of *T. urticae* and *F. occidentalis* in whole plant assays (Zhang et al., 2019b) •Reduced attraction of *B. tabaci* and *F. occidentalis* in both whole-plant cage experiments and Y-tube assays (Bouagga et al., 2017) •No difference in green peach aphid (*Myzus persicae*; Hemiptera: Aphididae) performance or attraction (whole plant) (Zhang et al., 2018) •Enhanced attraction of parasitoid *E. formosa* in Y-tube assays (Bouagga et al., 2017)

Orius laevigatus (Hemiptera: Anthocoridae)	Tomato (*Solanum lycopersicum*)	Direct, via oviposition	•Accumulation of H_2O_2 in response to ovipositor puncture and egg deposition •Upregulation of three JA-related genes (prosystemin, allene oxide synthase (AOS) and proteinase inhibitor I (PI-I)) •Upregulation of PI–II protein (De Puysseleyr et al., 2011)		•Reduced feeding damage by *F. occidentalis* in plants exposed to oviposition (De Puysseleyr et al., 2011)
Orius laevigatus (Hemiptera: Anthocoridae)	Sweet pepper (*Capsicum annuum*)	Direct, via feeding	•Upregulation of JA-related PIN2 and SA-regulated PR1 genes; no change in expression of ABA-regulated *ASR1* gene •Increased emission of 10 volatile compounds (terpenoids, green leaf volatiles, methyl salicylate, unknown compound) (Bouagga et al., 2018)		•Reduced attraction of *Bemisia tabaci* (Hemiptera: Aleyrodidae) and *F. occidentalis* in Y-tube assays •Enhanced attraction of parasitoid *E. formosa* in Y-tube experiments (Bouagga et al., 2018)
Nesidiocoris tenuis (Hemiptera: Miridae)	Tomato (*Solanum lycopersicum*)	Direct, via feeding	•Enhanced production of phytohormones JA-Ile, OPDA, and ABA (Perez-Hedo et al., 2015a) •Upregulation of *PIN2* and *ASR1* genes (Perez-Hedo et al., 2015a; Perez-Hedo et al., 2015b) •Plants exposed to volatiles from N. tenuis-induced plants (but not exposed themselves) had increased levels of *PIN2*, but not *ASR1* (Perez-Hedo et al., 2015a)		•Reduced attraction of *B. tabaci* and *T. absoluta* herbivores in Y-tube assays (Perez-Hedo et al., 2015a; Perez-Hedo et al., 2015b) •Enhanced attraction of parasitoid *E. formosa* (Perez-Hedo et al., 2015a) •Plants exposed to volatiles from *N. tenuis*-induced plants (but not exposed themselves) also more attractive to *E. formosa,* but no difference in attractiveness to *B. tabaci* (Perez-Hedo et al., 2015a)
Nesidiocoris tenuis (Hemiptera: Miridae)	Sweet pepper (*Capsicum annuum*)	Direct, via feeding	•Increased levels of phytohormones JA-Ile, JA OPDA, ABA, but not SA (Bouagga et al., 2017) •Induction of PIN2, ASR1, and PR1 genes. This effect persisted at 2 weeks after predator removal (Bouagga et al., 2017) •Upregulation of emission of 11 volatile compounds (terpenoids, green leaf volatiles, methyl salicylate) (Bouagga et al., 2017)		•Reduced attraction of *B. tabaci* and *F. occidentalis* in both cage experiments and Y-tube assays (Bouagga et al., 2017) •Repellent effect of plants on *B. tabaci in* Y-tube assays persisted at 4 and 7 days after *N. tenuis* removal, but not 14 days (Bouagga et al., 2017) •Enhanced attraction of parasitoid *E. formosa* in Y-tube assays at 4, 7, and 14 days after *N. tenuis* removal (Bouagga et al., 2017)

Dicyphus maroccanus (Hemiptera: Miridae)	Tomato (*Solanum lycopersicum*)	Direct, via feeding	•Induction of JA-related gene *PIN2*; no change in expression of ABA-related *ASR1* gene (Perez-Hedo et al., 2015b)		•Attraction of pest *T.* absoluta in Y-tube assays (Perez-Hedo et al., 2015b) •Attraction of parasitoid *E.* formosa in Y-tube assays (Perez-Hedo et al., 2015b) •No difference in attraction of *B. tabaci* in Y-tube assays (Perez-Hedo et al., 2015b)
Frankliniella occidentalis (Thysanoptera: Thripidae)	Cotton (*Gossypium hirsutum*)	Direct, via feeding	•Elevated peroxidase activity and density of gossypol glands (Spence et al., 2007)		
Parasitoids					
Gregarious endoparasitoid *Cotesia glomerata* (Hymenoptera: Braconidae) and symbiotic polydnavirus	Wild population 'Kimmeridge' *Brassica oleracea*	Indirect, via venom- and polydnavirus-mediated modification of host (*Pieris rapae* and *Pieris brassica*) salivary elicitors. However, virus-genes were detected in host salivary glands, indicating plant direct detection of parasitoid symbiont could also modulate plant defenses (Zhu et al., 2018)	•Parasitized larvae consumed more plant tissue compared with healthy caterpillars (Poelman et al., 2011a, 2012) •Plants treated with regurgitant from caterpillars that were parasitized or injected with calyx fluid and venom had reduced expression of myrosinase gene compared with plants treated with regurgitant from unparasitized or PBS-injected caterpillars (Cusumano et al., 2018) •Feeding by parasitized *Pieris rapae or Pieris brassica* upregulated genes involved in glucosinolate biosynthesis compared with unparasitized larvae (Zhu et al., 2015) •Different volatile blends emitted from plants fed on by parasitized and unparasitized larvae (Zhu et al., 2015; Poelman et al., 2012)	•Mortality of parasitoid *C. glomerata* developing within *P. rapae* hosts was lower when feeding on plants previously induced by *C. glomerata*-parasitized hosts, compared with undamaged plants, but not different compared to plants damaged by healthy larvae (Poelman et al., 2011a)	•*Plutella xylostella* laid fewer eggs on plants treated with regurgitant from parasitized larvae compared with unparasitized (Poelman et al., 2011b) •*P. xylostella* laid fewer eggs on plants fed on by parasitized larvae (or injected with polydnavirus-containing calyx fluid +venom or calyx fluid+venom+eggs (Cusumano et al., 2018) •Plant volatiles induced by parasitized larvae feeding, application of oral secretions of *C. glomerata*-parasitized larvae, or injection of polydnavirus+venom were more attractive to hyperparasitoid *Lysibia nana* compared with plants induced by feeding or oral secretions of healthy larvae in Y-tube assays; cocoons attached to plants damaged by parasitized larvae had higher parasitism rates in field and garden experiments (Poelman et al., 2012; Zhu et al., 2015; Zhu et al., 2018; Cusumano et al., 2019) •No difference in mortality of parasitoid *C. rubecula* developing within *P. rapae* hosts feeding on plants that were previously induced by C. glomerata-parasitized larvae, compared with undamaged plants or plants damaged by healthy larvae (Poelman et al., 2011a)

Solitary endoparasitoid *Cotesia rubecula* (Hymenoptera: Braconidae)	Wild population 'Kimmeridge' *Brassica oleracea*	Indirect, via modification of host (*Pieris* spp.) oral secretions AND/OR due to reduction of host feeding rate	•Parasitized larvae consumed less plant tissue compared with healthy caterpillars (Poelman et al., 2011a, 2012) •No difference in the composition of volatile blends emitted by plants damaged by healthy or *C. rubecula*-parasitized larvae (Poelman et al., 2012)	•No difference to performance of *C. rubecula* developing within *P. rapae* hosts feeding on plants previously induced by *C. rubecula*-parasitized larvae, compared with undamaged plants or plants damaged by healthy larvae (Poelman et al. 2011a)	•Mortality of parasitoid *C. glomerata* developing within *P. rapae* hosts was higher on plants previously induced by *C. rubecula*-parasitized larvae, compared with undamaged plants or plants damaged by healthy larvae (Poelman et al. 2011a) •Plants fed on by *C. rubecula*-parasitized *Pieris rapae* and *Pieris brassicae* larvae were equally attractive to hyperparasitoid *Lysibia nana* as plants fed on by healthy larvae (Cusumano et al., 2019; Poelman et al., 2012)
Gregarious endoparasitoid *Copidosoma floridanum* (Hymenoptera: Encyrtidae)	*Brassica* spp.	Indirect, via increasing host (*Trichoplusia ni*) plant consumption	•Feeding by parasitized larvae induced higher levels of the indole glucosinolates glucobrassicin and neoglucobrassicin (Ode et al., 2016)	•Fitness parameters (development time, brood size) were negatively correlated with glucobrassicin and neoglucobrassicin, which were produced in higher quantities in plants fed on by parasitized larvae (Ode et al., 2016)	•Although aliphatic glucosinolates were correlated with poorer performance of healthy *T. ni*, these compounds were not induced by herbivory from healthy or parasitized larvae (Ode et al., 2016)
Solitary endoparasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae)	Wild lima bean (*Phaseolus lunatus*)	Indirect, via modification of host (*Spodoptera latifascia*) feeding rate	•Significantly reduced leaf damage and improved seed production in plants fed on by parasitized larvae compared to unparasitized larvae •No difference in time to flowering or pod ripening time, or germination rate of seeds •Seed concentration of the cyanogenic glycosides linamarin and lotaustralin were reduced in parasitized treatment compared with unparasitized, and similar compared to undamaged control plants; there was no difference in seed phenolic content between treatments (Bustos-Segura et al., 2019)		•Emergence of seed beetles (*Zabrotes subfasciatus* and *Acanthoscelides obtectus*) was significantly lower in plants fed on by parasitized larvae than undamaged control plants, but not different to plants fed on by unparasitized larvae •Parasitism rates of seed beetle larvae by *Stenocorse bruchivora* and *Chryseida* sp. were similar for all treatments (Bustos-Segura et al., 2019)

Egg parasitoid *Anagrus nilaparvatae* (Hymenoptera: Mymaridae)	Rice (*Oryza sativa*)	Unknown. Indirect via modification of host *Nilaparvata lugens* eggs cues OR direct, via wasp factors applied to eggs (e.g. marking pheromones) or mechanical damage from wasp ovipositor	•JA and JA-Ile levels higher in plants exposed to parasitized eggs compared with plants exposed to unparasitized eggs; no difference in SA •Plants exposed to parasitized eggs emitted higher levels of four volatile chemicals (linalool, methyl salicylate, α-zingiberene and an unknown compound) compared with plants exposed to unparasitized eggs (Li et al., 2020)	•In Y-tube assays, *A. nilaparvatae* were less attracted to plant volatiles from parasitized eggs compared to unparasitized eggs, suggesting this could help parasitoid locate susceptible hosts •Exposure to dispensers of α-zingiberene and methyl salicylate, but not linanool, repelled wasps in Y-tube assays, •In field studies, adding dispensers with α-zingiberene, but not linanool or methyl salicylate, to host egg masses on rice plants reduced parasitism rates (Li et al., 2020)	
Parasitic fly *Compsilura concinnata* (Diptera: Tachinidae)	Wild population 'Kimmeridge' *Brassica oleracea*	Indirect, via modification of host (*Pieris* spp.) regurgitant	•Parasitized larvae induced higher levels of several JA-related genes (Poelman et al.,2011b)		•Reduced oviposition by herbivore *Plutella xylostella* on mechanically damaged plants treated with regurgitant from parasitized larvae compared with unparasitized (Poelman et al., 2011b)
Solitary endoparasitoid *Hyposoter ebeninus* (Hymenoptera: Ichneumonidae)	Wild population 'Kimmeridge' *Brassica oleracea*	Indirect, via modification of host (*Pieris* spp.) regurgitant	•Parasitized larvae induced higher levels of several JA-related genes (Poelman et al., 2011b)		•Reduced oviposition by herbivore *Plutella xylostella* on mechanically damaged plants treated with regurgitant from parasitized larvae compared with unparasitized (Poelman et al., 2011b)
Solitary endoparasitoid *Platygaster* sp. (Hymenoptera: Platygastridae)	*Euonymus* spp.	Unknown. Possibly indirect, via modification of host gall midge *Masakimyia pustulae* cues	*M. pustulae g*alls parasitized by *Platygaster* sp. had significantly thicker upper and lower gall walls and were thicker overall (Fujii et al., 2014)	Parasitoids may be more protected from hyperparasitization, but this was not tested (Fujii et al., 2014)	

Early endoparasitic-late ectoparasitic parasitoid *Parnips nigripes* (Hymenoptera: Figitidae)	Poppy (*Papaver rhoea*)	Unknown. Possibly indirect, via modification of host gall wasp *Barbotinia oraniensis* cues	*B. oraniensis* galls parasitized by *P. nigripes* were significantly thicker than unparasitized galls (Ronquist et al., 2018)	Parasitoids may be more protected from hyperparasitization, but this was not tested (Ronquist et al., 2018)
Solitary endoparasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) and symbiotic polydnavirus	Tomato (*Solanum lycopersicum*)	Indirect, via polydnavirus-mediated modification of host (*Helicoverpa zea* and *Heliothis virescens*) salivary elicitor glucose oxidase levels	•Feeding by parasitized larvae, application of saliva from parasitized larvae, or saliva from virus-injected larvae induced lower production of defense proteins polyphenol oxidase (PPO) (Tan et al., 2018), peroxidase, and trypsin inhibitor (Tan et al., 2020) •Lower production of defense genes *PPOB* and CysPI (Tan et al., 2020) •Plants treated with saliva from parasitized larvae had greater numbers of flowers and heavier fruit than plants treated with saliva from unparasitized larvae (Tan et al., 2020) •Offspring of plants treated with parasitized larvae saliva produced greater PPO levels in response to herbivory compared with the offspring of plants treated with unparasitized larvae saliva; differences in transgenerational effects were not observed for other defense proteins (Tan et al., 2020)	Enhanced pupation rate, pupal weight, and survival rate when parasitoid developed in larvae feeding on plants treated with parasitized larvae saliva compared to plants treated with healthy larvae saliva (Tan et al., 2018)
Solitary endoparasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) and symbiotic polydnavirus	Tobacco (*Nicotiana tabacum*)	Indirect, via modification of host *Helicoverpa zea* salivary elicitor glucose oxidase levels	•Herbivory or application of parasitized larvae saliva induced higher levels of PPO and trypsin inhibitor compared to unparasitized larvae; there was no difference in peroxidase between these treatments (Tan, 2019)	•Parasitoids developing in larvae feeding on plants treated with saliva from parasitized larvae experienced increased development times, higher mortality, lower cocoon weight, and reduced survival (Tan, 2019)

Entomopathogenic nematodes (EPNs)					
Heterorhabditis bacteriophora and symbiotic bacterium *Photorhabdus luminescens*	Potato (*Solanum tuberosum*)	Indirect, via volatile cues from EPN-killed host (*Galleria mellonella)*cadavers. However, volatiles could have also been produced directly by EPNs or symbiotic bacteria	•Although undamaged plants exposed to EPN cadaver cues did not show differences in SA or JA compared with control cadaver-exposed plants, following damage by *L. decemlineata* larvae plants had stronger induction of SA and JA, indicating that defenses were primed (Helms et al., 2019)		•Colorado potato beetle (*Leptinotarsa decemlineata*) gained less weight and consumed less plant tissue when feeding on plants previously exposed to EPN cadaver volatiles compared with control cadavers •CPB adults laid fewer eggs on plants exposed to EPN-killed cadavers (Helms et al., 2019)
Steinernema carpocapsae and symbiotic bacterium *Xenorhabdus nematophila*	Potato (*Solanum tuberosum*)	Likely direct, via infective juvenile EPN cues interacting with plant roots	•Undamaged plants exposed to EPN infective juveniles had higher SA levels and higher levels of the SA-regulated *PR-1(P4)* gene, but no difference in levels of JA or the JA-associated (LoxD) gene (Helms et al., 2019)		
Steinernema carpocapsae and symbiotic bacterium *Xenorhabdus nematophila*	Hosta *Hosta* sp.	Direct and indirect via *Galleria mellonella* hosts, Plant roots exposed to live EPN infective juveniles (Jagdale et al., 2009) or via application of host *G. mellonella* cadavers to roots (Jagdale and Grewal, 2008; Jagdale et al., 2009) or cadaver extracts (Jagdale and Grewal, 2008)	•Application infective juveniles, EPN-killed cadavers, or *X. nematophila* bacteria alone upregulated peroxidase and catalase activity, and the effects lasted up to two weeks (Jagdale et al., 2009) •Preventative applications of EPN cadavers to soil enhanced plant biomass, leaf size, and mean number of leaves (Jagdale and Grewal, 2008)		•Curative applications of EPN cadavers or cadaver extracts to soil reduced multiplication of foliar nematode pest *Aphelenchoides fragariae* in hosta leaves and the size of leaf lesions for at least 45 days (Jagdale and Grewal, 2008) •Preventative applications of EPN cadavers to soil reduced multiplication of foliar nematode pest *Aphelenchoides fragariae* in hosta leaves and the size of leaf lesions (Jagdale and Grewal, 2008)

Steinernema carpocapsae and symbiotic bacterium *Xenorhabdus nematophila*	*Arabidopsis thaliana*	Direct cues from EPN infective juveniles AND indirect and/or direct cues from EPNs and symbiotic bacteria in EPN-killed host (*Galleria mellonella*) cadavers	•Application of infective juveniles and EPN-killed cadavers to plant roots induced *PR1* gene expression relative to unexposed plants •Application of infective juveniles (but not cadavers) enhanced catalase activity (Jagdale et al., 2009)		
Steinernema carpocapsae and symbiotic bacterium *Xenorhabdus nematophila*	Tomato (*Solanum lycopersicum*)	Indirect, via cues from EPN-killed host (*Galleria mellonella*) cadavers OR direct cues from EPNs or symbiotic bacteria	•No difference in plant biomass between cadaver-treated plants and control plants, suggesting no trade-off between induction of systemic resistance and growth (An et al., 2016)		•On EPN cadaver treated plants, compared with control-cadaver treated plants, *Spodoptera exigua* larvae developed slower, *B. tabaci* had reduced egg hatch, and there was reduced disease severity (lesion formation) of pathogen *Pseudomonas syringae*. These effects were observed at 3 and 7 days after treatment, but not at 14 days (An et al., 2016)
Entomopathogenic microbes					
Autographa californica multiple nucleocapsid nucleopolyhedrovirus (AcMNPV; Baculoviridae)	Tomato (*Solanum lycopersicum*)	Indirect, due to the effects of immune challenge on host *Helicoverpa zea* larvae by sublethal dose of AcMNPV. Experiments indicate that plant defense modulation was likely due to pathogen-mediated modification of the volume of host secretions	•Plants damaged by AcMNPV-infected *H. zea* induced higher trypsin inhibitor activity, and higher expression of JA-associated genes *Pin2* and *CysPI* compared with feeding by healthy larvae; there was no difference in peroxidase or PPO activity between treatments (Pan et al., 2019)		•Healthy *H. zea* larvae consumed less leaf tissue and exhibited lower relative growth rates when they fed on plants previously damaged by AcMNPV-infected larvae compared with healthy larvae (Pan et al., 2019)

1 Direct = NE produces cues that plants detect directly, indirect = NE modulates host cues

2 In comparison with undamaged plants (for zoophytophagous bugs) , in comparison with feeding by unparasitized herbivores (for parasitoid studies)

interactions have been reviewed elsewhere (Bamisile et al., 2018; Dara, 2019; Jaber and Ownley, 2018; Ownley et al., 2010), and I do not provide an exhaustive list of these studies in Table 1.1.

It is also likely that entomopathogenic bacteria modulate plant defense responses during soil-inhabiting or endophytic phases. One interesting study found that entomopathogenic *B. thuringiensis kurstaki* strain HD1 bacteria were translocated from cotton and cabbage roots to plant leaves and caused mortality in lepidopteran larvae feeding on these leaves—although the authors did not investigate whether the bacteria also influenced plant defenses (Monnerat et al., 2009). Endophytic or rhizosphere-dwelling *Bacillus* and *Serratia* isolates have been shown to induce plant resistance by altering plant hormone signalling pathways and inducing defense-related genes (Beneduzi et al., 2012; Choudhary and Johri, 2009; Glick, 2012; Hyakumachi et al., 2013). Although these bacteria are related to entomopathogenic *Bacillus* and *Serratia*, most studies did not specifically identify whether the isolates used were entomopathogens (but see Qi et al., 2016). There is considerable variation in pathogenicity of bacteria, even among strains or isolates of the same species (Pineda-Castellanos et al., 2015). For example, not all *Bacillus thuringiensis* isolates produce insecticidal Cry proteins and some may produce inactive proteins (Ehling-Schulz et al., 2019). Because I cannot be sure whether the bacteria modulating plant defenses in most studies are NEs of herbivores, I do not include examples of bacterial entomopathogens in Table 1.1.

EPNs are another type of NE that interact with plant roots in the rhizosphere. The motile infective juveniles are associated with cues such as secreted metabolites and symbiotic bacteria, both of which may play a role in altering plant defense responses (An et al., 2016; Jagdale and Grewal, 2008; Jagdale et al., 2009). After location and infection of a host, EPNs and their symbiotic bacteria overcome host immunity and break down host tissues (Ciche et al., 2006)—presenting further cues for plants (*see Indirect cues*).

Volatile cues produced by NEs could also be detectable by plants. Plants are known to respond to volatile compounds produced by plants (Dudareva et al., 2006) and insect herbivores (Helms et al., 2013, 2017), suggesting that olfactory cues emitted by arthropod parasitoids and predators (e.g. pheromones) and entomopathogenic microbes (Bojke et al., 2018; Crespo et al., 2008) could similarly elicit plant responses. Although volatile compounds from *Bacillus* and *Serratia* spp. have been shown to affect plant growth (Wenke et al., 2019) and resistance

(Rudrappa et al., 2010; Tahir et al., 2017), as mentioned above there is no indication that the isolates used in these studies are entomopathogenic.

NE indirect cues

Unlike predators that typically kill their prey immediately, parasitoids, entomopathogens, and EPNs may live within their hosts for days or weeks before causing death. NEs often significantly alter host physiology and behavior during this infection period (Abram et al., 2019; Clem and Passarelli, 2013). By modifying host cues that plants use to detect herbivores, NEs can indirectly modulate plant defense responses.

Parasitoids are found in several insect orders including Diptera, Coleoptera, Lepidoptera, and Strepsiptera, although this life history is particularly common in Hymenoptera (Eggleton and Belshaw, 1992; Pennacchio and Strand, 2005). Idiobiont parasitoids halt development of their hosts and often attack egg and pupal hosts, whereas koinobionts allow their hosts to continue to feed and grow and usually attack larval insects (Pennacchio and Strand, 2005; Pennacchio et al., 2014). Along with eggs, parasitoids inject other factors such as venom, teratocytes, ovarian fluids, and—in the case of many braconid and ichneumonid wasps—symbiotic polydnaviruses (PDVs). These factors suppress immunity and modify physiology of hosts and benefit the developing parasitoid (Pennacchio and Strand, 2005; Pennacchio et al., 2014).

These parasitoid-induced host modifications can include cues that plants use to detect herbivores. For example, the symbiotic PDV associated with the parasitoid wasp *Microplitis croceipes* suppressed levels of glucose oxidase in *Helicoverpa zea* saliva. Because glucose oxidase is an important elicitor of plant defenses in tomato, feeding by parasitized larvae induced lower defense responses compared with healthy larvae (Tan et al., 2018). Parasitoids could also modulate plant defenses indirectly through modification of cues associated with host eggs (Li et al., 2020). Another study found that volatile profiles of cabbage plants induced by *Cotesia glomerata*-parasitized *Pieris rapae* and *Pieris brassicae* caterpillars were more similar than volatiles induced by healthy caterpillars, indicating that the parasitoid substantially alters host physiology and plant responses (Zhu et al., 2015). Although plant responses are thought to be driven primarily through indirect host modulation by the parasitoid and PDV, possible genes from *C. glomerata* PDV (CgPDV) were found in saliva of host caterpillars (Zhu et al., 2018), suggesting plants could directly detect parasitoid symbionts.

During infection or parasitization, NEs could also alter the quantity of host secretions. Feeding by *H. zea* larvae that were immune challenged by sublethal doses of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) induced higher levels of plant defenses in tomato, compared with feeding by healthy larvae (Pan et al., 2019). However, when equal volumes of saliva, ventral eversible gland secretion, or regurgitant from healthy and immune-challenged larvae were applied to mechanically-damaged plants, there was no difference in plant defense responses. These findings suggest that baculovirus infection did not alter the chemical composition of host secretions; instead, the authors suggest that higher plant defense responses elicited by immune-challenged *H. zea* feeding was due to baculovirus-mediated changes to the volume of secretions (Pan et al., 2019). Although another explanation could be that baculovirus infection altered some other host cue that was not tested.

After EPN infective juveniles penetrate an insect host, they release venom proteins and symbiotic bacteria that induce physiological changes in the host (Lu et al., 2017). This process allows infective juveniles to transition to an active parasitic lifestyle, where they initiate feeding, development, and reproduction—eventually killing the host 2–5 days after infection (Alonso et al., 2018; Lu et al., 2017). The incubation period between host death and release of infective juveniles can be up to 20 days; during this time the symbiotic bacteria help suppress growth of other microorganisms in the cadaver and prevent decomposition (Jones et al., 2015). EPN-killed cadavers applied to plant roots (An et al., 2016; Jagdale and Grewal, 2008; Jagdale et al., 2009) and volatile compounds released from cadavers can affect plant defense responses (Helms et al., 2019), although specific compounds that mediate these plant responses have not been identified.

NEs could also modulate plant defenses indirectly through their effects on host behavior (Bernardo and Singer, 2017; Roy et al., 2005; Shikano and Cory, 2016). Although idiobiont parasitoids attack non-feeding egg or pupal host stages or prevent further feeding by paralyzing larval hosts, koinobiont parasitoids can cause their hosts to feed more or less (Rahman, 1970; Rossi et al., 2014) compared with healthy larvae. Changes in feeding rate can affect plant responses to infected herbivores (Bustos-Segura et al., 2020; Ode et al., 2016). For example, *Trichoplusia ni* caterpillars parasitized by the gregarious parasitoid *Copidosoma floridanum* consumed more plant tissue than healthy caterpillars and altered expression of cabbage defense compounds (Ode et al., 2016). Parasitoid-mediated modification of host feeding preferences for protein (Mason et al., 2014) or other nutrients could also alter the location of herbivory (e.g.

flowers, seeds, old or young tissue) and modify plant defenses (Bustos-Segura et al., 2020)—although this has not been tested.

Additionally, predators can alter host behaviour and physiology through anti-predator responses that herbivores initiate to avoid consumption (Hermann and Landis, 2017). Such predation-risk effects can alter how prey organisms interact with plants, which could influence induction of plant defences. For example, when exposed to predators, Colorado potato beetle reduces feeding and oviposition on potato plants (Hermann and Thaler, 2014, 2018), both of which are behaviors that are known to affect plant defenses (Balbyshev and Lorenzen, 1997; Chung et al., 2013). Although the indirect impacts of NEs on plant defenses are currently understudied, these interactions are likely important to consider to capture the complexity of plant–herbivore–NE interactions.

Section 2: Plant responses to NEs

Plants respond to NEs with induction or suppression of JA, SA and other plant-hormone signalling pathways, leading to differential production of defense-related genes and volatile emissions (Helms et al., 2019; Pappas et al., 2015; Pérez-Hedo et al., 2015a; Zhang et al., 2018). Such modifications to plant defense phenotypes influence herbivores, pathogens, NEs, and other trophic levels (Table 1.1). For example, feeding by zoophytophagous predators on tomato and sweet pepper plants induced JA- and abscisic acid–mediated plant defences which negatively affected performance of herbivorous spider mites and thrips (Pappas et al., 2015; Pérez-Hedo et al., 2015a; Zhang et al., 2018). Predator oviposition can also alter plant responses. *O. laevigatus* oviposition elicited a JA-mediated defense response and subsequently increased plant resistance to the western flower thrips (De Puysseleyr et al., 2011). Similarly, fungal entomopathogens alter plant defenses and enhance resistance to insect herbivores (Ahmad et al., 2020; Shrivastava et al., 2015) and disease-causing plant pathogens (Jaber and Ownley, 2018; Ownley et al., 2010).

Changes in plant volatile emissions in response to NEs have been reported to reduce attraction of subsequent herbivores. When given a choice, spider mites and thrips preferred undamaged plants over plants that were previously wounded by their zoophytophagous predators (Bouagga et al., 2017, 2018; Pérez-Hedo et al., 2015a, 2015b; Zhang et al., 2019b, 2019a). Similarly, the herbivore *Plutella xylostella* laid fewer eggs on cabbage plants fed on by *C. glomerata*-parasitized larvae, compared with plants fed on by healthy larvae (Cusumano et al.,

2018; Poelman et al., 2011b). However, one study found that attraction or deterrence of herbivores depended on the identity of the NE; while some natural enemy species may elicit plant responses that can deter subsequent herbivory, other species may induce responses that render the plant more attractive to other herbivores (Pérez-Hedo et al., 2015b).

Induction of plant defenses in response to NEs can be both local, at the site of damage, and systemic, showing in distal plant tissues (Pappas et al., 2015), with immediate or long-lasting effects. Feeding by the mirid bug *Macrolophus pygmaeus* induced systemic defenses in tomato, an effect that lasted for at least two weeks after predator removal (Pappas et al., 2015). In *Hosta* sp. plants, belowground application of EPNs reduced multiplication of the foliar nematode pest *Aphelenchoides fragariae* and the size of leaf lesions for at least 45 days after application (Jagdale and Grewal, 2008). These studies indicate that some NEs could benefit plants not only through reduction of herbivores, but also by activating defense responses that provide ongoing protection against herbivores and pathogens (Pappas et al., 2015; Pérez-Hedo et al., 2015a).

Potential benefits to plants from perception of NEs is likely to depend on the composition of the ecological community. Because JA- and SA-mediated defenses often exhibit negative crosstalk, induction of one suite of defenses can suppress the other. For example, induction of JA-related defenses typically associated with resistance against chewing herbivores can downregulate SA-mediated defenses and make plants more vulnerable to plant pathogens or phloem-feeding insects (Karban and Kuć, 1999; Pappas et al., 2016). However, trade-offs between JA–SA defenses are not always clear. Entomopathogenic *Beauveria bassiana* increased levels of several defense compounds in groundnut (*Arachis hypogaea*) and enhanced resistance to both leafminer insects and collar rot disease (Senthilraja et al., 2013). Similarly, root applications of EPN-killed cadavers enhanced tomato resistance to insect pest species (*Spodoptera exigua* caterpillars and *Bemisia tabaci* whiteflies) and the bacterial pathogen *Pseudomonas syringae* (An et al., 2016). These examples indicate that induction of defenses in response to certain NEs can provide resistance to multiple pests.

Understanding the effects of plant defense modulation likely also depends on the presence or absence of herbivores. Allocation of plant resources to defense induction can lead to trade-offs with growth and reproduction (Coley et al., 1985), suggesting that unnecessary induction of plant defenses in absence of herbivores could have negative effects on plant fitness.

Indeed, indirect suppression of defenses by the parasitoid wasp *M. croceipes* enhanced plant fitness measures in tomato, likely because plants could reallocate resources from defenses to reproduction (Tan et al., 2020). However, this study was performed in the absence of additional herbivore pressure. In nature, plants are often exposed to multiple simultaneous attacks, and downregulation of plant defenses could make plants more susceptible to other herbivores.

One strategy that plants may use to balance resource allocation to defense and growth is defense priming (Karasov et al., 2017). Priming is a state where plants prepare their defense system for a faster or stronger response to future attack, without actually inducing defenses (Mauch-Mani et al., 2017). Potato plants exposed to volatiles from EPN-killed wax worm cadavers did not show differences in levels of SA or JA compared to undamaged plants in the absence of herbivory (Helms et al., 2019). However, after applying Colorado potato beetles, plants that were previously exposed to the EPN cues had higher levels of SA and JA and subsequently reduced herbivore feeding and oviposition, indicating that EPN cues primed potato plants for a stronger response to herbivory (Helms et al., 2019).

There is evidence that NE-mediated plant defense modulation can have positive impacts on NEs. For example, male *Macrolophus caliginosus* (Hemiptera: Miridae) were attracted to plants previously fed on by female conspecifics, suggesting that these plant bugs use NE-induced plant volatiles for mate location (Moayeri et al., 2007). Induction of defenses that delay herbivore development could also benefit NEs by extending the period of host susceptibility (Chen and Chen, 2018). For example, parasitoid wasps often prefer early instar larval hosts (Li et al., 2006; Saini et al., 2019), and baculoviruses are typically more virulent when infecting younger caterpillars (both within and between instars) (Cory and Myers, 2003). The baculovirus AcMNPV indirectly induced higher tomato defenses via host *H. zea* larvae (Pan et al., 2019). Although the authors did not investigate the consequences of this interaction, they suggest that increased defenses could delay larval development and extend the period of susceptibility to infection, thus increasing the likelihood of viral transmission (Pan et al., 2019). Alternatively, increased levels of phytochemicals could reduce virus-induced mortality (Pan et al., 2019). For example, plant peroxidases in cotton strongly inhibit baculovirus infectivity in the caterpillar midgut (Hoover et al., 1998, 2000).

Other negative impacts of plant defense induction on NEs have been reported. For example, sweet pepper plants exposed to feeding by *M. pygamaeus* produced fewer flowers than control plants, and flower number was negatively correlated with survival of nymphs and adults (Zhang et al., 2019a). As described above, emission of volatiles from NE-exposed plants can repel herbivores (Bouagga et al., 2017; Helms et al., 2019; Pérez-Hedo et al., 2015b), thus potentially hindering NE performance by reducing the availability of hosts/prey (Helms et al., 2019). More studies are needed to assess the preference and performance of NEs on induced plants.

Modulation of plant volatiles by NEs can also affect other trophic levels. Induction of JA-regulated plant volatiles by several species of predatory bug were attractive to another beneficial organism, the whitefly parasitoid *Encarsia formosa* (Bouagga et al., 2017; Pérez-Hedo et al., 2015a), and neighboring undamaged plants exposed to these volatiles also activated JA-related responses and attracted *E. formosa* (Pérez-Hedo et al., 2015a). This example suggests that not only could plants benefit from recruiting an additional NE, but this effect could provide additional protection for neighboring plants as well. Although herbivore- and NE-induced plant volatiles can benefit plants, hyperparasitoids in the fourth trophic level also use these cues to locate prey. Cabbage plants fed on by *C. glomerata*-parasitized *P. rapae* caterpillars produced a blend of volatiles distinct from plants fed on by healthy caterpillars (Poelman et al., 2012). Although the former plants were less attractive for *P. xylostella* herbivores (Poelman et al., 2011b), they were also attractive to the parasitoid's enemy, the hyperparasitoid *Lysibia nana* (Poelman et al., 2012). Because *L. nana* attacks parasitoid pupae after they emerge from the caterpillar, hyperparasitization does not interfere with initial parasitization and is unlikely to have immediate negative consequences for plants. However, long-term top-down pressure from hyperparasitoids could negatively affect parasitoid populations, disrupting the ecological services they provide to plants, and leading to pest outbreaks (Nenzén et al., 2018).

Studying effects across multiple generations is likely to be important for understanding the ecology and evolution of NE modulation of plant defenses. *T. ni* caterpillars parasitized by the gregarious parasitoid *C. floridanum* grew approximately 50% larger than unparasitized larvae, consumed more plant tissue, and induced higher levels of glucosinolate defense compounds (Ode et al., 2016). Although it is likely that the parasitoid decreases plant performance in the short term, high parasitism levels could reduce *T. ni* populations and benefit

plants across seasons or generations (Ode et al., 2016). Similarly, gall midge (*Masakimyia pustulae*) galls parasitized by *Platygaster* sp. wasps were thicker than unparasitized galls (Fujii et al., 2014). The authors suggest that the wasp modifies its dipteran host to indirectly alter plant development, producing thicker galls that protect it from hyperparasitization (Fujii et al., 2014). Gall inducers manipulate plants to produce galls (Larson and Whitham, 1991), often with negative effects on plant growth and reproduction (Fay and Hartnett, 1991). Although plants could expend more resources producing thicker parasitized galls (Fujii et al., 2014), protection of parasitoids from hyperparasitoids could result in better long-term control of herbivorous gall midge populations and ultimately benefit plants.

Through modulating plant production of secondary chemicals and emission of volatiles, NEs can influence bottom-up plant defenses against herbivores and pathogens and affect higher trophic levels. However, in most systems, the implications of these interactions on plant and NE fitness is unknown. A clearer view of the ecology of plant–NE interactions is likely to emerge from examining multitrophic responses in different contexts and across multiple time scales. Likewise, understanding the evolution of these responses and whether they are adaptive will require considering fitness perspectives of both plant and NE.

Section 3: Why do plants respond to NEs?

Plants are under strong selection pressure by insect herbivores and pathogens, and these interactions drive evolution of plant defense traits (Hare, 2012; Huot et al., 2013; Mauricio and Rausher, 1997). NEs may also influence plant evolution through their impacts on herbivore populations (Hare, 2002; Ode, 2006). For example, ants act as agents of selection for the evolution of EFN. Plants with EFN attract ants that consume herbivores, reducing herbivore damage and improving plant fitness (Kessler and Heil, 2011; Nogueira et al., 2012; Rudgers, 2004). In addition to consuming herbivores, NEs interact with plants in a variety of other ways (*see Section 1*). Plants respond to NE cues by modulating defense responses, which consequently affects plant, herbivore, and NE performance (*see Section 2*). However, whether NE–plant interactions are adaptive for either participant and which selection pressures shape these interactions have yet to be unravelled.

There are several non-mutually exclusive reasons for the evolution of plant–NE interactions. First, plant detection of NEs could have evolved as mutualisms between plants and

NEs. Second, plants could benefit from detecting NE cues without corresponding benefits to NEs. Third, plant responses could represent NE manipulation of plant defenses, providing benefits to NEs without benefitting plants. Fourth, plant detection of NEs could simply be due to similarities between NE and herbivore or pathogen cues. Finally, modulation of plant defenses through NE cues could be a by-product of some other essential biological function.

Mutualisms

NEs are often presumed to be mutualists with plants and part of the defense responses that plants have to herbivory (Price et al., 1980), and numerous studies have empirically shown that attraction of NEs improves plant fitness by reducing herbivory (Gómez and Zamora, 1994; Heil et al., 2001; Tooker and Hanks, 2006). The ability of plants to recognize and respond to NEs could have evolved as mutualisms between plants and NEs. For example, indirect suppression of tomato defenses by the *M. croceipes* through modification of its host *H. zea* led to improved performance of developing parasitoid larvae (Tan et al., 2018). If these benefits translate to enhanced parasitoid populations and better regulation of herbivores, plants could benefit in the long-term.

Regulation of plant defenses could also improve NE foraging. For example, the egg parasitoid *Anagrus nilaparvatae* discriminated between plant volatiles induced by healthy host plant hopper (*Nilaparvata lugens*) eggs or parasitized eggs, suggesting that modulation of plant volatiles could aid the location of suitable hosts and improve parasitization efficiency (Li et al., 2020). By alerting plants that they have “arrived”, NEs could also allow plants to attenuate defenses and reallocate resources to growth and reproduction (Cusumano et al., 2018). Indeed, downregulation of defenses by *M. croceipes*-parasitized larvae was associated with higher fitness measures such as greater flower numbers and heavier fruit (Tan et al., 2020).

Benefits to plants

Plant detection of NEs could have evolved as an indication of the presence of herbivores (Helms et al., 2019), where induction of defenses in response to NEs may allow plants to induce or prime defenses for impending herbivore attack. Several studies have shown that induction of plant defenses in response to EPNs and zoophytophagous bugs benefit plants by increasing resistance to herbivore feeding or repelling herbivores (An et al., 2016; Bouagga et al., 2017, 2018; Pappas et al., 2015; Pérez-Hedo et al., 2015a, 2015b) or priming plants to mount a stronger

response to subsequent herbivory (Helms et al., 2019). Plant defense induction by NEs could lower plant quality as a food resource for omnivorous predators, prompting them to consume less plant tissue and more herbivores (Zhang et al., 2019a). Plant responses to NEs often include altered volatile emissions that render plants less attractive to subsequent herbivores (Bouagga et al., 2017; Pérez-Hedo et al., 2015b; Zhang et al., 2019b; Table 1.1). While repelling herbivores is likely to benefit plants, NE performance could suffer due to reduced availability of hosts/prey, although this has not been tested (Helms et al., 2019).

From a plant perspective, induction of defenses is expected to be energetically costly (Bazzaz et al., 1987; Coley et al., 1985). Therefore, taking into account the net fitness gains or consequences of detecting and responding to NEs can help determine whether these responses are adaptive. Nonetheless, induction of defenses in response to EPN-killed cadavers improved plant resistance to insects and pathogens without affecting tomato biomass (An et al., 2016), and sweet pepper plants exposed to feeding by *M. pygmaeus* predators produced more seeds than unexposed plants (Zhang et al., 2019a). In the same study, sweet pepper plants produced fewer flowers than undamaged plants, which was correlated with reduced survival of *M. pygmaeus* nymphs and adults (Zhang et al., 2019a), suggesting that plant responses do not benefit this predator.

By-products of NE biology

If plant recognition of NEs has negative consequences for NEs, why does natural selection not favour reduction of cues that plants use to detect NEs? One possibility is that that these cues are by-products of some other important physiological or ecological function. If these cues are crucial for NE survival or reproduction, then selection against their production would be limited (Helms et al., 2019). For example, the gregarious parasitoid *C. glomerata* indirectly modulated cabbage volatile profiles by altering *Pieris* spp. salivary elicitors, making plants more attractive to the hyperparasitoid *L. nana* (Poelman et al., 2012; Zhu et al., 2018). Given that attraction of hyperparasitoids could have negative consequences for both the parasitoid and the plant (Poelman et al., 2012), natural selection might be expected to reduce parasitoid-mediated host changes that are responsible for production of these plant volatiles. Indeed, the solitary parasitoid *C. rubecula* also parasitizes *Pieris* spp., although cabbage volatiles induced by *C. rubecula*-parasitized larvae are equally attractive to *L. nana* as those induced by healthy larvae, suggesting that this parasitoid species could have evolved to avoid detection by hyperparasitoids

(Poelman et al., 2012). Given that *C. glomerata* is a gregarious parasitoid, with multiple larvae developing as broods within each host, modification of certain host cues could be important for its development (Poelman et al., 2012). If this is the case, plant responses to parasitized larvae could be an unavoidable consequence of parasitism—that hyperparasitoids exploit.

Manipulation of plant defenses by NEs

The relationship between plants and NEs may not always be mutualistic; NEs may actually have conflicting interests with plants (Van Der Meijden and Klinkhamer, 2000). Although some studies have empirically shown that some NEs reduce herbivory and improve plant fitness (Cardinale et al., 2003; Gómez and Zamora, 1994; Romero et al., 2008), NEs that do not kill their hosts immediately can cause herbivores to consume more plant tissue compared to their healthy counterparts (Gols et al., 2015; Ode et al., 2016). NE modulation of plant defenses could be an adaptive trait for NEs, and these interactions could represent NE manipulation of plants.

Indirect modulation of plant defenses by NEs may be particularly likely to have evolved as NE manipulation of plant defenses. NEs such as parasitoids or baculoviruses that interact with plants indirectly through their hosts may not present unique or reliable cues to plants that signal the presence of the NE. Instead, these NEs seem to alter the quantity of herbivore-associated cues (Cusumano et al., 2018; Pan et al., 2019; Tan et al., 2018), leading to modulation of plant defenses. In absence of direct interaction of NE cues with the plant, it seems unlikely that NEs would impose selection pressure on plants to evolve responses to these unspecific cues. Instead, manipulation of plant defenses via host modification could have evolved as an adaptive trait in NEs if it improved NE fitness. For example, because saliva from *M. croceipes*-parasitized *H. zea* larvae suppressed tomato defenses compared to that of healthy larvae, parasitoid performance was improved when larvae developed in hosts that fed on plants previously treated with saliva from parasitized larvae (Tan et al., 2018).

Parasitoids of gall-inducing insects may also manipulate plant regulation of galls to their own benefit. Many insect species induce plants to produce tumor-like swellings known as galls that provide both nutrition and protection to the gall-inducer. Although the factors that insects use to induce galls and the mechanisms by which they are formed are not well known, the gall formation process involves phytohormones that regulate the development of gall tissue and the

suppression of plant defenses (Tooker and Helms, 2014). Parasitoids of gall-inducing herbivores may manipulate plants to produce thicker galls that protect them from hyperparasitoids (Fujii et al., 2014; Ronquist et al., 2018). Thus, gall parasitoids could have negative effects on plant performance because thicker galls likely use more plant resources.

Manipulations of plant defenses by NEs could facilitate the transition of NEs to herbivores. The hymenopteran family Cynipidae is exclusively herbivorous, including gall-inducers and inquilines which feed on galls created by other cynipids (Ronquist, 1994). Although these wasps are endophytic herbivores, evidence indicates that they evolved from parasitoids (Ronquist, 1995). The ancestors of cynipids were likely parasitoids of stem-boring or gall-inducing insects. These parasitoids may have developed the ability to facultatively feed on plant tissue, eventually developing into obligate herbivores (Ronquist, 1995). The mechanisms that parasitoids use to manipulate herbivore host physiology could be preadaptations that enabled the manipulation of plant physiology and gall induction (Ronquist, 1995). Acquisition of genes encoding effectors that modulate plant development and suppress plant defenses were likely key developments allowing parasitoids to exploit plants as gall-inducers (Cambier et al., 2019; Hearn et al., 2019). Gall-inducing wasps have also been described in several other hymenopteran lineages (La Salle, 2005; Ranjith et al., 2016), suggesting that transition from parasitoid to herbivore may be common; manipulation of plant defenses by NEs may enable such transitions.

Mistaken identity and specificity of cues

Another possible explanation for evolution of plant responses to NEs is "mistaken identity" where plant recognition of NEs is based on broadly conserved cues that signal the presence of herbivore or pathogen threats (Helms et al., 2019). For example, some of the volatile compounds emitted by EPN-killed cadavers are also produced by bacteria and herbivore-damaged plants (Helms et al., 2019). EPN symbiotic bacteria could also produce volatile or non-volatile cues that are detected by plants directly (Jagdale et al., 2009). EPNs and plant-parasitic nematodes may also share other nematode-associated molecular patterns that elicit plant responses (Choi and Klessig, 2016; Kaplan et al., 2012; Manosalva et al., 2015). Similarly, microbial NEs could be detected by plants on the basis of broadly conserved MAMPs. Some fungal genera (e.g. *Colletotrichum* and *Verticillium*) contain both entomopathogens and plant pathogens (Elliot et al., 2000), indicating that there could be overlap in cues between pathogenic and beneficial fungi.

On the other hand, NEs could possess unique cues that allow plants to differentiate between "bodyguard" and threat. Feeding by zoophytophagous bugs can damage plants and cause economic losses of crops (Castañé et al., 2011), but they also benefit plants by reducing herbivore densities. It may be advantageous for plants to be more tolerant of omnivore feeding (Spence et al., 2007). Ideally, plant responses to these omnivorous insects would strike a balance between reducing plant quality to encourage predation on herbivores (Zhang et al., 2019a), but not repelling them entirely. Plants could differentiate between herbivorous and predaceous bugs on the basis of variation in stylet morphology (Cobben, 1978; Wang et al., 2019) and salivary enzyme profiles (Torres and Boyd, 2009), which have been observed between these groups. Tomato defenses differed in response to feeding by three zoophytophagous mirid species (Pérez-Hedo et al., 2015b), providing evidence that plants could differentiate between even closely related species. However, cotton plants responded similarly to herbivorous and omnivorous thrips species (Spence et al., 2007). More studies are needed to better understand whether plant recognition of NEs is based on cues that are specific to NEs.

Moving forward

To understand whether evolution of plant detection of NEs evolved as adaptive traits for plants or NEs, several key criteria should be addressed: 1) Is there variation in plant responses to NEs or in NE modulation of plant defenses? 2) Are traits heritable? 3) Do NE–plant interactions affect plant or NE fitness? (Karban, 2015). Answering these questions would inform whether there is variation in NE cues or plant responses for natural selection to act upon and whether these interactions impose strong enough selection pressure on plant and/or NE fitness to drive evolution of traits that mediate these interactions. These questions should be evaluated with and without the presence of herbivores and other community members because the evolution of plant–NE interactions can likely not be fully understood in a simple pairwise context (Strauss and Irwin, 2004). Instead, multispecies interactions among herbivores, plants, NEs, and other trophic levels may combine to exert selective pressures on plants via "diffuse coevolution" (Levin et al., 1990).

Section 4: Biological control: challenges, opportunities, and future directions

Biological control and host plant resistance

Invertebrate and microbial NEs are used for biological control of herbivore pest species in crop fields, greenhouses, orchard, and forests worldwide (De Bach and Rosen, 1991; Lacey et al., 2015; van Lenteren, 2012; van Lenteren et al., 2018). Together, biological control and host plant resistance comprise two of the cornerstones of integrated pest management (IPM) programs (Panda and Khush, 1995; Stern et al., 1959). These components can interact synergistically or antagonistically (Bergman and Tingey, 1979; Peterson et al., 2016; Stenberg, 2017). For example, plant defense traits can both positively and negatively affect NE recruitment and success (Hare, 2002), and NEs can slow or accelerate evolution of herbivore resistance to plant defenses if they preferentially attack resistant or susceptible herbivores, respectively (Peterson et al., 2016). Accumulating evidence that NEs can directly and indirectly affect plant defenses (*see Section 1 and 2*) introduces an additional component to interactions between plant resistance and biological control. Better understanding of how plants respond to NEs could be harnessed to maximize biological control and plant resistance in IPM programs.

"Vaccinating" plants

One strategy to improve plant resistance to biotic threats is "vaccination", where plants are exposed to non-pathogenic microbes, minor herbivores, or elicitor applications that induce defense responses (Kessler and Baldwin, 2004; Stenberg, 2017). Induction or priming of systemic defense responses can increase plant resistance to subsequent attack from more damaging herbivores or pathogenic microbes (Kessler and Baldwin, 2004). Zoophytophagous predators, EPNs, and entomopathogenic microbes have also been shown to induce systemic plant defenses and enhance plant resistance to herbivores and pathogens (An et al., 2016; Pappas et al., 2015), suggesting that these NEs could be used to vaccinate plants (Pappas et al., 2016). In effect, NEs may be a better choice of plant vaccine than herbivores or pathogens because they can provide dual benefits to plants through consumption/infection of herbivores along with induction of defenses (Pappas et al., 2016).

Although use of NE vaccinations could benefit most biological control programs, it is likely particularly suited for augmentative strategies. For example, *M. pygmaeus* induced systemic resistance in tomato and pepper plants, and the effects lasted for several weeks, suggesting that early-season inoculative applications of this predator could be used to vaccinate greenhouse plants (Pappas et al., 2015). However, it is important to consider how defense induction affects NEs if the biological control program relies on growth and reproduction of NE

populations. One tactic to mitigate potential negative effects on NEs and improve compatibility of biological control with host-plant resistance could be to select for NEs that are better able to cope with plant defenses (Pappas et al., 2017).

Another approach that could enhance both biological control and plant resistance is NE-mediated plant priming. EPN-killed cadavers applied to roots have been shown to prime plant defenses (Helms et al., 2019) and induce resistance to multiple threats without reducing plant growth (An et al., 2016). Defense priming by NEs could be a promising tool in agriculture for enhancing resistance to subsequent herbivore attack without compromising crop yield or NE performance (Pappas et al., 2017). Identifying NE cues and plant responses involved in priming should be a focal area for future research.

Plant growth–promoting bacteria and fungi

Plant growth–promoting bacteria (PGPB) and fungi (PGPF) are root-colonizing and endophytic microbes that provide multiple benefits to plants by directly inhibiting pathogens, improving nutrient uptake, modulating plant hormone levels, and inducing systemic resistance to pathogens and herbivores (Bent, 2006; Glick, 2012; Hossain et al., 2017). Numerous studies have demonstrated that some entomopathogenic fungi also have PGPF effects, highlighting the multi-functional roles these NEs could perform in agricultural applications (Dara, 2019; Jaber and Ownley, 2018; Vega et al., 2009). Currently, few studies have identified bacteria with both entomopathogenic and PGPB effects (Qi et al., 2016), but this is likely to be a productive area for future research (Azizoglu, 2019). Compared with typical applications of entomopathogens as topical sprays, use of plant-colonizing entomopathogens could provide other advantages such as protection from damaging environmental conditions on plant surfaces (e.g. UV radiation, rainfall), control of cryptic herbivores, and ongoing plant protection without the need to synchronize applications to pest populations (Jaber and Ownley, 2018). Currently, most entomopathogens are applied as inundative biological controls, providing immediate action against herbivores without relying on successive generations (Lacey et al., 2001, 2015). However, because plant-colonizing entomopathogens can propagate in plants and be vertically transmitted (Lefort et al., 2016; Quesada-Moraga et al., 2014), there is potential for microbial NEs to be used in biological control programs that rely on their growth and reproduction such as in inoculative and classical strategies.

Despite numerous potential benefits of PGPF and PGPB, a major challenge in implementing wide-scale use is inconsistency of results across systems (Ahemad and Kibret, 2014; Hossain et al., 2017). Identifying fungi and bacteria that survive in different soil types and environments and are able to colonize multiple plant species is likely to enhance commercial use of these microbial applications (Glick, 2012). Selecting strains with high virulence against one or multiple pests is an additional consideration for entomopathogenic plant growth–promoting microbes (Jaber and Ownley, 2018). Other characteristics such as plant- and tissue- specific colonization, persistence in plant tissues, and interactions with other plant-colonizing microbes will also influence the degree of protection against herbivores conferred by these microbes (Jaber and Ownley, 2018).

Integrating NE modulation of plant responses into IPM

Optimizing use of NEs for regulating plant defenses will depend on the ecology of the crop system and presence of pest and other NE species (Pappas et al., 2017; Peterson et al., 2016). NE species may complement each other, acting additively or synergistically to provide top-down control of pests. But NE species can also negatively affect each other through intraguild predation, facultative hyperparasitism, competition, or reduction of host quality (Letourneau et al., 2009), and as I discuss in *Section 2*, through modulation of plant defenses. In a comparative study of tomato responses to feeding by zoophytophagous mirid bugs, plant volatiles induced by *Nesidiocoris tenuis, M. pygmaeus* and *Dicyphus maroccanus* were more attractive to another beneficial organism, the parasitoid *E. formosa* (Pérez-Hedo et al., 2015b). Although attraction of other NEs could benefit plants, these volatiles may represent "dishonest" signals, and parasitoids could learn to ignore plant volatiles if they are not associated with a suitable host (Pappas et al., 2017). In the same study, *M. pygmaeus*- and *D. maroccanus*-induced tomato plants were also more attractive to the herbivore pest *Tuta absoluta* than undamaged plants, whereas *M. pygmaeus*-induced plants repelled this herbivore (Pérez-Hedo et al., 2015b). This research highlights the importance of considering multiple members of ecosystems when evaluating plant responses to NEs and suggests that some NEs could be more effective than others.

An obvious goal of plant resistance in IPM is achieving resistance to multiple plant pests. Entomopathogenic fungi and EPNs have been shown to induce SA-mediated defenses (Helms et al., 2019), whereas feeding by zoophytophagous predators induced JA- and abscisic acid–

mediated defenses (Pérez-Hedo et al., 2015b). Although SA- and JA-mediated defenses can be antagonistic, other phytohormones such as ethylene can act synergistically with JA (Erb et al., 2012). Moreover, other hormones such as auxins, cytokinins, gibberellins, and abscisic acid play important roles in modulating plant responses to herbivory and pathogen infection (Erb et al., 2012). Several studies have shown that NE-mediated modulation of plant defenses can provide protection against both pathogens and herbivores (Ahmad et al., 2020; An et al., 2016), suggesting that a single NE could induce broad spectrum resistance. Another approach to induce resistance to multiple threats could be simultaneous application of multiple NEs to vaccinate plants, although I am not aware of any studies that have investigated this to date. It would also be of interest to examine potential interactions between above- and belowground NEs and implications for plant resistance to biotic threats in both spheres.

Although diet flexibility of omnivorous NEs is one reason they are effective biological control agents because they can survive when prey are scarce, it also means that they walk a fine line between being beneficial organisms or plant pests (Dumont et al., 2018). Under certain circumstances, plant feeding by zoophytophagous bugs can cause economically significant crop damage (Castañé et al., 2011). In another example, the multicolored Asian lady beetle (*Harmonia axyridis*), a generalist predator, was introduced to North America for biocontrol of aphids and other herbivores (Koch, 2003) In addition to predation of non-target arthropods, *H. axyridis* has been reported to feed on fruits such as apples, peaches, pumpkins, and is a particular problem in grape production (Koch and Galvan, 2008). Interestingly, *H. axyridis* is not known to feed on fruits in its native range (Koch and Galvan, 2008). Little is known about the reasons *H. axyridis* is able to exploit fruit in North America (Koch and Galvan, 2008), but modulation of plant defenses by this NE could play a role. Frugivory by the herbivorous bug *Halyomorpha halys* has been shown to induce production of defense compounds in fruit and repel further feeding by conspecifics (Zhou et al., 2016), and plant defenses can be important for regulating the degree of phytophagy or carnivory in omnivorous insects (Agrawal and Klein, 2000). Exotic NEs may be able to tolerate or modulate defenses of native plants in novel ways, potentially allowing them to exploit plant resources and become pests. This example indicates that plant–NE interactions could be an important consideration in host testing to minimize non-target effects of biological control agents (Babendreier et al., 2005).

Parasitoids are often used in biological control programs for the important ecosystem services they provide in regulating herbivore populations. Conversely, hyperparasitoids can provide "disservices" by disrupting top-down control (Nenzén et al., 2018), and negative impacts of these fourth-trophic-level organisms have been documented in conservation, classical, and inoculative biological control programs (Tougeron and Tena, 2019). Because hyperparasitoids do not interfere with initial parasitization success, they are less likely to be a concern in inundative control efforts where NEs provide immediate suppression of pests, without the goal of NE reproduction (Eilenberg et al., 2001). Given that parasitoids can indirectly modulate plant defenses via their hosts, leading to emission of plant volatiles that attract hyperparasitoids (Poelman et al., 2012; Zhu et al., 2015), improved understanding of the volatile compounds that mediate hyperparasitoid behavior could be used to develop chemical repellents or lures for hyperparasitoids akin to push–pull methods for herbivore control (Tougeron and Tena, 2019). Parasitoids could also be chosen for biological control programs on the basis of their host-mediated effects on plant defenses and apparency to hyperparasitoids (Cusumano et al., 2019). Parasitoid/PDV traits that mediate plant responses could potentially be targeted to develop parasitoid lines that are less apparent to hyperparasitoids.

Overall, little is known about the molecular mechanisms governing NE-mediated induction and priming of plant defenses. Many studies have measured increased levels of plant hormones, transcription of defense genes, expression of defense proteins, and emission of volatiles in response to NEs, and some of these phenotypes were associated with improved plant resistance to pests (Table 1.1). Strategies have been proposed to breed or genetically modify plants to express traits that better support or attract NEs such as enhanced production of certain volatile attractants (Cortesero et al., 2000; Degenhardt et al., 2003; Pappas et al., 2017; Rasmann et al., 2005). More research on NE-induced defense compounds could identify new volatiles or blends of volatiles that repel pests or attract beneficial insects. This information could help inform development of products or facilitate breeding of plants with greater production of ecologically important compounds (Bouagga et al., 2018).

Before plants mount downstream defense responses, they detect and respond to NE cues (*see Section 1*). Plant defense responses to herbivores and microbes can be highly variable depending on species and genotype (Bruce, 2014). Work in predaceous mirid bug and parasitoid systems indicates that plant responses depend on plant and NE identity (Pérez-Hedo et al.,

2015b; Poelman et al., 2011b; Tan, 2019), although more comparative studies are needed to examine intraspecific variability in plant responses to NEs. If plants exhibit variability in these traits, plant varieties could be bred that respond more strongly or specifically to NE cues. However, as other authors have acknowledged, a challenge in traditional plant breeding of resistant plants is the complex genetic nature of plant resistance (Stenberg et al., 2015; Stout and Davis, 2009). Moreover, artificial selection for traits such as yield and taste in the domestication of crops has led to a loss of diversity in resistance traits and vastly altered tritrophic interactions (Chen et al., 2015). More studies of NE-modulation of plant defenses in natural systems and wild relatives of crop plants will be important to furthering our understanding of plant–NE interactions. This knowledge could eventually be used to reintroduce plant defense traits back into crop plants through breeding efforts (Chen et al., 2015).

Another opportunity to integrate biological control and plant resistance could be targeting cues that NEs use to modulate plant defenses. Few studies have specifically identified NE effectors and elicitors involved in these interactions (Table 1.1), and whether NEs possess unique cues or share them with herbivores and pathogens is still largely unexplored (see *Section 1 and 3*). Previous authors have suggested manipulating traits (e.g. fecundity, sex ratio, aggressiveness, responsiveness to plant volatiles) of NEs to make them more effective biological control agents (Dumont et al., 2018; Hiltpold et al., 2010; Hopper et al., 1993). If expression of NE elicitors and effectors are selectable traits, NEs could be bred to induce or prime plant defenses in ways that improve plant resistance or facilitate NE fitness. 'Omics technologies (e.g. proteomics, transcriptomics, genomics) may facilitate discovery of effectors and elicitors, and CRISPR could be used to confirm the role of specific genes in mediating NE–plant interactions or introduce specific genes to improve NEs (Basu et al., 2017). Identification of elicitors could also be used in applications of synthetic elicitors for crop protection (Bektas and Eulgem, 2015).

A further consideration for use of NEs in modulating plant defenses is crop yield. Although several studies have measured various plant fitness parameters such as seed production, flowering time, number of flowers, and plant biomass (An et al., 2016; Tan et al., 2020; Zhang et al., 2019a), it is not clear whether these traits were desirable from a crop production perspective. Growers are likely to favor improvements to crop yield and aesthetics, which may not necessarily correlate with plant fitness measures (Pappas et al., 2017). Variability between systems and geographical locations means that making global recommendations for

implementation of NE modulation of plant defenses in IPM is likely to be a challenge (Peterson et al., 2016). More field studies are needed to understand how NE modulation of plant defenses affects pest pressure and crop yield in realistic settings and comparative studies on different crops will be required to make variety-specific recommendations (Stout and Davis, 2009).

Conclusions

NEs are typically appreciated as vital components of ecosystems and biological control programs for their role in regulating herbivore populations. Recent studies expand this view, identifying complex ways that NEs influence ecosystems through regulation of plant defenses. Many questions about the cues and evolution of these interactions still remain. Future studies that compare plant responses to related species in different feeding guilds (e.g. herbivorous, carnivorous, zoophytophagous) or specialists vs. generalists will likely shed light on evolution of plant–NE interactions and help identify whether they represent mutualisms, manipulations, or cases of “mistaken identity”. Identification of NE cues and genes involved in modulating plant defenses will also be important for advancing this field of study, likely aided by CRISPR and ‘omics tools. More studies investigating the effects of NE-modulated defenses on both plant and NE fitness over multiple generations will also be key to understanding the evolutionary nature of these interactions. Additionally, measuring how effects on plant defenses and fitness translate to crop yield will be necessary for implementing this information into IPM programs and making recommendations to growers. Future studies should consider the importance of crop and context specificity, because the effects of NEs on plant performance are likely to depend on plant species, environmental conditions, and herbivore and pathogen pressures.

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Chapter 2

Host plant and population source drive diversity of microbial gut communities in two polyphagous insects

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Abstract

Symbioses between insects and microbes are ubiquitous, but vary greatly in terms of function, transmission mechanism, and location in the insect. Lepidoptera (butterflies and moths) are one of the largest and most economically important insect orders; yet, in many cases, the ecology and functions of their gut microbiomes are unresolved. I used high-throughput sequencing to determine factors that influence gut microbiomes of field-collected fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*). Fall armyworm midgut bacterial communities differed from those of corn earworm collected from the same host plant species at the same site. Corn earworm bacterial communities also differed between collection sites. Subsequent experiments using fall armyworm evaluating the influence of egg source and diet indicated that that host plant had a greater impact on gut communities. I also observed differences between regurgitant (foregut) and midgut bacterial communities of the same insect host, suggesting differential colonization between gut regions. These findings indicate that host plant is a major driver shaping gut microbiota, but differences in insect physiology, gut region, and local factors can also contribute to variation in microbiomes. Additional studies are needed to assess the mechanisms that affect variation in insect microbiomes, as well as the ecological implications of this variability in caterpillars.

Introduction

Insect herbivores inhabit a diverse set of niches, and therefore face a wide variety of challenges such as nutritionally recalcitrant food sources, toxins, environmental extremes, and threats from parasites and pathogens. Insects have integrative strategies to contend with these challenges, which often include forming symbiotic associations with microbes (Douglas, 2015). Microbial associations are ubiquitous among animals, but vary along functional and ecological

continua (Pontes and Dale, 2006). Compared to endosymbiotic bacteria, the evolutionary trajectories and transmission strategies of facultative gut symbionts are far more variable (Douglas, 2015; Salem et al., 2015). Extracellular symbionts can be obtained through environmental sources (Kikuchi et al., 2007), shared food resources (Caspi-Fluger et al., 2012; Gonella et al., 2012), trophallaxis (Salem et al., 2015), deposition on egg surfaces, and copulation (Gonella et al., 2012).

The insect gut can be rich in microbial symbionts, and the associations, locations, and functions of these associates vary considerably. Some insects possess special gut modifications or structures such as paunches, diverticula, and caeca to house symbionts (Dillon and Dillon, 2004), while others lack such modifications. The roles of insect gut symbionts are diverse. For example, insect gut symbionts can be involved in metabolism of recalcitrant food sources (Kaufman and Klug, 1991; Salem et al., 2017), provisioning of vitamins (Salem et al., 2014) and nutrients (Hu et al., 2018; Scully et al., 2014), and metabolism of plant allelochemicals (Douglas, 2015; Mason et al., 2014; Pontes and Dale, 2006). Oral bacteria found in the regurgitant can also be involved in manipulating plant responses to herbivore feeding, thereby suppressing induction of plant defenses and leading to increases in herbivore fitness (Chung et al., 2013; Wang et al., 2016).

Lepidoptera (butterflies and moths) is the third largest insect order with over 200,000 described species. Larval lepidopteran guts are characterized by a simple, tube-like morphology that facilitates the rapid transit of food associated with high consumption rates (Dow, 1987). These insect guts represent extreme environments for microorganisms due to their high alkalinity ($pH > 10$) (Appel and Martin, 1990; Harrison, 2001; Johnson and Felton, 1996).

Commonly, caterpillar gut microbiomes are simple and variable, usually being comprised of relatively few dominant taxa (Broderick et al., 2004; Chen et al., 2016; Tang et al., 2012; Xiang et al., 2006), and appear to be predominantly shaped by dietary and environmental sources (Chaturvedi et al., 2017; Mason and Raffa, 2014; Xiang et al., 2006). Bacterial communities have also been described from lepidopteran eggs (Chen et al., 2016; Staudacher et al., 2016), suggesting there is potential for maternal transmission of microbiota. The roles of lepidopteran oral and gut bacteria in facilitating plant-insect interactions remain unclear, but they likely have facultative functions, particularly in relation to plant defenses (Acevedo et al., 2017; Mason et

al., 2014). The sources of gut microbiota in lepidopterans has received little attention; understanding the factors that influence bacterial community composition may shed light upon symbiont-host co-adaptation and the strategies insects use to acquire their microbial partners.

The lepidopteran family Noctuidae includes many polyphagous agricultural pests that can cause significant economic losses. *Spodoptera frugiperda* (fall armyworm) is a highly polyphagous noctuid that is a major agricultural pest in South America, the Caribbean, and most recently in Africa (Goergen et al., 2016; Stokstad, 2017). In the northern United States and Canada, sporadic fall armyworm infestations occur from populations that annually migrate from southern Texas and Florida as this species cannot overwinter in the cooler northern climates (Nagoshi et al., 2012). Although fall armyworm develops faster and prefers grasses such as maize and wheat, it can also complete development on broadleaf crops such as soybean and cotton (Silva et al., 2017). Corn earworm (*Helicoverpa zea*) is another agricultural noctuid pest that is distributed throughout the American continent (Bentivenha et al., 2016). Its major host plant is maize, although it will also feed on other crops such as tomato, cotton and soybean (Reisig et al., 2017).

Despite the economic importance of corn earworm and fall armyworm, little is known about the composition of their midgut and oral bacteria and how these communities are shaped by transmission from eggs and host plant feeding. I used bacterial 16S-rRNA sequencing to determine if there were differences in gut communities from different population and/or host plant sources. Specifically, my objectives were to: i) characterize and compare the midgut bacterial communities of wild-collected fall armyworm and corn earworm from different locations in Pennsylvania, ii) determine whether egg source (wild-collected or laboratory-reared) or diet source (soybean leaves or corn silk) were more important in shaping fall armyworm midgut bacterial communities, and iii) compare bacterial communities present in the regurgitant with those of the midgut in fall armyworm. This latter objective is of interest because specific bacteria from oral secretions (regurgitant) of lepidopterans have been shown to influence plant defenses (Acevedo et al., 2017), but whether these bacteria are a subset of those found in the midgut or comprise a unique community is unknown. To address these objectives, I used culture-independent high throughput sequencing methods. I also used traditional culture-based and 16S sequencing methods to examine bacteria present in the regurgitant of fall armyworm collected from Pennsylvania and Puerto Rico.

Methods

Insect and plant sources

Plant and insect material were collected from two sites in Centre County, PA in September 2016: a local farm in State College, PA (HF) and the Pennsylvania State University Russell E. Larson Research Farm (RS). These two sites are separated by approximately 11 km. I obtained fall armyworm from HF, and corn earworm from HF and RS. Insects were collected from the ears of sweet corn (*Zea mays*) (Appendix A). At the time of insect collection, I also collected soybean (*Glycine max*) foliage and maize silk and stored them at 4 °C. Fall armyworm from HF were used for culture-based sequencing of bacteria in regurgitant and bacterial community analysis of regurgitant and midguts. Corn earworm collected from HF and RS were used for bacterial community analysis of midguts.

Fall armyworm larvae were also collected from three sites in southern Puerto Rico in February 2017, which included two local farms (PRL and PRS) and the Juana Diez Experimental Research Station (PRJ) (Appendix A). Fall armyworm from the Puerto Rico sites were used for culture-based analysis of regurgitant. Upon removal from the field, larvae were placed in individual plastic cups with maize silk from the plant they were collected from for food, until sample collection. For larvae from Site HF and RS, regurgitant and midgut samples were collected the following day. For larvae collected in Puerto Rico, due to shipping time back to the United States, regurgitant was collected three days after larval collection.

A laboratory colony of fall armyworm was obtained from Benzon Research (Carlisle, PA), and maintained at Pennsylvania State University. To determine the contributions of host plant and egg source on gut bacterial composition in fall armyworm I used egg masses produced by 1) the Benzon laboratory colony and 2) field-collected fall armyworm. The field-collected eggs were produced by the moths that developed from caterpillars originally collected from Site HF on September 7, 2016. These caterpillars were final instars when collected and were fed exclusively on maize silk collected from the same site until they pupated, with the aim of maintaining their natural gut microbiota. Upon hatching, neonate larvae were placed in individual cups with either soybean leaves (FS Hisoy HS33A14-98SB132B) or maize silk (var. Providence), from plant material collected from Site RS. Larvae were fed *ad libitum* and leaves

were replaced every 2–3 days until larvae reached the final instar. Regurgitant and midgut collections were conducted on the second day of the final instar.

Culture-based sequencing of regurgitant bacteria

Regurgitant was collected by gently squeezing larvae with soft forceps until they regurgitated. The regurgitation droplet was collected directly from the caterpillar's oral cavity using a sterile pipette tip (Acevedo et al., 2017). One µl of regurgitant was diluted with sterile Milli-Q water. Serial dilutions were plated on 2xYT media and the number of colony-forming units (CFUs) per ml of regurgitant was quantified. Colonies that displayed unique morphology were subcultured and these pure cultures were stored as glycerol stocks at -80 °C. Pure cultures were prepared from glycerol stocks on solid media for 48 h. DNA was extracted using the CTAB protocol (Chen and Kuo, 1993) and PCR was performed on extracted DNA using the 16S primers 27F (5' AGAGTTTGATYMTGGCTCAG 3') and 1392R (5' ACGGGCGGTGTGTRC 3') with GoTaq® Green Master Mix (Promega, Madison, WI, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min; followed by 30 cycles of 95 °C for 1 min, 50 °C for 45 s, 72 °C for 1 min; then 72 °C for 5 min. PCR products were purified using Exo-SAP-it (Affymetrix. Santa Clara, CA) following manufacturer's instructions. Sanger Sequencing of purified DNA products was performed at the Penn State Genomics Core Facility, University Park, PA.

Contigs for bacterial sequences were assembled and trimmed using SeqMan Pro (DNASTAR, Madison, WI). Consensus sequences were used to search the Ribosomal Database Project database for bacterial type strains with similar (0.80-1.00 match) sequences (Cole et al., 2014; Wang et al., 2007). The Muscle algorithm within MEGA7 software (Kumar et al., 2016) was used to align the bacterial type strains and samples. The aligned sequences were then used to construct an unrooted phylogenetic tree using the Maximum Likelihood method based on the Jukes-Cantor (Jukes and Cantor, 1969) model in MEGA7.

Sample collection and DNA extraction

Regurgitant was collected from larvae as described above. Approximately 20 µl of regurgitant was collected from each larva and stored at -80 °C until needed. After regurgitant collection, caterpillars were starved for 2–3 h, surface sterilized in 10% Coverage Plus NPD (Steris, Mentor, OH, USA), and rinsed twice in sterile molecular grade water. Midguts were

dissected under sterile conditions and stored at –80 °C until DNA extraction. DNA extractions were performed using the Quick-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions.

Generation and sequencing of 16S amplicons

Primers used for bacterial V4 16S-rRNA amplification were 515F and 806R (Caporaso et al., 2012). Amplicons were generated in 25 µL volumes using Phusion Hi-Fidelity Polymerase (New England BioLabs, Ipswich, MA, USA) containing 0.5 µM of forward and reverse primers and 25 ng of template DNA. Reaction conditions for 16S amplification were: 94 °C 3 min, 30 cycles of 94 °C for 45 sec, 50 °C for 60 sec, and 72 °C for 90 sec, followed by a final extension of 72 °C for 10 min. Indices and Illumina sequencing adapters were added to amplicon pools with 5 additional cycles of PCR. Barcoded products were pooled and sequenced on an Illumina MiSeq using 2×300 bp paired end reads. Generation of amplicon pools and sequencing of products was completed by the PSU Hershey Genomics Facility.

Processing of sequencing data

Bacterial amplicons were processed and analyzed using mothur v. 1.37 (Schloss et al., 2009). The recommended workflow was modified such that the mothur command 'pcr.seqs' was implemented with pdiffs = 2 after the command 'make.contigs' to remove primer sequences from the reads. Bacterial operational taxonomic units (OTUs) were picked at 97% similarity and used for subsequent 3000 analyses. I used mothur to conduct OTU subsampling to OTUs. Taxonomies were determined using a mothur-formatted version of the Ribosomal Database Project (v. 9). Prior to subsequent analysis, samples were evaluated against a negative sequencing control, and two OTUs were removed from subsequent analyses.

Statistical analyses

Analyses of the bacterial communities were conducted using non-metric multidimensional scaling (nMDS) ordination and permutation-based multivariate analysis of variance (PerMANOVA). Heat maps were generated using the 20 most abundant OTUs by performing a $\log_2$ [x] transformation. I used Bray-Curtis dissimilarities generated from standardized data that incorporated relative abundance of OTUs to assess community structure; Jaccard dissimilarities were generated using subsampled data incorporating presence/absence to assess composition. PerMANOVA and nMDS was conducted in PRIMER-E (v. 7.0) using these

dissimilarities. PERMANOVA was also run in PRIMER-E using 999 iterations of the model. First, I assessed if there were differences between corn earworm and fall armyworm collected from the field using a one-way analysis. Then, I evaluated if insect egg source and plant sources have impacts on fall armyworm gut communities using a two-way analysis. Finally, I determined if there were differences between gut and regurgitant communities, and if there was an impact of host plant using a two-way analysis. Following this last test, I used similarity percentages (SIMPER) analysis to identify the OTUs that drive the differences, and then conducted non-parametric Wilcoxon rank sum analysis on the relative abundances.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Short read sequences have been submitted to NCBI SRA under accession number PRJNA507591. Sanger sequences of individual bacterial isolates have been submitted to NCBI Genbank under submission number SUB4856027.

Results

Comparisons of midgut microbiota between fall armyworm and corn earworm

There were significant differences in the bacterial communities inhabiting the midguts of fall armyworm and corn earworm (Figure 2.1a,b; Figure 2.2). Multivariate analyses revealed significant differences between fall armyworm and corn earworm microbiota based on Bray-Curtis (Figure 2.1a; PerMANOVA $p < 0.001$) and Jaccard (Figure 2.1b; PerMANOVA $p < 0.001$) similarity indices. In addition to the differences between these two species collected at the same location, corn earworm collected from different locations also exhibited divergent communities (Figure 2.1). I found substantial differences in the bacterial midgut communities of corn earworm obtained from the two sites in Pennsylvania, despite the fact they were collected from the same host plant species. Alpha diversity metrics also differed between lepidopteran species, with fall armyworm exhibiting marginally significantly lower Simpson ($p = 0.053$) and Shannon ($p = 0.033$) metrics than corn earworm (Figure 2.2; Table 2.1). This suggests that corn earworm midgut microbiota exhibited greater levels of diversity and greater evenness.

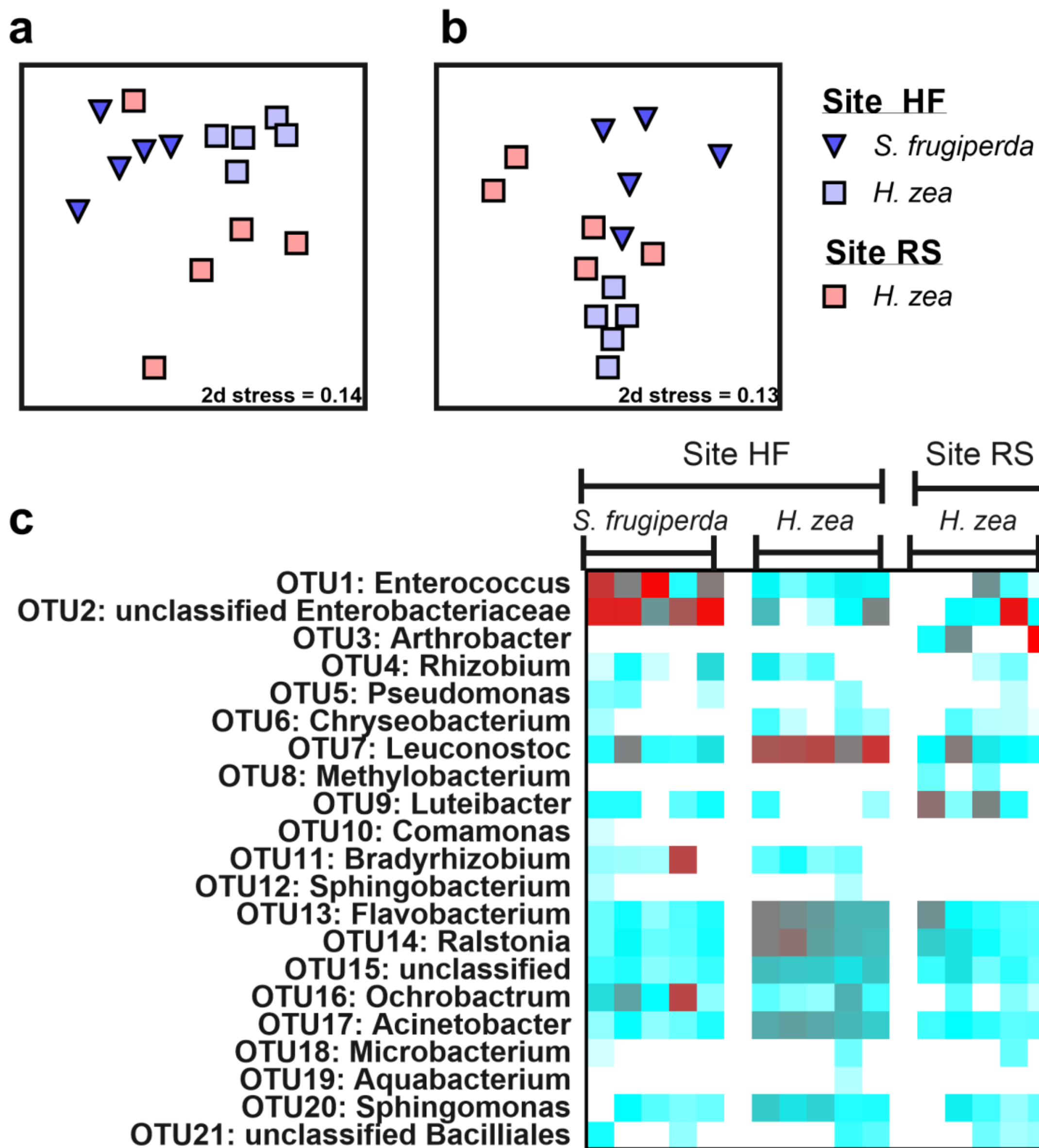


Figure 2.1: Differences between fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*) midgut bacterial communities collected from sweet corn ears in Pennsylvania. Corn earworm was collected from Site HF (Harner Farm) and Site RS (Rock Springs), while fall armyworm was collected only from Site HF. Non-metric multidimensional scaling (nMDS) plots were constructed using Bray-Curtis (A) and Jaccard (B) dissimilarities. Stress values indicate a good fit in two dimensions. Heat maps (C) show relative abundance ($\log_2$ transformed) of OTUs (operational taxonomic units). Red boxes indicate greater relative abundances, blue boxes correspond to lower values, and white boxes correspond to no detection of that particular OTU.

Heat maps of midgut microbiota indicated that conspecific individuals exhibited a high degree of variability in terms of both membership and abundance (Figure 2.1c). Among the most enriched OTUs (operational taxonomic units), fall armyworm had the greatest relative abundances of *Enterococcus* and unclassified Enterobacteriaceae. Corn earworm from the HF population had lower relative abundances of *Enterococcus* and Enterobacteriaceae, but were enriched for *Leuconostoc*. Corn earworm from the RF site were highly variable as to which OTUs were present in high relative abundances, and had fewer *Leuconostoc* than corn earworm from the HF site.

Table 2.1: Comparisons between alpha diversity of field-collected fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*) Numbers represent means (std. errors).

	S. frugiperda	*H. zea*	t-value	p-value
OTUs	85.4 (10.6)	190.2 (77.5)	1.15	0.281
1 / Simpson	2.23 (0.2)	10.7 (6.2)	2.26	0.053
Shannon	1.4 (0.2)	2.7 (0.8)	2.45	0.033

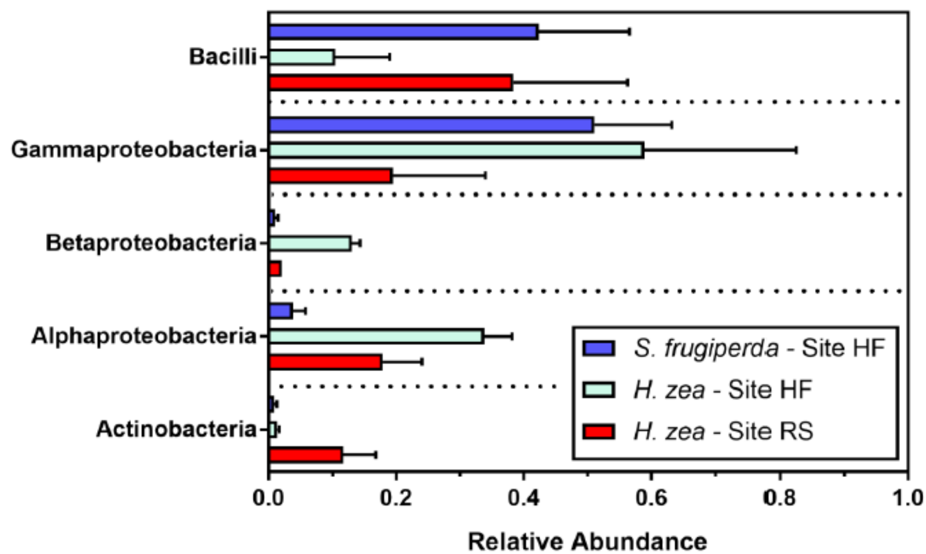


Figure 2.2: Relative abundance of the most common bacterial orders present in fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa* zea) midguts (> 95% of the abundances). Bars represent means with standard errors.

Influence of egg population source and plant diet on fall armyworm midgut bacterial communities

Multivariate analyses revealed there was a significant impact of host plant on midgut bacterial communities in terms of both Bray-Curtis (Figure 2.3a) and Jaccard (Figure 2.3b) similarities (Table 2.2). There was no impact of egg population source or its interaction with host plant on midgut bacterial communities (Figure 2.3a, b; Table 2.2). At both the order and individual OTU levels, there was substantial variation in abundance of bacterial taxa between individual fall armyworm larvae within treatments (Figure 2.3c; Figure 2.4). In general, larvae feeding on maize had high relative abundances of OTUs corresponding to unclassified Enterobacteriaceae, *Rhizobium,* and *Chryseobacterium*. Soybean-fed larvae generally had higher relative abundances of *Enterococcus,* unclassified Enterobacteriaceae, *Chryseobacterium*, and *Pseudomonas* (Figure 2.3c).

Alpha-diversity metrics indicated that, in general, gut bacterial communities of larvae fed on soybean were more diverse than those fed on maize (Figure 2.5, Table 2.3). Midguts from insects reared from field-collected and laboratory (Benzon) egg sources generally had the same levels of OTU richness. However, maize-fed larvae from the field population had higher bacterial gut diversity than those from the laboratory population (Figure 2.5).

Differences in fall armyworm bacterial communities in midgut and regurgitant

I collected paired samples of fall armyworm regurgitant and midguts to determine if there were differences between the microbiota that reside in the foregut versus the midgut. There were substantial differences in the communities present, and like in prior analyses, there was a major impact of host plant on the midgut bacterial communities using both Bray-Curtis (Figure 2.6a) and Jaccard (Figure 2.6b) similarities (Table 2.4). In addition to these differences between host plants, there was a substantial impact of whether the community of origin was from the midgut or regurgitant (Table 2.4; $p = 0.013$). However, I observed no interaction between the host plant and the physical origin of the bacterial community. These trends were consistent for both Bray-Curtis and Jaccard dissimilarities.

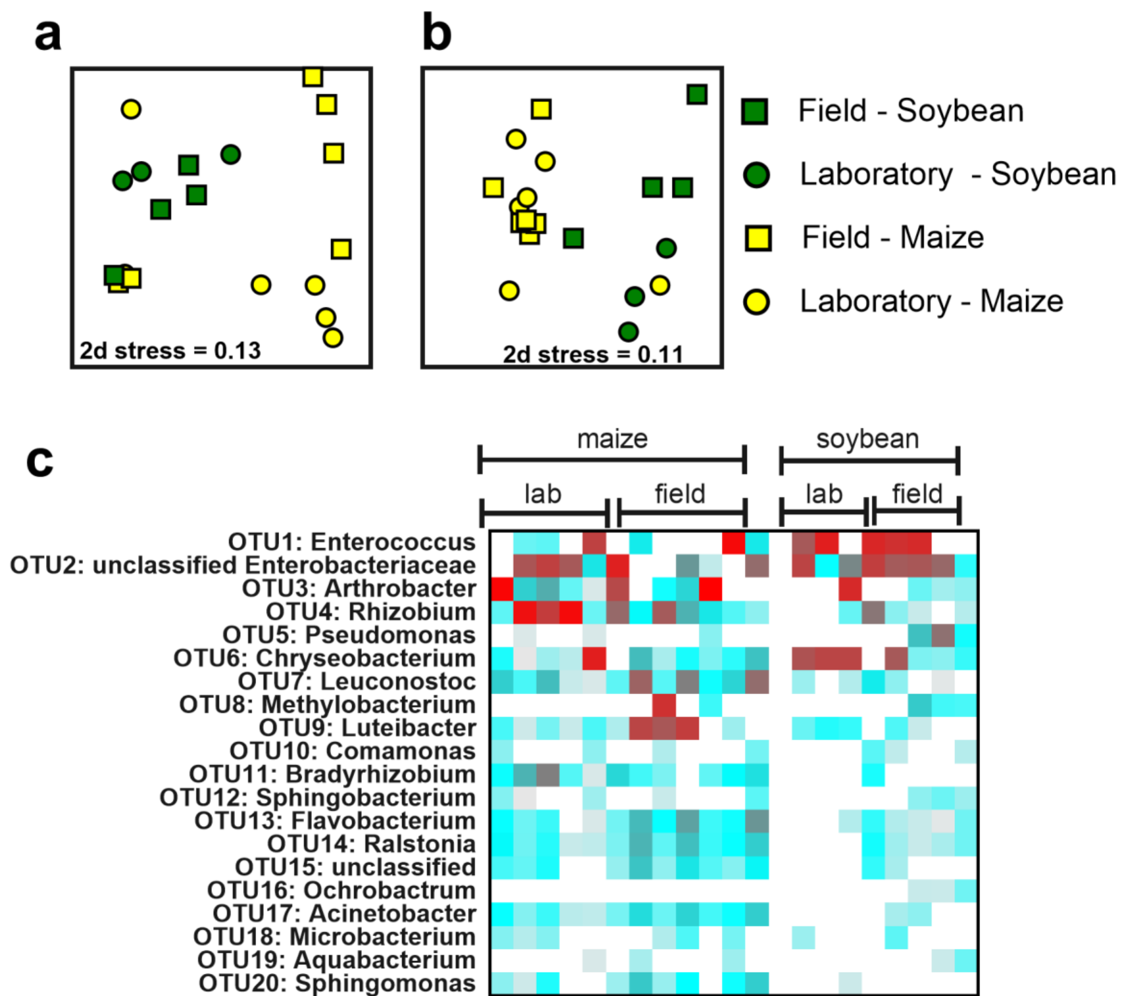


Figure 2.3 Influence of diet (maize or soybean) and egg population source (field-collected or laboratory) on fall armyworm (*Spodoptera frugiperda*) midgut bacterial communities. Non-metric multidimensional scaling (nMDS) plots were constructed using Bray-Curtis (A) and Jaccard (B) dissimilarities. Stress values indicate a good fit in two dimensions. Heat maps (C) show relative abundance (log2 transformed) of OTUs (operational taxonomic units). Red boxes indicate greater relative abundances, blue boxes correspond to lower values, and white boxes correspond to no detection of that particular OTU.

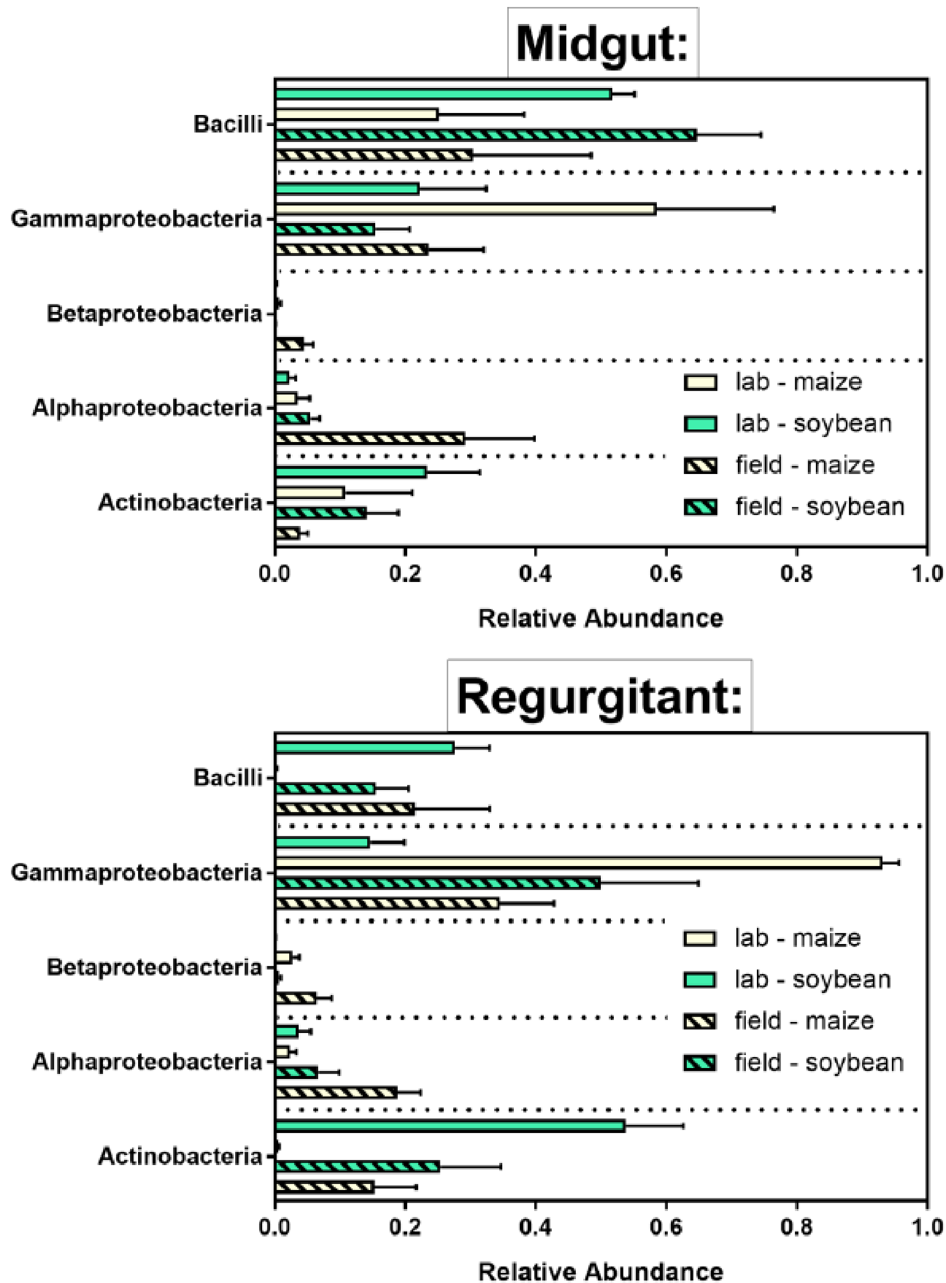


Figure 2.4: Relative abundance of the most common bacterial orders present in fall armyworm midguts (top) and regurgitant (bottom) (> 95% of the abundances). Different colors represent different host plants (soybean or maize). Open bars indicate lab populations; hatched bars indicate field populations. Bars represent means with standard errors

Table 2.2: PERMANOVA output assessing differences between fall armyworm (*Spodoptera frugiperda*) midgut bacterial communities between egg population source (field or laboratory) and host plant (maize or soybean). PERMANOVAs were generated using 999 permutations, and the individual insect was included in the model as a random effect. Bold values indicate significant (p<0.05) differences.

Source	df	SS	MS	Pseudo-F	p-value
Bray-Curtis Similarity					
Plant	1, 19	8057	8057	4.15	**0.029**
Population	1, 19	3407	3407	1.75	0.156
Plant*Population	1, 19	1447	1447	0.74	0.512
Jaccard Similarity					
Plant	1, 19	8025	8025	2.07	**0.002**
Population	1, 19	4381	4381	1.13	0.181
Plant*Population	1, 19	4167	4167	1.07	0.297

Table 2.3: ANOVA of midgut and regurgitant alpha diversity in fall armyworm (*Spodoptera frugiperda*)

		Plant		Egg source		Interaction	
		F-value	P-value	F-value	P-value	F-value	P-value
Gut :	OTUs	8.83	**0.009**	1.59	0.226	0.107	0.748
	1 / Simpson	1.25	0.282	3.97	0.065	2.3	0.15
	Shannon	2.45	0.155	2.98	0.105	1.87	0.192
Regurgitant:	OTUs	4.73	**0.053**	6.23	**0.025**	0.032	0.86
	1 / Simpson	0.26	0.642	11.2	**0.004**	3.46	0.083
	Shannon	1.09	0.313	11.5	**0.004**	5.93	**0.028**

Figure 2.5: Alpha diversity indices of midgut (a–c) and regurgitant (d–f) bacterial communities in fall armyworm (*Spodoptera frugiperda*).

Using Bray-Curtis similarities, I conducted a two-way SIMPER analysis to identify the major OTUs that contributed to the differences between host plant and regurgitant, and then conducted paired statistical tests. While host plant had a significant impact on the relative abundance of several of the OTUs that inhabited the midgut and regurgitant (Table 2.5), the effects of regurgitant were far more limited. Only *Enterococcus* exhibited significant differences between midgut and regurgitant from fall armyworm feeding on maize ($p = 0.045$) and soybean ($p = 0.001$).

<u>16S sequencing of individual isolates from Puerto Rico and Pennsylvania</u>

Culture-dependent sequencing of bacteria isolated from the regurgitant of fall armyworm collected in both Puerto Rico and Pennsylvania support the findings of the community analysis data reported above. I found that bacterial taxa in the Enterobacteriaceae, Pseudomonadaceae and Enterococcaceae and Microbacteriaceae were commonly identified based on sequencing of the V4 region of the bacterial 16S gene (Figure 2.7). Common bacterial genera that were isolated include *Pantoea* (Figure 2.7, clade ii), *Klebsiella* (Figure 2.7, clade iii), *Enterobacter* (Figure 2.7, clade iv), *Pseudomonas* (Figure 2.7, clade v), *Ochrobactrum* (Figure 2.7, clade vii), *Enterococcus* (Figure 2.7, clade viii), *Mycetocola* (Figure 2.7, clade x) and *Curtobacterium* (Figure 2.7, clade xi). In some cases, the first six of these genera were detected in larvae collected in both Pennsylvania and Puerto Rico.

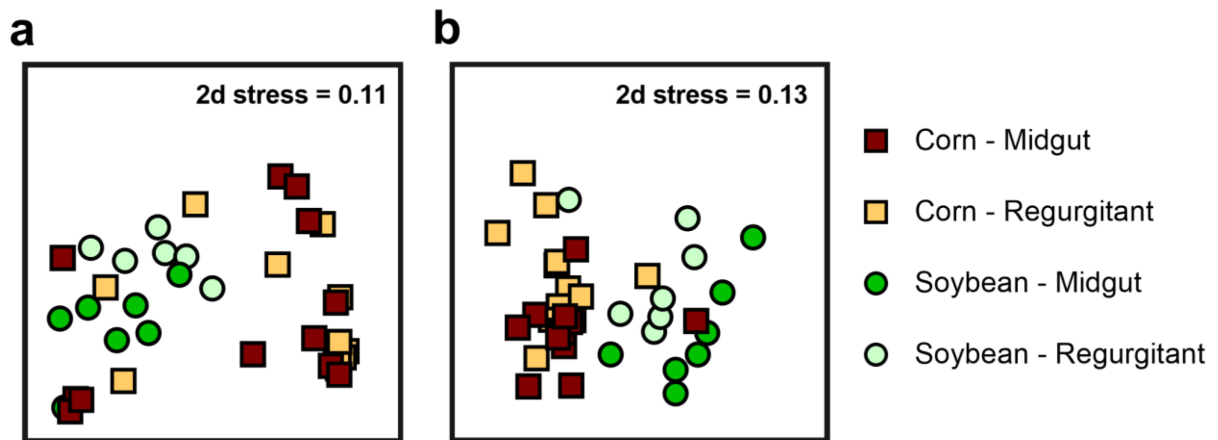


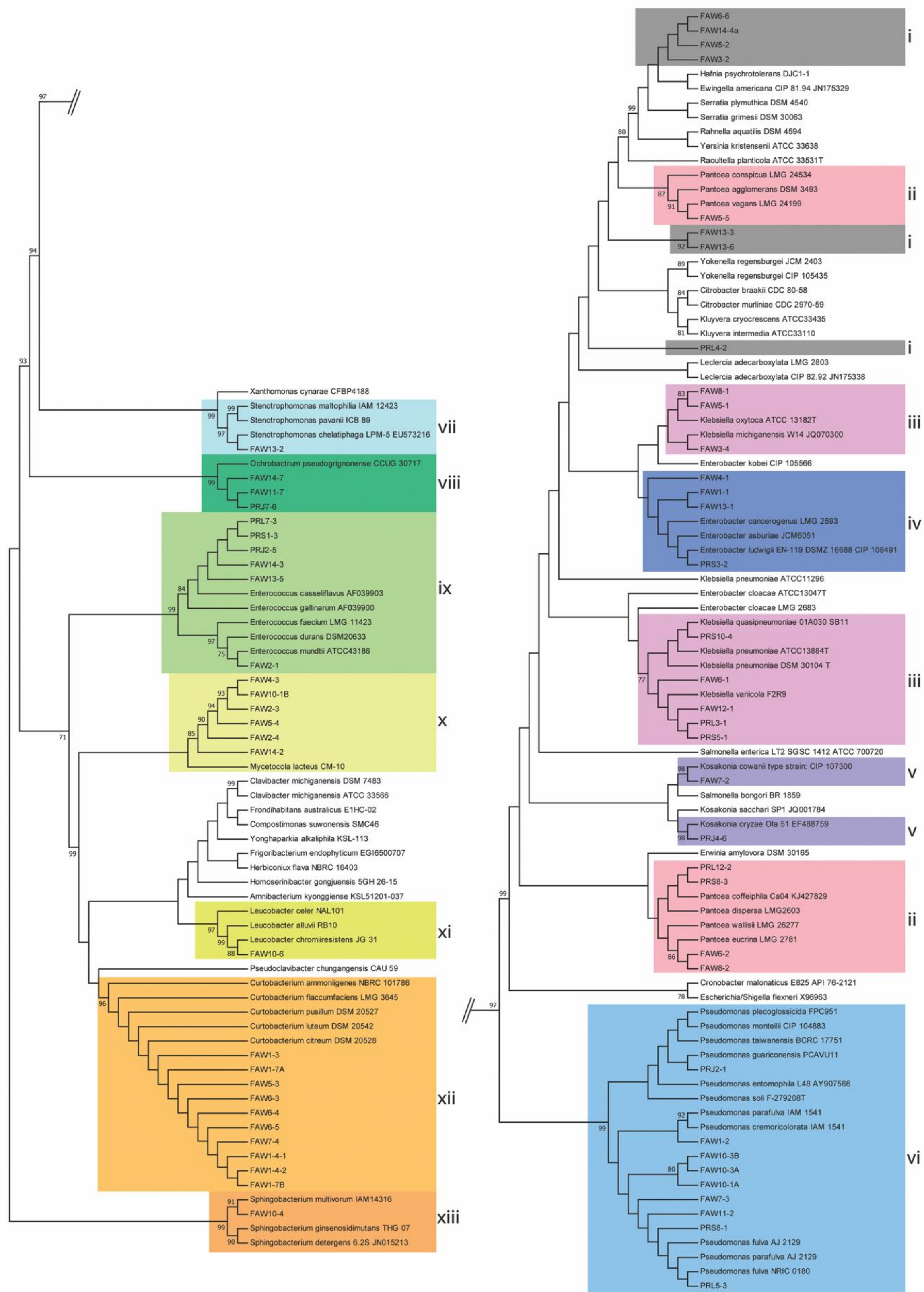
Figure 2.6: Influence of sample type (midgut or regurgitant) and diet (maize or soybean) on fall armyworm (*Spodoptera frugiperda*) bacterial communities. Non-metric multidimensional scaling (nMDS) plots were constructed using Bray-Curtis (A) and Jaccard (B) dissimilarities. Stress values indicate a good fit in two dimensions.

Table 2.4: PERMANOVA output assessing differences between fall armyworm (*Spodoptera frugiperda*) bacterial communities between sample type (midgut or regurgitant) and host plant (maize or soybean). PERMANOVAs were generated using 999 permutations, and the individual insect was included in the model as a random effect. Bold values indicate significant (p<0.05) differences.

Source	df	SS	MS	Pseudo-F	p-value
Bray-Curtis Similarity					
Sample type	1, 39	7191	7191	3.82	**0.013**
Plant	1, 39	16257	16257	8.64	**0.001**
Sample type*Plant	1, 39	2075	2075	1.10	0.331
Jaccard Similarity					
Sample type	1, 39	4772	4772	1.22	**0.020**
Plant	1, 39	11526	11526	2.95	**0.001**
Sample type*Plant	1, 39	4222	4222	1.08	0.169

Table 2.5: Pairwise comparisons of abundances of individual bacterial OTUs (operational taxonomic units) between host plant (soybean or maize) and sample type (midgut or regurgitant) in fall armyworm (*Spodoptera frugiperda*). Bold values indicate significant ($p<0.05$) differences. OTUs were selected using a SIMPER analysis.

	Maize		Soybean		Plant		Sample type (maize)		Sample type (soybean)	
OTU	Gut	Regurgitant	Gut	Regurgitant	W	p - value	W	p-value	W	p-value
OTU001: *Enterococcus*	27.48 ± 10.8	9.82 ± 6.2	32.94 ± 5.95	15.43 ± 2.69	427	**0.0055**	185	**0.0449**	98	**0.0006**
OTU002: Enterobacteriaceae	39.93 ± 10.9	55.15 ± 9.46	22.25 ± 5.52	27.74 ± 9.76	253	**0.0387**	131	0.2913	59	0.3823
OTU003: *Arthrobacter*	5.33 ± 4.66	4.14 ± 3.1	24.49 ± 4.97	34.01 ± 7.78	455	**0.0003**	159.5	0.5968	50	0.065
OTU004: *Rhizobium*	6.87 ± 3.44	2.31 ± 0.85	0.91 ± 0.36	1.24 ± 0.63	348	0.5944	143	0.7125	57	0.2786
OTU005: *Pseudomonas*	0.97 ± 0.4	1.44 ± 0.58	0.07 ± 0.03	0.09 ± 0.05	192	**<0.0001**	140	0.5899	65	0.798
OTU008: *Methylobacterium*	1.46 ± 0.64	1.73 ± 0.81	0.04 ± 0.02	0.03 ± 0.01	222	**0.0028**	155.5	0.7679	65	0.798
OTU011: *Bradyrhizobium*	0.98 ± 0.38	0.91 ± 0.42	0.04 ± 0.03	0.01 ± 0	191	**<0.0001**	158	0.6707	61.5	0.5169
OTU015: Unclassified	3.85 ± 2.42	1.25 ± 0.47	0.02 ± 0.01	0.01 ± 0.01	181.5	**<0.0001**	157	0.7122	58	0.2821
OTU016: *Ochrobactrum*	1.43 ± 0.79	0.5 ± 0.16	0.76 ± 0.33	1.15 ± 0.61	328	1.000	154	0.8426	59	0.3667
OTU018: *Microbacterium*	0.48 ± 0.25	1.25 ± 0.7	0.56 ± 0.17	0.7 ± 0.31	364.5	0.3207	128	0.2184	62	0.5737
OTU033: *Bacillus*	0.02 ± 0.02	0.29 ± 0.2	2.1 ± 1.56	2.11 ± 1.39	468.5	**<0.0001**	116.5	0.3205	63	0.6454



Xanthomonas cynarae CFBP4188
Stenotrophomonas maltophilia IAM 12423
Stenotrophomonas pavanii ICB 89
Stenotrophomonas chelatiphaga LPM-5 EU573216
FAW13-2
vii
Ochrobactrum pseudogrignonense CCUG 30717
FAW14-7
FAW11-7
PRJ7-6
viii
PRL7-3
PRS1-3
PRJ2-5
FAW14-3
FAW13-5
Enterococcus casseliflavus AF039903
Enterococcus gallinarum AF039900
Enterococcus faecium LMG 11423
Enterococcus durans DSM20633
Enterococcus mundtii ATCC43186
FAW2-1
ix
FAW4-3
FAW10-1B
FAW2-3
FAW5-4
FAW2-4
FAW14-2
Mycetocola lacteus CM-10
x
Clavibacter michiganensis DSM 7483
Clavibacter michiganensis ATCC 33566
Frondihabitans australicus E1HC-02
Compostimonas suwonensis SMC46
Yonghaparkia alkaliphila KSL-113
Frigoribacterium endophyticum EGI6500707
Herbiconiux flava NBRC 16403
Homoserinibacter gongjuensis 5GH 26-15
Amnibacterium kyonggiense KSL51201-037
Leucobacter celer NAL101
Leucobacter alluvii RB10
Leucobacter chromiiresistens JG 31
FAW10-6
xi
Pseudoclavibacter chungangensis CAU 59
Curtobacterium ammoniigenes NBRC 101786
Curtobacterium flaccumfaciens LMG 3645
Curtobacterium pusillum DSM 20527
Curtobacterium luteum DSM 20542
Curtobacterium citreum DSM 20528
FAW1-3
FAW1-7A
FAW5-3
FAW6-3
FAW6-4
FAW6-5
FAW7-4
FAW1-4-1
FAW1-4-2
FAW1-7B
xii
Sphingobacterium multivorum IAM14316
FAW10-4
Sphingobacterium ginsenosidimutans THG 07
Sphingobacterium detergens 6.2S JN015213
xiii
FAW6-6
FAW14-4a
FAW5-2
FAW3-2
i
Hafnia psychrotolerans DJC1-1
Ewingella americana CIP 81.94 JN175329
Serratia plymuthica DSM 4540
Serratia grimesii DSM 30063
Rahnella aquatilis DSM 4594
Yersinia kristensenii ATCC 33638
Raoultella planticola ATCC 33531T
Pantoea conspicua LMG 24534
Pantoea agglomerans DSM 3493
Pantoea vagans LMG 24199
FAW5-5
ii
FAW13-3
FAW13-6
i
Yokenella regensburgei JCM 2403
Yokenella regensburgei CIP 105435
Citrobacter braakii CDC 80-58
Citrobacter murliniae CDC 2970-59
Kluyvera cryocrescens ATCC33435
Kluyvera intermedia ATCC33110
PRL4-2
i
Leclercia adecarboxylata LMG 2803
Leclercia adecarboxylata CIP 82.92 JN175338
FAW8-1
FAW5-1
Klebsiella oxytoca ATCC 13182T
Klebsiella michiganensis W14 JQ070300
FAW3-4
iii
Enterobacter kobei CIP 105566
FAW4-1
FAW1-1
FAW13-1
Enterobacter cancerogenus LMG 2693
Enterobacter asburiae JCM6051
Enterobacter ludwigii EN-119 DSMZ 16688 CIP 108491
PRS3-2
iv
Klebsiella pneumoniae ATCC11296
Enterobacter cloacae ATCC13047T
Enterobacter cloacae LMG 2683
Klebsiella quasipneumoniae 01A030 SB11
PRS10-4
Klebsiella pneumoniae ATCC13884T
Klebsiella pneumoniae DSM 30104 T
FAW6-1
Klebsiella variicola F2R9
FAW12-1
PRL3-1
PRS5-1
iii
Salmonella enterica LT2 SGSC 1412 ATCC 700720
Kosakonia cowanii type strain: CIP 107300
FAW7-2
v
Salmonella bongori BR 1859
Kosakonia sacchari SP1 JQ001784
Kosakonia oryzae Ola 51 EF488759
PRJ4-6
v
Erwinia amylovora DSM 30165
PRL12-2
PRS8-3
Pantoea coffeiphila Ca04 KJ427829
Pantoea dispersa LMG2603
Pantoea wallisii LMG 26277
Pantoea eucrina LMG 2781
FAW6-2
FAW8-2
ii
Cronobacter malonaticus E825 API 76-2121
Escherichia/Shigella flexneri X96963
Pseudomonas plecoglossicida FPC951
Pseudomonas monteilii CIP 104883
Pseudomonas taiwanensis BCRC 17751
Pseudomonas guariconensis PCAVU11
PRJ2-1
Pseudomonas entomophila L48 AY907566
Pseudomonas soli F-279208T
Pseudomonas parafulva IAM 1541
Pseudomonas cremoricolorata IAM 1541
FAW1-2
FAW10-3B
FAW10-3A
FAW10-1A
FAW7-3
FAW11-2
PRS8-1
Pseudomonas fulva AJ 2129
Pseudomonas parafulva AJ 2129
Pseudomonas fulva NRIC 0180
PRL5-3
vi

Figure 2.7: Phylogenetic tree of near full-length 16S rRNA gene sequences from bacterial isolates cultured from fall armyworm (*Spodoptera frugiperda*) regurgitant collected in Pennsylvania, United States (indicated by FAW prefix) Puerto Rico (indicated by PR prefix), and selected type strains from the RDP database. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Jukes-Cantor model in MEGA7. The tree with the highest log likelihood (-5718.26) is shown. Bootstrap values with values of 70 or greater are shown. Annotations correspond to clades that are identified as likely the following genera i) unresolved ii) *Pantoea* iii) *Klebsiella* iv) *Enterobacter* v) *Kosakonia* vi) *Pseudomonas* vii) *Stenotrophomonas* viii) *Ochrobactrum* ix) *Enterococcus* x) *Mycetocola* xi) *Leucobacter* xii) *Curtobacterium* and xiii) *Sphingobacterium.*

Discussion

Bacterial gut communities of two lepidopteran species, fall armyworm and corn earworm, were variable and influenced by multiple factors. Bacterial communities were distinct depending on insect species and collection location. I also found that host plant was much more important in shaping midgut bacterial communities in fall armyworm than the population source of the eggs. Midgut and regurgitant bacterial composition of fall armyworm were also distinct, suggesting that the physiology of different gut regions may affect bacterial composition or abundance.

I found high variability in gut bacterial composition and abundance between individuals of the same species, even from those feeding on the same food source. This is consistent with reports of several other lepidopteran species that possess microbial gut assemblages that differ between individuals (Hammer et al., 2017; Priya et al., 2012; Staudacher et al., 2016). The high variability in lepidopteran gut bacterial assemblages and the apparent lack of a resident microbiota has fueled speculation that lepidopteran gut associates lack functional importance in these insects (Appel, 1994; Douglas, 1992; Hammer et al., 2017; Visôtto et al., 2009). Such suggestions likely stem from the limited research into functional roles of gut bacteria in Lepidoptera. However, demonstrated roles of lepidopteran oral and gut bacteria include suppression (Acevedo et al., 2017) and detoxification (Mason et al., 2014) of plant defenses. Moreover, these associates can mediate other ecological associations, such as interactions with entomopathogens (Broderick et al., 2006; Caccia et al., 2016; Jakubowska et al., 2013). Future studies manipulating the presence/absence and assemblage of gut bacterial associates in

controlled studies are needed to address functions of these associates and the consequences of variations in community composition/abundance.

Although I observed high intraspecific variation in bacterial gut composition and structure, I also found that corn earworm and fall armyworm had distinct midgut bacterial communities despite feeding on the same host plant species at the same location. This suggests that acquisition of bacteria from the environment is not a completely stochastic process and that bacterial communities are not merely a reflection of the host plant microbiome. There are likely gut physiological mechanisms that select for certain taxa, which may help shape bacterial composition in these different species. Competition among taxa may also play a role and it is likely that microbial community composition is dynamic. The ability of insect guts to filter certain taxa from the wider environmental pool of bacteria has been observed in several systems including cockroaches (Mikaelyan et al., 2016), bean bugs (Byeon et al., 2015; Futahashi et al., 2013; Ohbayashi et al., 2015), and bumble bees (Näpflin and Schmid-Hempel, 2017). There is little known about the mechanisms involved in bacterial acquisition and establishment in lepidopteran guts, but variation in physiochemical properties and competition among taxa are likely important for shaping these communities.

Host plant (maize or soybean) played a much bigger role in influencing the composition of midgut bacteria in fall armyworm than egg source (Figure 2.3). These results are comparable to studies on other folivores showing that host plant can shape gut bacterial communities, including in *Spodoptera littoralis* (Tang et al., 2012)*, Helicoverpa* spp. (Priya et al., 2012; Tang et al., 2012; Xiang et al., 2006), *Lymantria dispar* (Broderick et al., 2004; Mason and Raffa, 2014), and *Leptinotarsa decemlineata* (Chung et al., 2017). Pre- and post-digestive interactions between the plant and microbe may affect the composition of bacterial communities. For example, differences in leaf surface, wax composition, availability of sugars, and interactions with other bacterial species can alter the bacterial community composition present on plant leaves (Lindow and Brandl, 2003), thereby altering the composition and quantity of bacteria that insects encounter. There are also chemical interactions occurring in the gut that may mediate these interactions; for example, foliar concentrations of plant secondary compounds can affect bacterial composition in insect guts (Mason et al., 2015). To what degree fall armyworm gut bacterial composition reflects that of their host plant was not investigated in this study, but is

likely influenced by a combination of phyllosphere bacteria and chemical composition of plant tissues.

Corn earworm midgut bacterial communities from two sites in Pennsylvania were distinct, even though the host plant (sweet corn) was the same at both sites. These differences were likely due to variation in phyllosphere bacteria inhabiting host plants at each site. Several studies investigating factors that influence phyllosphere bacteria found that geographical location of plants altered bacterial community composition (Knief et al., 2010; Rastogi et al., 2012).

Although fall armyworm individuals possessed different overall bacterial communities in paired regurgitant and midgut samples, pairwise comparisons revealed that this variation was primarily driven by a single *Enterococcus* OTU. There are several physiological and morphological differences between the midgut and foregut that may differentially impact microbial gut associates. The foregut is covered in a cuticular lining while midgut cells are protected by the peritrophic membrane lining. Additionally, the pH of the lepidopteran alimentary canal changes across its length; the midgut is highly alkaline, while the foregut is usually closer to neutral (Appel and Maines, 1995; Dow, 1992). These differences may provide differential binding and colonization affinities for microbiota, resulting in greater abundances of certain microbial taxa. The foregut may also pose greater disturbances to bacterial communities, because the cuticular lining is replaced during each molt. It is unclear if and how microbes recolonize the foregut after molting, although in *S. littoralis* fluorescently-labeled bacteria were observed in the foregut throughout different instars (Teh et al., 2016).

Relatively few bacterial taxa were associated with the regurgitant of fall armyworm. In contrast, a recent study of the “regurgitome” of Mexican bean beetle (*Epilachna varivestis*) revealed a highly diverse microbiome with 1230 bacterial species in 577 genera (Gedling et al., 2018). In this study I observed less diversity and richness; culture-independent sequencing detected about 100 OTUs in larvae fed on maize and soybean. In both plant species, *Enterococcus* and an unclassified Enterobacteriaceae OTU comprised the majority of the taxa in this study. These results paired well with the culture-dependent survey, which I conducted to obtain isolates for future physiological and ecological studies (Mason et al., 2019, 2020). Culture-dependent 16S sequencing of bacteria from regurgitant of fall armyworm larvae collected in Pennsylvania and Puerto Rico showed that isolates belonged to genera including

Pantoea, Enterobacter, Klebsiella, Pseudomonas, Curtobacterium, *Mycetocola*, and *Enterococcus*. These genera are commonly associated with many different insect groups (Engel and Moran, 2013), and have documented roles in mediating fall armyworm-plant interactions (Acevedo et al., 2017).

Based on the phylogenetic tree constructed from sequencing near-full length 16S-rRNA, I found some bacterial isolates that could not be reliably identified (Figure 2.7, i), and several genera including *Pantoea, Klebsiella, Enterobacter,* and *Kosakonia* were non-monophyletic. The 16S rRNA gene is widely used to study bacterial ecology, but can have limitations distinguishing closely related taxa due to high sequence similarities (Větrovský and Baldrian, 2013). The Enterobacteriaceae tends to have poor resolution using hypervariable regions of the 16S rRNA gene, including the broadly used V4 region (Chakravorty et al., 2007; Jovel et al., 2016). It is likely that the unclassified Enterobacteriaceae OTU in the culture-independent analyses actually contains several bacterial genera, and thus underestimated the diversity in these samples.

This study contributes to understanding of the factors that influence gut microbiomes in two lepidopteran insects and offers insight into the composition of bacterial communities in fall armyworm regurgitant, which are likely to be relevant for mediating plant-insect interactions. I show that variation in insect species, gut region, and host plant can affect the composition of bacterial gut communities of lepidopteran larvae. Further research will be required to identify the factors that alter bacterial communities at each of these levels of scale.

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Chapter 3

Effects of baculovirus-killed cadavers on plant defenses and insect behavior

Abstract

Baculoviruses (Baculoviridae) are a group of entomopathogenic viruses that are important natural enemies of insects, particularly lepidopteran larvae. Baculoviruses are transmitted in the environment as occlusion bodies (OBs) that are released from liquified virus-killed cadavers. An important component of baculovirus transmission efficiency is the frequency with which hosts encounter patchily distributed OBs on plants, yet little is known about the ecology of virus-killed cadavers. I used the generalist baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and host *Trichoplusia ni* caterpillars to study the effects of virus-killed cadavers on tomato (*Solanum lycopersicum*) defenses and *T. ni* behavior. Because both OBs and cadaver-associated bacteria could play a role in these interactions, I used high-throughput Illumina amplicon sequencing of the V4 region of the 16S rRNA gene to characterize the bacterial communities of virus-killed cadavers in comparison with uninfected (freeze-killed) cadavers. The most represented bacterial orders in these communities were Lactobacillales, Enterobacteriales, Pseudomonadales, and Bacillales, in agreement with other studies in Lepidoptera. In terms of overall bacteria community composition and membership, there was no significant difference between tomato-fed virus-killed or freeze-killed cadavers. Comparison of virus-killed cadavers from two separate experiments revealed significant differences in bacterial community composition, suggesting that host plant could play a more important role in shaping bacterial communities than virus infection status. Culture-dependent experiments showed that virus-killed cadavers had significantly higher bacterial titers compared with uninfected cadavers. To examine the effects of cadavers on tomato defenses, I applied virus-killed cadavers, freeze-killed cadavers, or water to undamaged or mechanically wounded plants. I found that virus-killed cadavers suppressed polyphenol oxidase (PPO) activity, an important plant defense protein, in damaged plants. Although cadavers did not influence plant defenses induced by healthy or infected *T. ni* herbivory, this study provides the first evidence that baculoviruses could influence plant defenses through host cadavers. When applied to intact plants, virus-killed or freeze-killed cadavers did not influence oviposition or larval choice, indicating that *T. ni* did not discriminate cadaver cues. Virus-killed cadavers could play

important roles in mediating interactions between plants, herbivores, and other trophic levels, with potential implications for viral transmission dynamics.

Introduction

A major branch of ecological theory seeks to understand the forces that shape ecological communities and regulate herbivore consumption of plants. The relative importance of "top-down" regulation by natural enemies and "bottom-up" effects of plant defenses on herbivore populations is often debated (Hairston et al., 1960; Power, 1992; White, 1978), although additive and interactive relationships between these forces are also appreciated (Hunter et al., 1997; Mooney et al., 2012; Vidal and Murphy, 2018; Walker and Jones, 2001; Wilkinson and Sherratt, 2016). Inducible plant defenses are produced in response to herbivory, regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Erb et al., 2012; Karban and Baldwin, 1997). Chemical defenses affect not only herbivores, but can attract and sustain natural enemies in the third trophic level (Heil, 2008; McCormick et al., 2012) and alter natural enemy performance (Cory and Hoover, 2006; Ode, 2006). In turn, natural enemies shape plant–herbivore interactions through regulation of herbivore populations (Letourneau et al., 2009) and modulation of plant defenses (*see Chapter 1*). The third trophic level is therefore critical to understanding plant–herbivore interactions in both evolutionary and biological control contexts (Hawkins et al., 1997; Price et al., 1980).

An additional layer of complexity to understanding multitrophic interactions is the influence of microbes. Microbes act on all components of plant–herbivore–natural enemy tripartite systems—as symbionts, plant pathogens, and as natural enemies themselves (i.e. entomopathogens) (Franco et al., 2017; Mason et al., 2018; Shikano, 2017). Plant pathogens typically elicit induction of SA-mediated plant defenses, whereas chewing herbivores tend to induce defenses via JA/ET pathways. Due to mutual antagonism between the JA and SA pathways, upregulation of one suite of defenses can suppress the other in many plant species (Erb et al., 2012; Li et al., 2019; Thaler et al., 2012). Some chewing herbivores exploit this crosstalk by using orally secreted bacteria to suppress JA-regulated plant defenses that impair insect growth (Acevedo et al., 2017; Chung et al., 2013; Wang et al., 2016). Other microbes such as plant growth–promoting bacteria and fungi can provide benefits to plants through induction of defenses that confer resistance to pathogens and herbivores (Ahmad et al., 2020; Bent et al.,

2006). Another example of microbes that mediate complex multitrophic interactions is the symbiotic polydnaviruses associated with some parasitoid wasps. The polydnavirus of *Microplitis croceipes* was shown to suppress glucose oxidase levels in host *Helicoverpa zea* saliva. Because glucose oxidase is an important elicitor of tomato defenses, feeding by parasitized larvae induced lower defense responses compared to healthy larvae and improved performance of wasps developing in hosts that fed on these plants (Tan et al., 2018).

An important group of microbes that influence plant–herbivore interactions is the baculoviruses (Baculoviridae), entomopathogenic DNA viruses that predominantly target lepidopteran larvae (Harrison et al., 2018). Naturally occurring epizootics of baculoviruses can cause high mortality of larval hosts, particularly in forest systems (Cory and Myers, 2003). The high host specificity of baculoviruses makes them good candidates for biological control, and commercially produced baculovirus formations are used as agricultural biopesticides in many countries (Lacey et al., 2015; Moscardi et al., 2011). Although there is a wealth of knowledge on the molecular biology and pathology of baculoviruses (Slack & Arif, 2006), less is known about their ecological interactions with plants and insects (Cory, 2010; Cory and Hoover, 2006; Shikano, 2017).

Lepidopteran baculoviruses spread through the environment as occlusion bodies (OBs), proteinaceous structures that contain virus particles (virions). Caterpillars ingest OBs on plant foliage, and occlusion-derived virions (OVs) are released in the midgut lumen where they initiate infection through epithelial cells (Harrison and Hoover, 2012). Secondary infection between host tissues is carried out by non-occluded budded virus (BVs), and the resulting systemic infection eventually kills the insect. OBs are produced at the end of the infection cycle, and when the insect dies, millions of OBs are released into the environment as virus-killed cadavers characteristically liquify or "melt" (Harrison and Hoover, 2012).

Plants mediate several aspects of baculovirus–host dynamics (Cory and Hoover, 2006). Because OBs are sensitive to degradation by UV irradiation, plant shape and structure can affect baculovirus persistence on plant leaves by providing varying degrees of shading (Cory and Hoover, 2006). Phylloplane properties such as pH and ion concentration can also inactivate OBs on plant surfaces (Young et al., 1977). After baculovirus OBs are ingested by an insect, phytochemicals can influence viral infectivity of midgut epithelial cells. For example, plant

peroxidases can participate in redox cycling of phenolics and other phytochemicals, which can damage the insect midgut, increasing sloughing of infected midgut cells and eliminating virus particles (Hoover et al., 2000). Plants can also alter host susceptibility to viral infection (Shikano et al., 2017). For example, plant defenses that inhibit insect growth can prolong the period of larval susceptibility to baculovirus infection (Shikano et al., 2018), and plant quality can affect caterpillar resistance by influencing insect immunity (Martemyanov et al., 2012; Shikano et al., 2010).

While it is clear that plant defenses affect baculovirus–insect interactions, less is known about the influence of baculoviruses on plants (Shikano, 2017). Baculoviruses substantially modify physiology and behavior of their hosts (Wang & Hu, 2019), potentially influencing caterpillar interactions with plants. A recent study found that *H. zea* caterpillars infected with a sublethal dose of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) induced higher defense responses in tomato compared with healthy caterpillars, suggesting that the virus indirectly affected plants through modification of its host (Pan et al., 2019).

Baculoviruses interact with plants directly when liquified cadavers contact plant surfaces. An important component of baculovirus transmission efficiency is the frequency with which hosts encounter OBs in the environment (Harrison & Hoover, 2012). Caterpillar corpses containing high concentrations of OBs are distributed patchily on plants (Goulson, 1997). Baculoviruses often manipulate host behavior, inducing caterpillars to climb to the top of plants prior to death—leading to the term “tree top disease” (Hoover et al., 2011). This behavior is thought to improve the likelihood of ingestion by hosts because rainfall and wind distribute low concentrations of OBs across plants (D’Amico and Elkinton, 1995). Additionally, baculoviruses could actively enhance transmission rates by manipulating plant defenses or insect behavior via host cadavers. Virus-killed cadavers contain not only OBs, but high concentrations of bacteria, although the bacterial communities associated with these cadavers have not been described previously. Both OBs and cadaver-associated bacteria could mediate interactions of cadavers in food webs.

The aim of this study was to investigate effects of virus-killed cadavers on plant defenses and insect behavior. I used *Trichoplusia ni* caterpillars, tomato plants (*Solanum lycoperscium*), and AcMNPV, a generalist baculovirus that infects more than 30 caterpillar species in several

genera (Mcintosh et al., 2005). The first objective of this study was to quantify and characterize bacterial communities associated with virus-killed *T. ni* cadavers in comparison with control (freeze-killed) cadavers, with the hypothesis that bacterial communities would differ among treatments in terms of abundance, composition, or membership. The second objective was to examine how cadavers influenced plant defenses. I hypothesized that virus-killed cadavers would suppress plant defenses, whereas freeze-killed cadavers would have no effect. The final objective was to measure impacts of cadavers on larval and adult *T. ni* behavior, with the hypothesis that virus-killed cadavers would attract or repel healthy insects (Capinera et al., 1976; Parker et al., 2010; Rebolledo et al., 2015). Improved understanding of baculovirus ecology in multitrophic contexts could expand our view of natural enemies and microbes in multitrophic systems and have practical implications for the development of these viruses as biopesticides.

Methods

Plants and insects

I used tomato (*Solanum lycopersicum* cv. Better Boy) at the four-leaf-stage for all experiments (approximately 24–28 days post-planting). After transplanting seedlings to individual pots, I fertilized each plant with 3 g of Osmocote (ICL Specialty Fertilizers, Dublin, OH). I grew tomato plants in a temperature-controlled greenhouse at Penn State University (University Park, PA) equipped with high-pressure sodium and metal halide lights (400 W). I initially obtained *T. ni* caterpillars from Benzon Research (Carlisle, PA) and maintained subsequent generations on pinto bean–based artificial diet (Shorey and Hale, 1965) in a growth chamber (16:8 (L:D) h, 27°C) at Penn State University (University Park, PA). I supplied adult moths with 10% sugar solution and collected eggs every 24 h.

Production of virus-killed cadavers

I obtained wild-type AcMNPV (strain C6) OBs from Dr. Robert Harrison (ARS, USDA). After propagating the virus in *T. ni* caterpillars, I semi-purified the OBs according to previously described methods (Eberle et al., 2012). Briefly, I homogenized liquified cadavers with sterile water and centrifuged tubes at 500 x g for 10 min to pellet cadaver debris. After removing the supernatant, I repeated this centrifugation step several times. To pellet the OBs, I centrifuged tubes at 10,000 x g for 1 hour. After rinsing and resuspending the OBs in sterile water, I repeated this step once more. Finally, I resuspended semi-purified OBs in sterile 60% glycerol and stored

tubes at –20 °C. To estimate virus concentration, I counted OBs with an improved Neubauer brightline haemocytometer (0.1 mm deep; Hausser Scientific, Horsham, PA, USA) at 400 × magnification.

To produce virus-killed cadavers, I orally administered AcMNPV to newly molted fourth instar *T. ni* larvae that had previously been reared on artificial diet. I applied 1 µl of 60% glycerol containing 1000 OBs to a small disc (Ø 4mm, h = 1mm) of artificial diet. After larvae had consumed the entire piece of diet (within 12 h), I transferred them to new cups containing tomato foliage. I replaced the foliage every 1–2 days. Most larvae died from virus infection after 5–6 days.

To produce control freeze-killed cadavers, I used a similar protocol as above except that larvae received 1 µl of 60% glycerol without viral OBs on a disc of artificial diet. As above, these larvae fed on tomato foliage for 5–6 days before being killed. I anesthetized larvae by placing them at –20°C for 5–10 min and killed them by crushing with a pestle in a 1.5 ml Eppendorf tube.

For plant defense and insect behavior experiments, I pooled 10–12 cadavers of each treatment in an Eppendorf tube. After measuring the fresh weight of total cadavers for each treatment, I added an equal amount of sterile Milli-Q water to the tube (mg cadaver = ml water) and homogenized the cadavers to create a cadaver slurry. I added sterile milliQ water to aid homogenization of cadavers and to ensure that the cadaver solution could be pipetted. In doing so, I was able to standardize the volume of cadaver solution used for experiments.

Location of virus-killed cadavers on plants

Because I was interested in measuring whether virus-killed cadavers influenced tomato defenses, I conducted an experiment to measure whether infected larvae died on plants and if they died close to feeding wounds. I infected *T. ni* larvae (n=11) with 1000 AcMNPV OBs as above. The following day, I placed each larva onto a tomato plant enclosed within a mesh tent (40 x 40 x 60 cm). I monitored larvae daily, and measured the location of death and distance to edge of the nearest feeding wound.

Bacterial and viral abundance in cadavers

To estimate bacterial concentrations in virus-killed and freeze-killed cadavers, I counted the number of colony-forming units (CFUs) per mg of cadaver. After weighing and

homogenizing individual cadavers in sterile PBS, I made serial dilutions and plated 50 µl onto 2xYT media. After incubating the plates for 3 d at 27 °C, I counted the number of CFUs. I used the same serial dilutions to estimate the number of viral OBs in each cadaver. As previously described, I used a haemocytometer to count the number of OBs. I used 10–19 cadavers for each treatment, and I repeated the experiment with two different sets of insects on different dates.

Bacterial community analysis of cadavers

Sample collection and DNA extraction

To compare bacterial composition between freeze-killed cadavers and virus-killed cadavers, I used high-throughput Illumina amplicon sequencing. After *T. ni* larvae hatched, they fed on artificial diet for 7 d. To produce virus-killed cadavers ('Virus' treatment; n=8), I orally administered viral OBs in glycerol as above. After larvae consumed the full dose, they fed on tomato foliage for 6 d until death. To produce freeze-killed cadavers ('Freeze' treatment; n=8), I gave larvae 60% glycerol without viral OBs. After larvae consumed this mock dose, they fed on tomato foliage for 6 d. To produce control diet-fed larvae ('Diet' treatment; n=5), I similarly fed larvae 60% glycerol without viral OBs. After consuming this dose, larvae continued to feed on artificial diet. These three treatments came from the same set of eggs, and the Virus and Freeze larvae fed on tomato foliage from the same plants. I also took samples from tomato plant foliage ('Tomato'; n=5) by taking leaf discs (~ 50 mg) from randomly-selected leaves of five different tomato plants.

Lepidopteran bacterial communities often lack resident associates (Hammer et al., 2017), can be highly variable between individuals (Paniagua Voirol et al., 2018), and are influenced by host plant and other environmental factors (Jones et al., 2019; Priya et al., 2012). If baculovirus infection significantly alters gut bacterial communities, I was interested in whether it would do so in consistent ways across experiment replication. To compare whether virus-killed cadaver bacterial communities were similar between experiments, I included a fourth treatment ('Virus2'; n=8); I used the same methods for the Virus treatment above, but I used a different batch of eggs and plants.

After collecting cadaver and leaf samples, I immediately froze tubes in liquid nitrogen and stored them at –80 °C until DNA extraction. I extracted DNA from samples using Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, CA, USA) according to the

manufacturer's instructions. To generate16S-rRNA V4 amplicons, I used the primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015; Parada et al., 2016). To conduct PCRs, I used Platinum HotStart MasterMix (ThermoFisher) with the following conditions: 94°C for 3 min; 30 cycles of 94°C for 45s, 50°C for 60s, 72°C for 90s; and a final annealing hold of 72°C for 10 mins. I cleaned products and added barcodes and adapters with PCR. Amplicons were pooled and sequenced using an Illumina MiSeq at the Pennsylvania State University Hershey Medical Campus Genomics Core (Hershey, PA, USA).

Processing of sequencing data

I processed sequencing data using mothur v.1.43.0 (Schloss et al., 2009). Briefly, I formed, trimmed, and aligned contigs against the SILVA Gold database. To detect and remove chimeric sequences from samples, I used VSEARCH (Rognes et al., 2016). To classify sequences, I used the Ribosomal Database Project (RDP) reference taxonomy (Cole et al., 2014). After removing sequences classified as eukaryotic, plastids, or "unknowns," I picked operational taxonomic units (OTUs) based on 97% similarity. To normalize data, I rarefied the number of reads per sample to 22000. I constructed an OTU table based on normalized data for downstream analyses.

Sanger sequencing of bacterial isolates

To further classify bacterial OTUs that were unable to be classified at the genus level, I constructed a phylogenetic tree using the 25 most abundant OTUs from Illumina sequencing above, bacteria isolated from insect cadavers with culture-based methods (see below), and type strains obtained from RDP (Cole et al., 2014).

To obtain bacterial isolates from virus-killed and freeze-killed cadavers, I plated serial dilutions of virus-killed and freeze-killed cadavers onto 2xYT media plates. I picked colonies with unique morphologies and subcultured with isolation streaks to obtain pure cultures. After growing bacterial isolates overnight in liquid 2xYT media, I centrifuged the tubes to remove media and obtain bacterial pellets. I used a CTAB protocol (Chen and Kuo, 1993) to extract DNA from the pelleted bacteria, and I performed PCR using the 16S primers 27F (5′AGAGTTTGATYMTGGCTCAG 3′) and 1392R (5′ ACGGGCGGTGTGTRC 3′) with GoTaq® Green Master Mix (Promega, Madison, WI, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 50 °C

for 45 s, 72 °C for 1 min, then 72 °C for 5 min. I purified PCR products with Exo-SAP-it (Affymetrix. Santa Clara, CA) following manufacturer's instructions. Sanger sequencing of purified DNA products was performed at Penn State Genomics Core Facility (University Park, PA).

I used SeqMan Pro (DNASTAR, Madison, WI) to assemble contigs for bacterial isolate sequences. I used these consensus sequences of isolates from Sanger sequencing and OTU V4 sequences from Illumina sequencing to search the Ribosomal Database Project Seqmatch tool for bacterial type strains with similar (0.8–1.00) sequences (Cole et al., 2014). I used the ClustalW tool in MEGA7 to align and trim the bacterial type strain sequences, isolate sequences, and OTU sequences. I used the resulting 253 bp sequences to construct an unrooted phylogenetic tree based on maximum likelihood Jukes-Cantor model in MEGA7 (Kumar et al., 2016).

Larval choice tests

To examine whether cadavers affected *T. ni* larval feeding preferences, I conducted two-choice feeding assays. I used a cork borer to cut leaf discs (1 cm diameter) from tomato plants and placed them on either side of a Petri dish lined with moistened 7.5 cm filter paper (VWR, West Chester, PA). For each assay, leaf discs were cut from the same leaf to reduce the effects of between-leaf variation in nutritional content or phytochemicals which could affect larval choice. I applied 10 µl of virus-killed cadaver solution, control cadaver solution, or sterile water to each leaf disc. I compared each of the three possible combinations of the three treatments (n=18 per combination). After allowing the solutions to dry for 1 h, I added a newly molted third instar *T. ni* to each Petri dish. In preliminary experiments, I found that if larvae were placed in the center of the dish, they moved rapidly and seemingly at random, eventually selecting whichever leaf disc they encountered first (within 10–20 seconds). Instead, I placed the larvae on the lid of the Petri dish until they gripped the plastic with their prolegs. Then I inverted the lid to close the dish, orienting the larva equidistant between the two leaf discs. These larvae moved slower and took much longer to make a choice, often stopping and waving their heads, before adjusting their direction. I identified a positive choice as the first leaf disc the larva contacted. I also measured the amount of each leaf disc consumed after 24 hours. After removing larvae from the leaf discs and brushing away frass with a fine paintbrush, I used clear tape to affix leaf discs to a piece of paper. I photographed each leaf disc with a scale bar and analyzed the photographs to estimate leaf area consumed with the mobile application LeafByte (Getman-Pickering et al., 2020).

Oviposition assays

To examine whether cadavers affected the attractiveness of tomato plants for *T. ni* oviposition, I conducted two-choice oviposition assays. To obtain mated female moths, I placed pairs of newly eclosed males and females into mating jars (473 ml plastic cups; Global Supply Store, Inc., Pomona, CA) lined with a paper towel and containing a 30 ml plastic cup (SOLO) with 10% sugar water and a cotton wick. I used moths for assays when they had produced at least 10 eggs in the mating jars (3–4 d after eclosion).

I placed two tomato plants at either side of mesh pop up tents (40 x 60 x 60cm) and the container with sugar water in the centre of the tent. To treat plants, I applied 20 µl of sterile water, virus-killed cadaver, or freeze-killed cadaver to the newest expanded tomato leaf. After allowing the cadaver solutions to dry for 1 hour, I released one moth pair to each tent at approximately 5 pm. The following morning at 9am, I removed moths and counted the number of eggs laid on each plant. Very few eggs were laid on the sides of tents and these eggs were not counted. I conducted oviposition assays over four consecutive nights, and I used a fresh batch of cadavers and moths each evening.

Influence of cadavers on plant defenses

Mechanical damage

To test whether cadavers influenced tomato plant defenses, I left plants undamaged or mechanically wounded plants to mimic herbivory. I used a custom tool that creates many small holes over a circular area (1-cm diameter) to damage the newest fully expanded leaf. To the damaged plants, I applied 10 µl of sterile water, virus-killed cadaver solution, or freeze-killed cadaver solution to the wound. To the undamaged plants, I applied these treatments to intact leaves on a similar part of the leaf as the damage treatment. I used 9–11 plants for each of the six treatment combinations (2x3 factorial design), and I repeated the experiment twice on two separate dates. Forty-eight hours after treating the plants, I harvested two 50 mg leaf samples from the treated leaf in liquid nitrogen.

Healthy larvae feeding damage

Although mechanical wounding can simulate caterpillar herbivory, plant responses are often different when exposed to feeding from insects (Waterman et al., 2019). In this experiment, I tested whether cadavers influenced tomato defense responses on leaves that had been induced

by healthy *T. ni* feeding. For the damage treatments, I added a newly molted fifth instar *T. ni* to a clip cage (1.5-cm diameter) applied to the newest fully expanded leaf. After larvae had finished feeding (2–3 h), I removed the clip cage and applied 10 µl of sterile water ('Dam+Water'), virus-killed cadaver solution ('Dam+Virus'), or freeze-killed cadaver ('Dam+Freeze') solution to the edge of the wound. For control plants ('Un+Water'), I applied empty clip cages to leaves followed by 10 µl of sterile water. I used 7–14 plants for each of the four treatments, and I repeated the experiment twice on two separate dates. Forty-eight hours after treating the plants, I harvested two 50 mg leaf samples from the treated leaf and froze them immediately in liquid nitrogen.

Healthy and infected larvae feeding damage

Because baculovirus-infected caterpillars could alter plant defense responses compared with healthy larvae (Pan et al., 2019), I measured plant defenses in response to feeding by infected and healthy larvae. I also measured plant defenses in response to cadaver applications on wounds from infected larvae. Infected larvae had received a dosage of 1000 OBs 48 h prior to the experiment. Both healthy and infected larvae fed on tomato foliage for at least 48 h prior to the experiment.

For the healthy larvae feeding treatment (Hel+Water), I added healthy fourth instar *T. ni* larvae in clip cages as above. Larvae fed from approximately 12–3pm. The following morning at 9 am, I applied 10 µl of sterile water to the edge of the wound. For the three treatments with infected larvae, I added fourth instar virus-infected larvae to plants in clip cages. These larvae fed on experimental plants from approximately 12–7pm. After removing infected larvae from clip cages, the following morning at 9 am I applied 10 µl of sterile water (Inf+Water), virus-killed cadaver (Inf+Virus), or freeze-killed cadaver (Inf+Freeze). Although I used clip cages to standardize damage, the amount of leaf area consumed still varied among larvae. I used LeafByte to measure the amount of herbivory damage (cm^2) on each plant. For each larva, I also measured larval weight (mg) and feeding duration (h). After removing larvae from clip cags, I placed them into cups with artificial diet and measured time until death or pupation to confirm their status of infected or healthy, respectively. For control plants ('Un+Water'), I applied empty clip cages to leaves followed by 10 µl of sterile water.

I used 10–14 plants per treatment, and this experiment was only performed once. Forty-eight hour after treating the plants with water or cadaver solutions, I harvested two 50 mg leaf samples from the treated leaf and froze them immediately in liquid nitrogen.

Plant defense proteins

I measured polyphenol oxidase (PPO) and trypsin protease inhibitor (TPI) according to previously described methods (Tan et al., 2018). I used a Geno/Grinder (SPEX SamplePrep, Metuchen, NJ) to grind frozen leaf samples into a fine powder.

For PPO assays, I added 1.25mLof phosphate buffer (0.1 M, pH 7) with 5% polyvinylpyrrolidone (PVP) (Alfa Aesar 41631) to the samples and vortexed thoroughly. After incubating samples on ice for 5 min, I centrifuged the tubes (4 °C, 11,000 × g, 10 min) and combined 5 µl of the supernatant from each sample with 200 µL of caffeic acid substrate (3 mM; Sigma C0625). I measured the change in absorbance over 5 min at λ450 with a Spectramax 190 plate reader (Molecular Devices Corporation, CA, Sunnyvale).

For TPI assays, I added 1.25 mL of extraction buffer (0.046 M Tris and 0.0115 M CaCl2; pH 8.1) with 5% PVP to each powdered sample and vortexed thoroughly. After incubating samples on ice for 5 min, I centrifuged the tubes (4 °C, 11,000 × g, 10 min) and combined 10 µl of the supernatant from each sample with 10 µL of trypsin (20 µg/mL; Sigma T1426) and 80 µL of extraction buffer. After a 10-min incubation period, I added 100 µL of p-toluene-sulfonyl-L-arginine methyl ester substrate (TAME: 0.002 M; Sigma T4626). I measured change in absorbance over 5 min at 247 nm with a Spectramax 190 plate reader. I also calculated the change in absorbance for a 'no inhibitor control' by combining trypsin, TAME, and extraction buffer. I calculated percentage inhibition of samples as: TPI % = (1 – sample slope / no inhibitor control slope) * 100.

For each sample I also measured the amount of protein with a Bradford assay (Bradford, 1976). I report PPO and TPI values as change in absorbance per min per mg of protein (mOD/min/mg protein).

<u>Statistical analysis</u>

Bacteria CFU and viral OB abundance in cadavers

To compare log (CFUs)/mg tissue between virus- and freeze-killed cadavers, I performed a one-way ANOVA with treatment as the factor and experimental replication as a blocking

variable. Because I did not detect OBs in freeze-killed cadavers, I compared OB concentrations between virus-killed cadavers across the two experimental replications with a t-test. These analyses were performed in SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

Cadaver bacterial communities

To compare bacterial community alpha diversity among cadaver treatments, I calculated number of OTUs, Chao1 estimate of species richness, and Shannon and Simpson diversity indices. To test for differences in the number of OTUs between treatments, I used a one-way ANOVA. Because the other three alpha diversity metrics did not meet the assumptions of ANOVA, I used non-parametric Kruskal-Wallis tests followed by pairwise Wilcoxon rank sum tests. I used R. 3.6.3 for these analyses (R Core Team, 2017).

To compare beta diversity of bacterial OTUs between treatments, I used R 3.6.3 with the packages 'vegan', 'RVAideMemoire', and 'phyloseq' (R Core Team, 2017). I used the vegdist command to calculate Bray-Curtis and Jaccard similarity distances for abundance and presence/absence, respectively. To visualize these multivariate data in two dimensions, I used the 'ggplot2' package to construct non-metric multidimensional scaling (nMDS) plots. To test for differences in bacterial community structure between cadaver treatments, I used the adonis function to perform permutational multivariate analysis of variance (PERMANOVA) with 1000 permutations. To test which treatments differed from each other, I used the pairwise.perm.manova function to conduct pairwise PERMANOVAS with a Wilks test statistic and Holm p-value correction for multiple comparisons. To compare variance of treatments based on Bray-Curtis distances, I performed a permutation test for homogeneity of multivariate dispersions with the betadisper function based on Bray-Curtis distances with 1000 permutations.

I conducted a similarity percentage analysis (SIMPER) to identify OTUs that contributed most to Bray-Curtis distances by using the simper function. For the four OTUs that this test identified, I used univariate non-parametric Kruskal Wallis rank sum tests to analyze the difference in relative abundance between treatments. I also generated heat maps of the 20 most abundant OTUs based on square root–transformed abundance by using the plot_heatmap function.

Larval choice and oviposition data

To evaluate larval choice, I used an exact binomial test. To compare leaf consumption, I used non-parametric paired Exact Wilcoxon Signed-Rank Test with a Pratt modification for zero data in the 'asht' package in R 3.6.3 (R Core Team, 2017).

To evaluate oviposition data, I performed a square-root transformation on number of eggs so that the data fulfilled the assumptions of normality. For each combination of treatments, I used a paired t-test to compare the difference in number of eggs laid on each plant. I used R. 3.6.3 to conduct these analyses (R Core Team, 2017).

Plant defense proteins

To compare PPO and TPI plant defensive protein data, I used ANOVAs after appropriate transformations to fulfil statistical assumptions. For the mechanical damage experiment, I performed a two-way ANOVA with damage and application as factors and experimental replicate as a blocking variable. For the healthy larvae feeding experiment, I used a one-way ANOVA with experimental replicate as a blocking variable. For the infected larvae feeding experiment, I used a one-way ANOVA. After removing the undamaged treatment, I performed additional ANCOVA analyses with herbivory area, larval feeding time, and time to death as covariates. I used SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) to conduct these analyses.

Results

Location of virus-killed cadavers on plants

All infected larvae died in 5–7 d. More than half (54.5%) of larvae died on the leaves, and these cadavers were located 0–8 mm from feeding wounds ($\mu = 3.67$ mm, SE = 1.31 mm). I found 45.5% of cadavers on the plant soil or bottom of the mesh tent.

Bacteria and virus abundance in cadavers

Virus-killed cadavers had significantly more bacterial CFUs per mg of tissue compared with freeze-killed cadavers (Figure 3.1A; $F_{1,54} = 30.39$, $p < 0.0001$). There was also a significant effect of the experimental replicate, with bacterial CFUs higher in the second experiment for both treatments (Figure 3.1A; $F_{1,54} = 16.78$, $p = 0.0001$).

As expected, I did not find viral OBs in freeze-killed cadavers. For virus-killed cadavers, there was a significant effect of experimental replicate on the number of OBs per mg of cadaver

tissue (Figure 3.1B; t_{27} = , p = 0.005) There was no correlation between OBs and CFUs in virus-killed cadavers (R^2= 0.01, p = 0.56).

Cadaver bacterial communities

After rarefication of sequencing data, I was unable to retain tomato foliage samples due to insufficient reads; these samples were excluded from further analysis. Across the other four treatments, I observed a total of 237 unique bacterial OTUs. The Good's coverage was high for all samples (> 0.999), indicating that sequencing was sufficient to characterize bacterial diversity.

At the phylum level, bacterial communities in diet-fed cadavers were composed predominantly of Firmicutes (Figure 3.2). Within the plant-fed treatments, freeze-killed and virus-killed cadavers were dominated by Firmicutes and Proteobacteria, with lower amounts of Actinobacteria. The composition of Bacteroidetes and Deinococcus-Thermus phyla was negligible in samples across treatments (Figure 3.2).

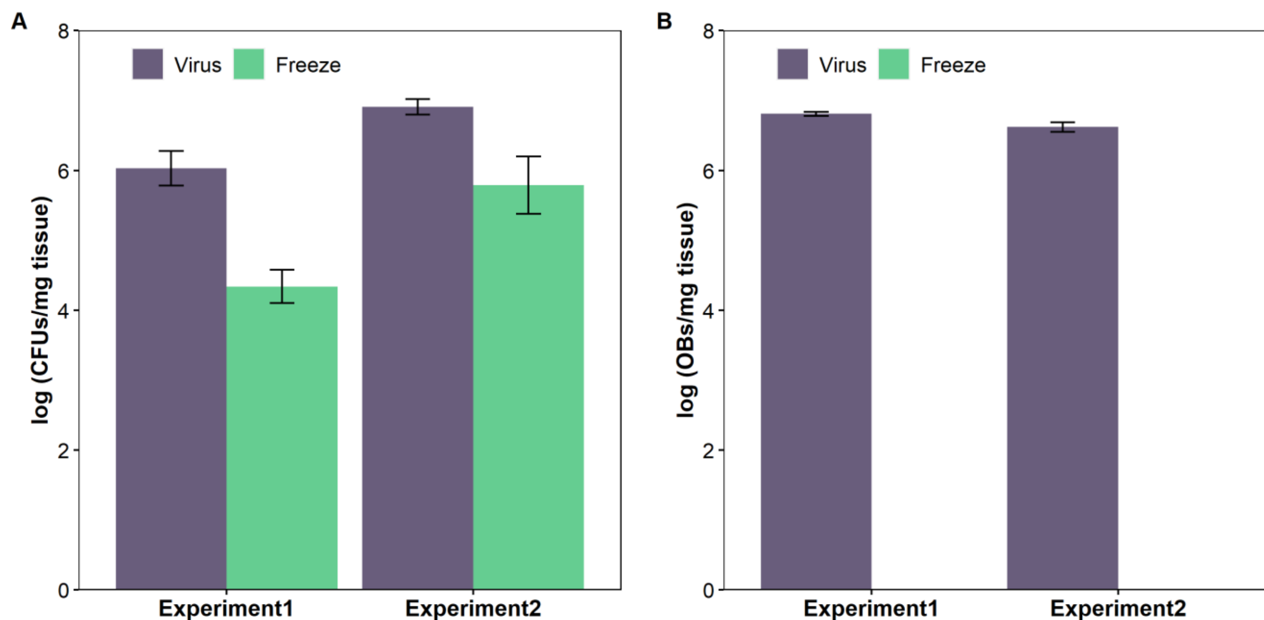

Figure 3.1: A) Bacterial colony-forming units (CFUs) and B) viral occlusion bodies (OBs) in virus-killed and freeze-killed cadavers from two separate experiments (n=10–19).

When comparing abundance of the six most abundant bacterial orders, I found that bacterial communities in diet-fed cadavers were composed predominantly of Lactobacillales (Figure 3.3). Virus-killed cadaver bacterial communities (Virus) were mainly composed of Lactobacillales and Enterobacteriales, with smaller amounts of Pseudomonadales and Actinomycetales. Virus2 bacterial communities similarly comprised mainly Lactobacillales and Enterobacteriales, with a larger proportion of Pseudomonadales than Virus samples. Freeze-killed cadaver bacterial communities mainly comprised Pseudomonadales, Lactobacillales, Enterobacteriales, and Bacillales (Figure 3.3).

Analysis of bacterial families present in *T. ni* cadavers showed that Enterococcaceae dominated diet-fed cadavers, whereas Enterococcaceae, Enterobacteriaceae, Pseudomonadaceae dominated virus- and freeze-killed cadavers. Freeze-killed cadavers also had high relative abundance of Staphylococcaceae (Figure 3.4).

Diet-fed cadaver bacterial communities had the lowest Chao1 and number of OTUs, indicating that these communities were the least rich (Figure 3.5), although these trends were not statistically significant (Table 3.1). Diet-fed cadaver bacterial communities also exhibited the lowest diversity and evenness, indicated by Shannon and inverse Simpson indices (Figure 3.5). Bacterial communities in the Virus2 treatments exhibited the highest number of OTUs, Chao1 richness, inverse Simpson diversity, and Shannon diversity. Although inverse Simpson and Shannon metrics differed significantly across treatments (Table 3.1), pairwise comparisons revealed that for inverse Simpson, only the Diet treatment differed significantly from the Virus2 treatment (Figure 3.5; Table 3.1). The Virus treatment had significantly higher Shannon diversity than Diet, and Virus2 had significantly higher Shannon diversity than the other three treatments (Figure 3.5; Table 3.1).

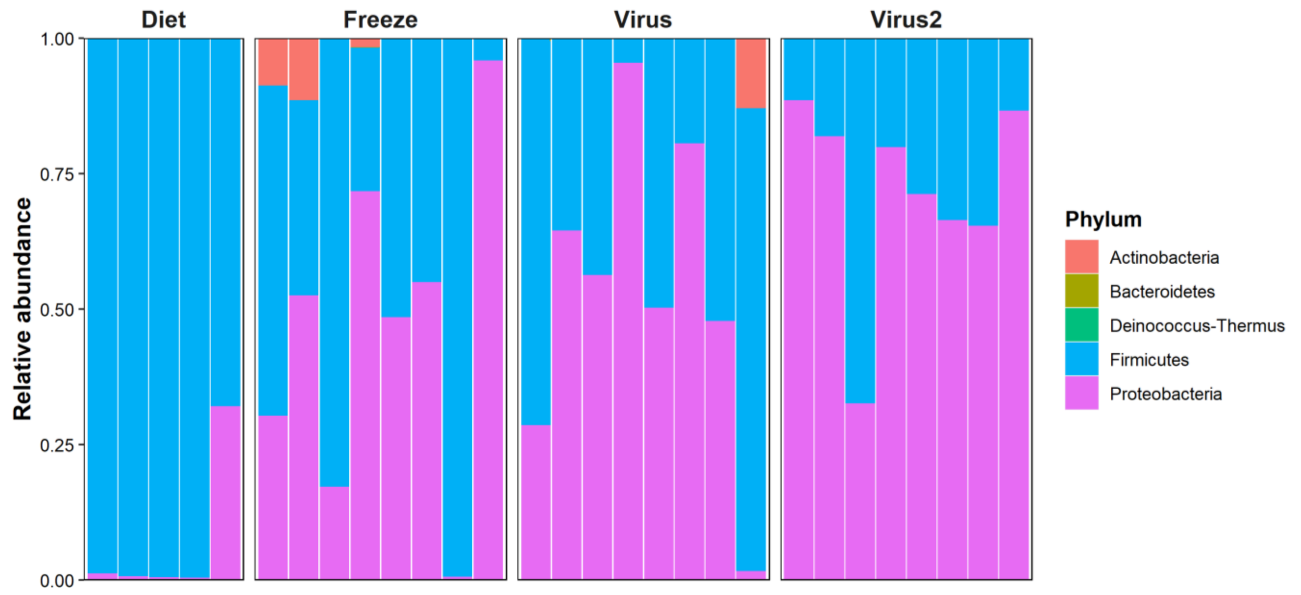

Figure 3.2: Relative abundance of bacteria phyla in *Trichoplusia ni* cadavers. Each column within a treatment represents an individual sample.

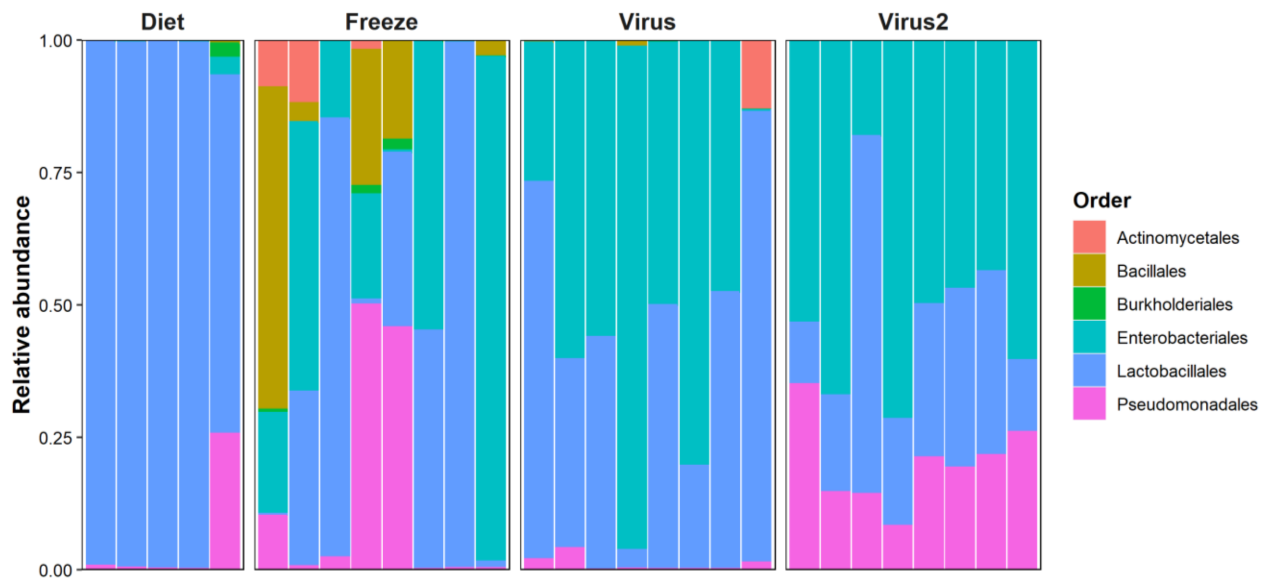

Figure 3.3: Relative abundance of the six most abundant bacteria orders in *Trichoplusia ni* cadavers. Each column within a treatment represents an individual sample.

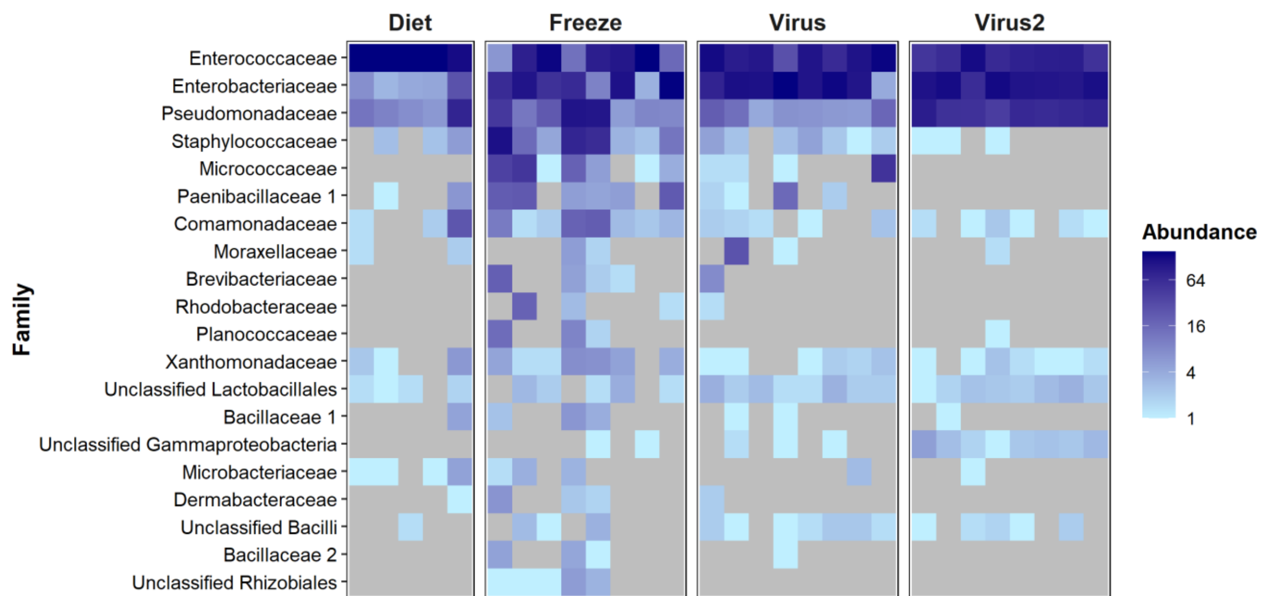

Figure 3.4: Heatmap showing 20 most abundant bacterial families in *Trichoplusia ni* cadavers. Each column within a treatment represents an individual cadaver sample. Grey boxes indicate the absence of the family from a sample. The scale bar indicates square–root transformed number of reads detected in the rarefied samples.

Table 3.1: Alpha diversity metrics of bacterial communities between different *T. ni* cadavers. Values represent mean (standard error). Values that share a letter are not statistically significantly different ($p < 0.05$).

	Diet	Freeze	Virus	Virus2	Test Statistic	p-value
OTUs	16.60 (7.67)	30.63 (5.57)	26.88 (2.17)	33.38 (2.71)	$F = 2.20$	0.11
Chao1	26.32 (10.85)	42.43 (8.42)	69.56 (17.34)	71.99 (9.84)	$\chi^2 = 8.92$	0.03[1]
Inverse Simpson	1.19 (0.18)[a]	2.13 (0.31)[ab]	1.81 (0.17)[ab]	2.33 (0.14)[b]	$\chi^2 = 10.19$	0.02
Shannon	0.22 (0.18)[a]	0.87 (0.19)[abc]	0.69 (0.09)[ab]	0.97 (0.04)[c]	$\chi^2 = 10.05$	0.02

[1]Despite a global significant difference, pairwise comparisons did not reveal significant differences between treatments

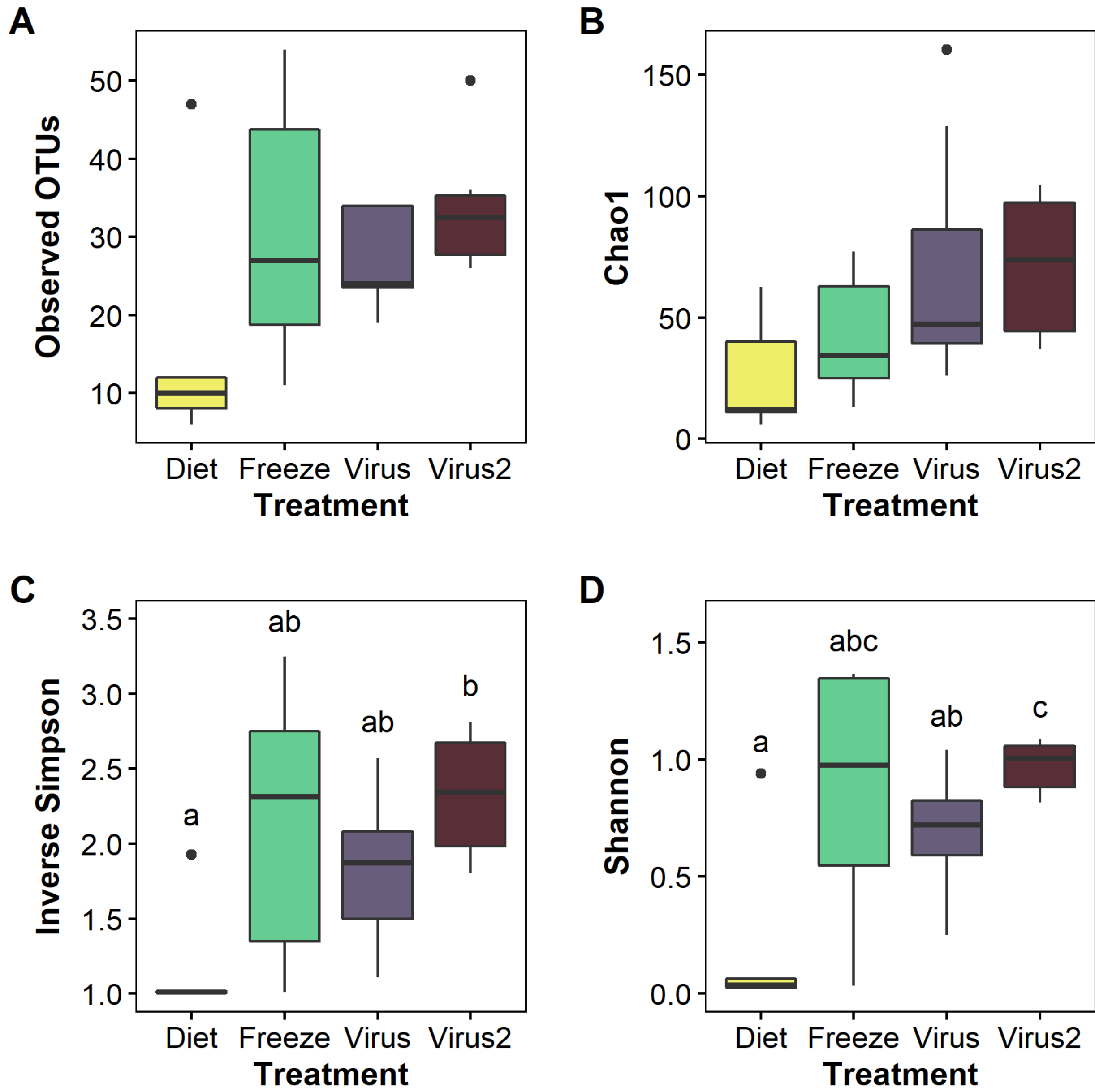

Figure 3.5: Alpha diversity metrics for cadaver bacterial communities. A) Number of observed OTUs in samples B) Chao estimate of species richness C) Inverse Simpson diversity index D) Shannon diversity index. Different letters denote statistically significant differences ($p < 0.05$).

nMDS plots of Bray-Curtis and Jaccard similarities indicate that samples largely clustered by treatment (Figure 3.6). PERMANOVA on Bray-Curtis similarities show that there was a significant effect of treatment on bacterial community composition (p = 0.002; Table 3.2). Pairwise comparisons show that the Diet and Virus2 treatments were significantly different from each other and from Freeze and Virus treatments. There was no significant difference between the Freeze and Virus treatments (Table 3.3). The Bray-Curtis plot shows that samples within the Virus2 treatment cluster closely together, whereas Freeze samples are further apart, suggesting that samples within these treatments exhibit less or more variation in bacterial community composition, respectively (Figure 3.6A). To test this, I performed a permutation test for homogeneity of multivariate dispersion. There was a significant effect of treatment on dispersion (p = 0.005). Pairwise comparisons indicated that the Freeze treatment was significantly more variable than the Diet and Virus2 treatments. The dispersion of Virus treatment samples was not significantly different compared to any of the treatments (Table 3.4).

Similarly, PERMANOVA on Jaccard similarities show that there was a significant effect of treatment on bacterial community membership (p = 0.001; Table 3.2; Figure 3.6B). Pairwise comparisons reveal that the Virus 2 treatments was significantly different from all other treatments. The Virus treatment was significantly different from Diet, but not from Freeze. Freeze and Diet treatments were not different from one another (Table 3.5).

Table 3.2: PERMANOVA outputs assessing differences in cadaver bacterial communities based on Bray-Curtis and Jaccard similarities. PERMANOVAs were generated with 1000 permutations.

Distance	**Source**	**df**	**SS**	**MS**	**Pseudo-F**	**p-value**
Bray-Curtis	Treatment	3	1.94	0.65	5.04	0.002
Jaccard	Treatment	3	1.93	0.64	3.34	0.001

Table 3.3: p-values for pairwise PERMANOVA comparisons of cadaver bacterial communities based on Bray-Curtis distance with 1000 permutations. p-values were correction for multiple corrections with a Holm correction.

	Diet	Freeze	Virus
Freeze	0.048		
Virus	0.020	0.321	
Virus2	0.006	0.028	0.048

Table 3.4: p-values for pairwise permutation test for homogeneity of multivariate dispersions based on Bray-Curtis distance. Differences in the variance were calculated through the betadisper function were tested for significance using a permutation test. The permutation tests for significant differences of median distance to centroid.

	Diet	Freeze	Virus
Freeze	0.0007		
Virus	0.0987	0.1284	
Virus2	0.2746	0.0006	0.1850

Table 3.5: p-values for pairwise PERMANOVA comparisons of cadaver bacterial communities based on Jaccard distance with 1000 permutations. p-values were correction for multiple corrections with a Holm correction.

	Diet	Freeze	Virus
Freeze	0.188		
Virus	0.015	0.118	
Virus2	0.010	0.006	0.010

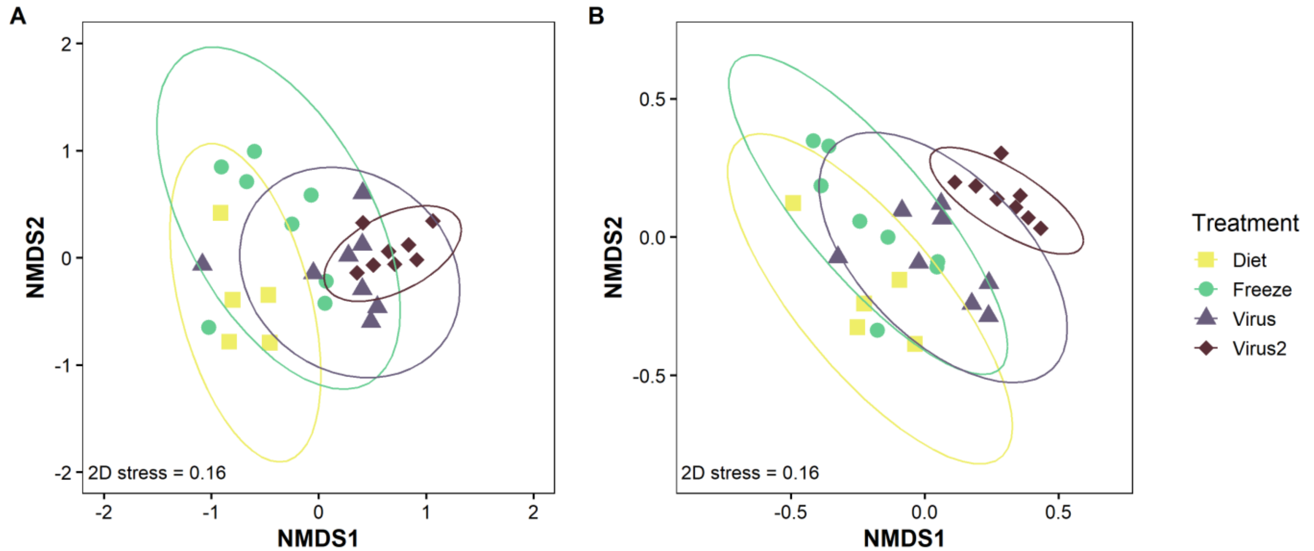

Figure 3.6: Non-metric multidimensional scaling (nMDS) plots of cadaver bacterial communities constructed from A) Bray-Curtis and B) Jaccard similarities. Stress values indicate an adequate fit of the data in two dimensions.

SIMPER analysis identified four OTUs that contributed most to Bray-Curtis similarity measures (Table 3.6). I also generated heat maps to visually show the abundance of the 20 most abundant OTUs in each sample (Figure 3.7). Diet-fed cadavers had the least diverse bacterial communities and were dominated by OTU001 (*Enterococcus* sp.) (Figure 3.7). Statistical analysis showed that this *Enterococcus* OTU was significantly more abundant in diet-fed cadavers compared to the other three treatments (Table 3.6). OTU002 (Unidentified Enterobacteriaceae) was present in high numbers in Freeze, Virus, and Virus2 samples, although only Virus2 was statistically significantly different compared to the Diet treatment (Table 3.6). Virus2 samples were also enriched for OTU003 (*Pseudomonas* sp.), and the abundance of this OTU was significantly greater in Virus2 compared to the other three treatments. Freeze samples had significantly higher levels of OTU006 (*Staphylococcus* sp.) than all other treatments, and Virus2 samples had the lowest abundance of this OTU (Table 3.6).

Table 3.6: Difference in the abundance of bacterial OTUs between treatments. OTUs were selected using a SIMPER analysis to detect those that contributed most to Bray-Curtis similarities. Analyses were performed using a Kruskal-Wallis rank sum test with a false discovery rate correction for multiple comparisons. Wilcoxon rank sum tests were performed to compare pairwise differences. Values represent means (standard errors), and those that share a letter do not differ significantly ($p < 0.05$).

	OTU001 Enterococcus sp.	OTU002 Enterobacteriaceae	OTU003 Pseudomonas sp.	OTU006 Staphylococcus sp.
Diet	20466.6 (1416.2) [a]	41.8 (19.9) [a]	8.8 (8.6) [a]	8.4 (5.4) [ab]
Freeze	8104.8 (2949.1) [b]	6811.9 (88) [ab]	204.0 (144.2) [a]	2818.9 (1592.8) [c]
Virus	9927.3 (2047.5) [b]	8750.3 (2436.9) [ab]	45.8 (44.8) [a]	8.6 (3.2) [a]
Virus2	6282.6 (1401.2) [b]	11265.0 (613.1) [b]	4331.5 (613.1) [b]	0.4 (0.2) [b]
W	10.92	10.54	18.88	17.03
p-value	**0.01**	**0.01**	**0.0003**	**0.0007**

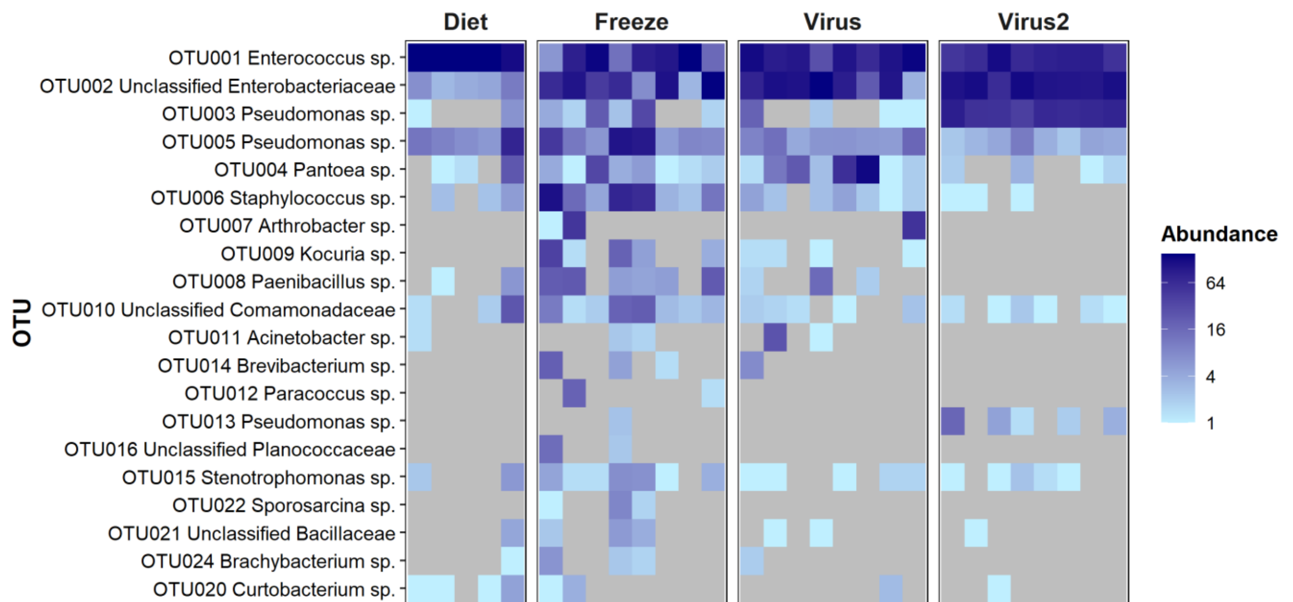

Figure 3.7: Heatmap showing 20 most abundant OTUs in *Trichoplusia ni* cadavers. Each column within a treatment represents an individual cadaver sample. Grey boxes indicate the absence of the OTU from a sample. The scale bar indicates square–root transformed number of reads detected in the rarefied samples.

I constructed a phylogenetic tree based on the 16S-rRNA V4 region with the top 25 OTUs identified from cadaver bacterial communities, bacterial isolates from culture-dependent sequencing, and bacterial type strains from RDP (Appendix B). I found that several taxa (e.g. *Pantoea, Enterococcus*, *Staphylococcus*, Enterobacteriaceae, *Curtobacterium*, *Pseudomonas*) were represented in both OTUs from culture-independent sequencing and bacterial isolates obtained from culture-dependent work (Appendix B). However, other taxa (e.g. *Paracoccus*, *Stenotrophomonas*, *Kocuria*, *Brachybacterium*) were only identified by culture-independent sequencing (Appendix B). For OTUs that were identified only to family level (OTU002, OTU010, OTU016, OTU021), I was unable to reliably classify further based on gene sequences from the 16S-rRNA V4 region (Appendix B).

Effect of cadavers on plant defenses

In plants subjected to mechanical damage, there was a significant effect of cadaver application ($F = 5.40$, $p = 0.006$), damage ($F = 4.92$, $p = 0.03$), and the interaction between cadaver application and damage ($F = 3.62$, $p = 0.03$) on PPO (Figure 3.8A). In undamaged plants, the applications of water or cadavers had no suppressing or inducing effects on PPO activity. While mechanical damage treated with water or freeze-killed cadavers induced PPO activity, the PPO activity of mechanically damaged plants treated with virus-killed cadavers was suppressed to a similar level as undamaged plants. For TPI, there was a significant effect of damage ($F = 35.64$, $p < 0.0001$), but not application ($F = 0.7$, $p = 0.50$), or the interaction of damage and application (Figure 8B; $F = 0.5$, $p = 0.61$).

In the experiment with healthy larvae feeding plus cadaver applications, there was a significant effect of treatment on PPO (Figure 3.9A; $F = 12.91$, $p < 0.0001$) and TPI (Figure 3.9B; $F = 33.5$, $p < 0.0001$). However, post-hoc pairwise comparisons revealed that the only significant differences were between undamaged plants and the other three treatments. Thus, applications of cadavers or water did not affect PPO or TPI in plants damaged by healthy larvae.

In the experiment with infected and healthy larvae feeding, there was a significant effect of treatment on PPO (Figure 3.10A; $F = 2.72$, $p = 0.04$) and TPI (Figure 3.10B; $F = 2.67$, $p = 0.04$). When I conducted pairwise comparisons of PPO between treatments with p-value adjustment for multiple comparisons, Un+Water treatment was only close to significantly different to Hel+Water ($p = 0.0507$) and In+Virus ($p = 0.0633$). Similarly, pairwise comparisons

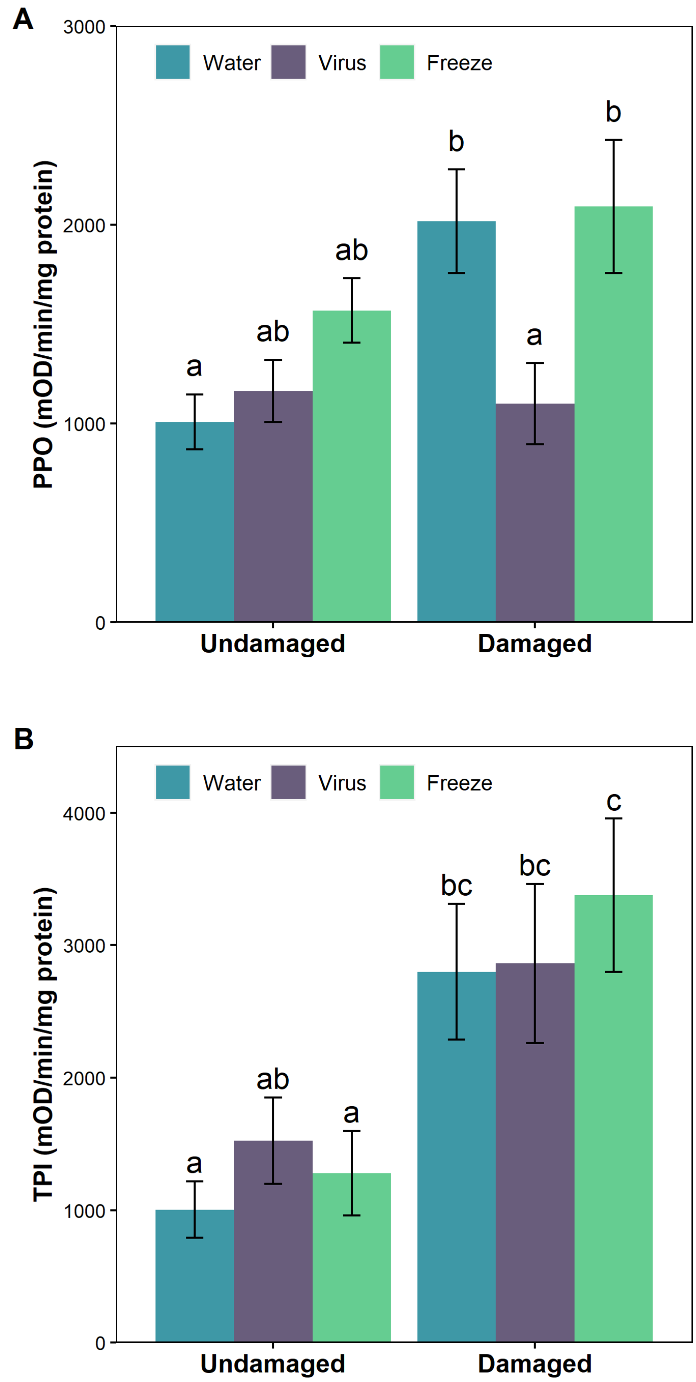

Figure 3.8: Influence of cadavers on A) polyphenol oxidase (PPO) and B) trypsin inhibitor (TPI) tomato defenses. Water, virus-killed cadaver, or freeze-killed cadaver were applied to undamaged, intact plants or mechanically damaged plants. Different letters denote statistically significant differences ($p < 0.05$).

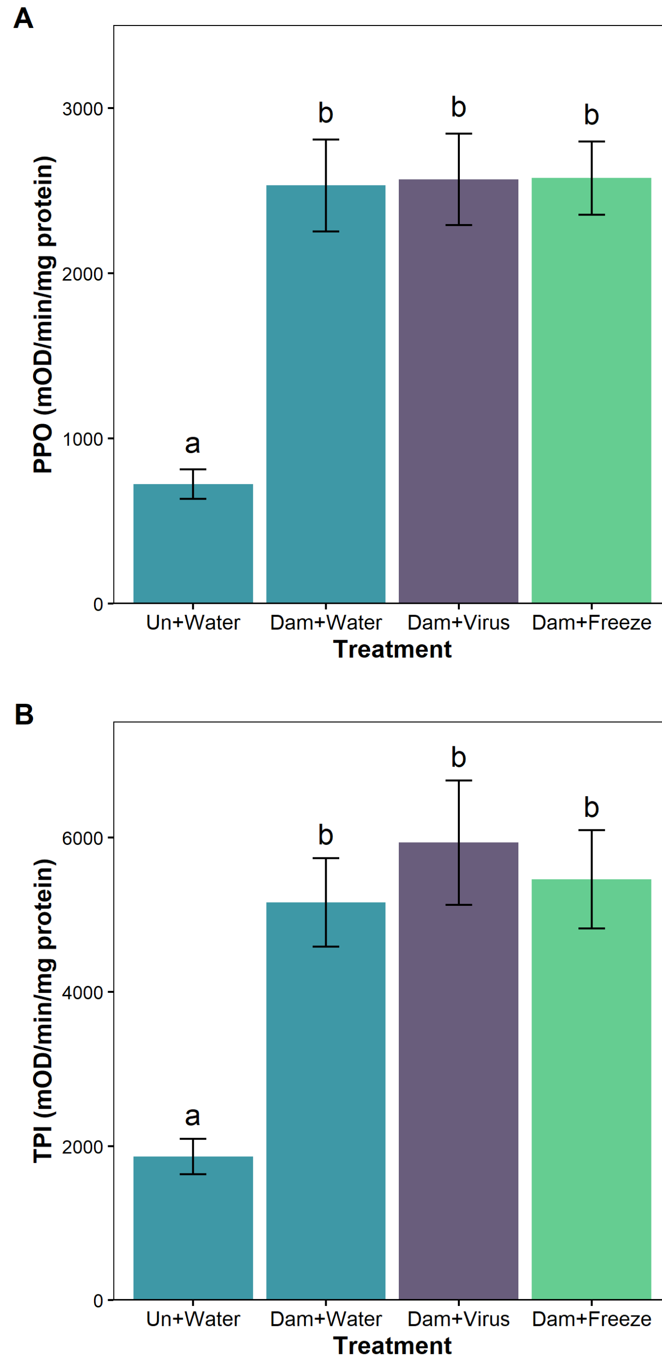

Figure 3.9: Influence of cadavers on A) polyphenol oxidase (PPO) and B) trypsin inhibitor (TPI) tomato defenses. Un+Water: Undamaged control plants; Dam+Water: Plants were damaged by healthy *T. ni* larvae followed by application of water; Dam+Virus: Plants were damaged by healthy *T. ni* larvae followed by application of virus-killed cadaver; Dam+Freeze: Plants were damaged by healthy *T. ni* larvae followed by application of freeze-killed control cadaver. Different letters denote statistically significant differences ($p < 0.05$).

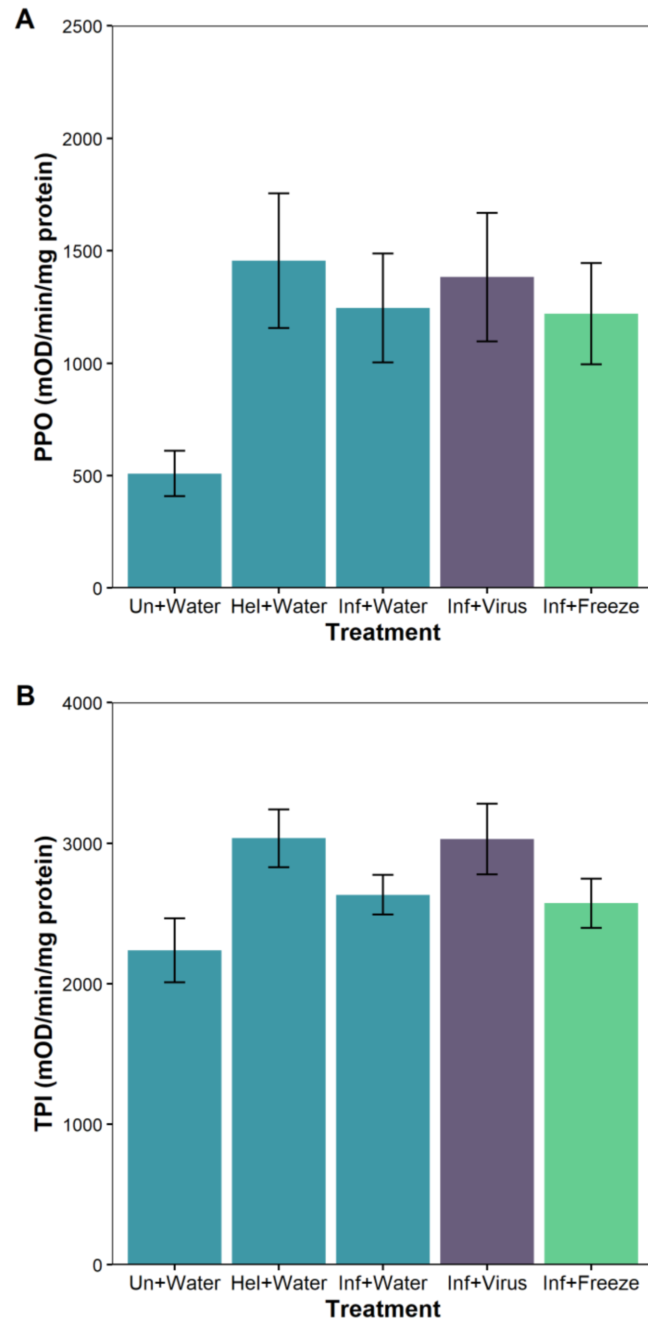

Figure 3.10: Influence of cadaver applications on A) polyphenol oxidase (PPO) and B) trypsin inhibitor (TPI) tomato defenses. Un+Water: Undamaged control plants; Hel+Water: Plants were damaged by healthy *Trichoplusia ni* larvae followed by application of water; Inf+Water: Plants were damaged by AcMNPV-infected *T. ni* larvae followed by application of water; Inf+Virus: Plants were damaged by AcMNPV-infected *T. ni* larvae followed by application of virus-killed cadaver; Inf+Freeze: Plants were damaged by AcMNPV-infected *T. ni* larvae followed by application of freeze-killed cadaver. Different letters denote statistically significant differences ($p < 0.05$).

for TPI revealed almost significant differences between Un+Water and Hel+Water (p = 0.0692), and between Un+Water and In+Virus (p = 0.0616). The addition of covariates (herbivory area, feeding weight, larval weight, days till death) into an ANCOVA model did not reveal differences between treatments for PPO or TPI.

Effect of cadavers on larval choice

The application of cadavers to leaf discs did not affect larval choice (Figure 3.11). There was no difference in the number of larvae that chose between leaf discs with freeze-killed and virus-killed cadavers (p = 0.383), between leaf discs with water and virus-killed cadavers (p = 1), or between leaf discs with water and freeze-killed cadavers (p = 1).

Similarly, cadaver application did not affect larval consumption. Leaf area consumption was not significantly different between leaf discs applied with virus-killed cadavers and freeze-killed cadavers (p = 0.517), between water and virus-killed cadavers (p = 0.422), and between water and freeze-killed cadavers (p = 0.714).

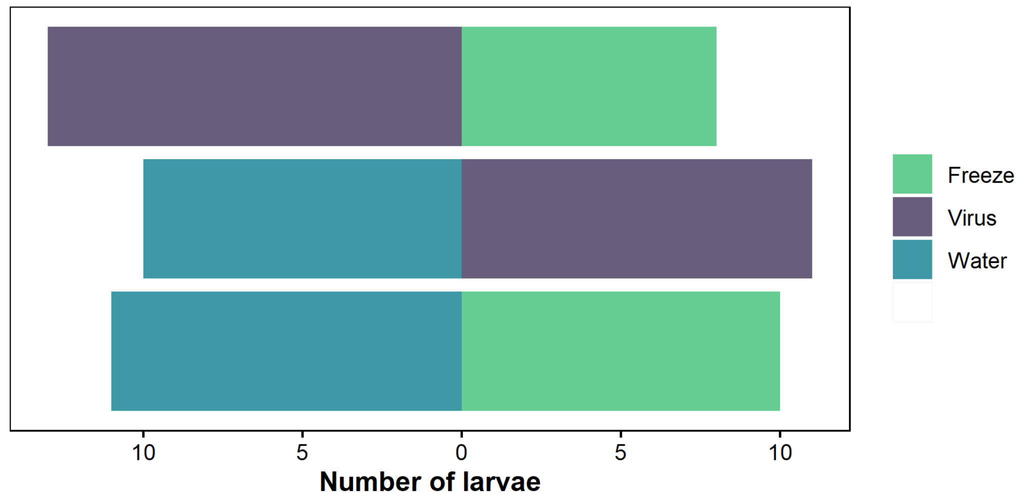

Figure 3.11: Influence of virus-killed or freeze-killed cadaver applications on *Trichoplusia ni* larval choice in two-way leaf disc Petri dish assays (n = 18 per treatment combination).

Effect of cadavers on oviposition

Female moths laid an average of 145.6 eggs (SE = 8.2) per tent. There was no difference in the number of eggs laid between plants in any of the three treatment combinations (Figure 3.12). Female moths laid similar numbers of eggs on plants treated with virus-killed cadavers compared to water (Figure 3.12A; $t = -0.56$, $p = 0.59$), on plants treated with freeze-killed cadavers compared to water (Figure 3.12B; -1.09, $p = 0.29$), and on plants treated with freeze-killed cadavers compared to virus-killed cadavers (Figure 3.12C; $t = 0.006$, $p = 0.995$). There was no significant difference in the number of eggs laid between plants on left and right sides of the tents.

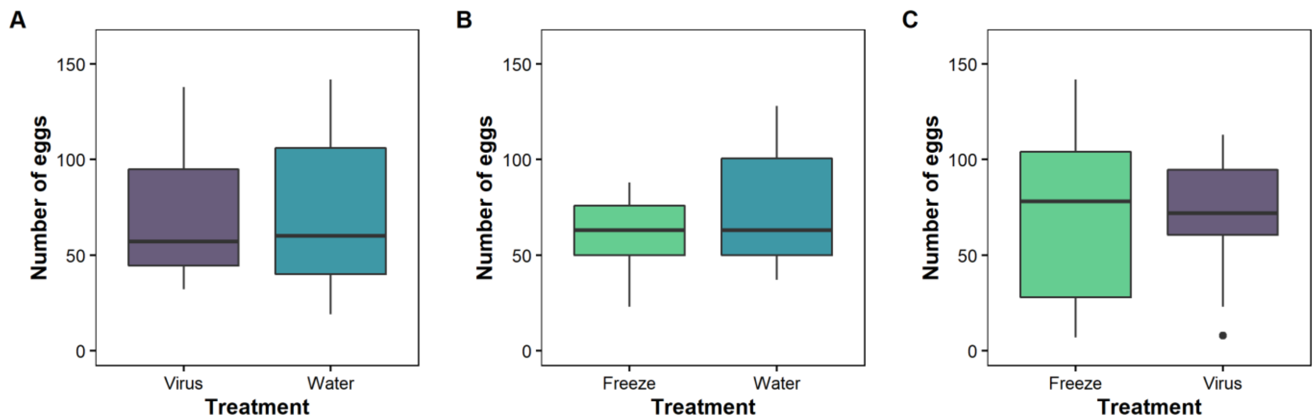

Figure 3.12: Influence of virus-killed or freeze-killed cadaver applications on *Trichoplusia ni* oviposition in two-way choice tests with tomato plants (n=18 per treatment combination). A) Virus-killed cadavers compared to water treatment, B) Freeze-killed cadavers compared to water treatment, and C) Freeze-killed cadavers compared to virus-killed cadavers. Analyses were performed using square root–transformed data.

Discussion

Baculoviruses are host-specific pathogens of lepidopteran insects with important applications for biological control of herbivore pests (Lacey et al., 2015). At the end of the baculovirus infection cycle, host tissues rupture, releasing viral OBs and bacteria from liquified cadavers. The interactions of these cadavers with plants and healthy insects are likely to be important in understanding virus persistence and transmission in the environment, yet relatively little is known about baculovirus cadaver ecology. To provide the first characterization of

bacterial communities associated with baculovirus-killed cadavers, I examined AcMNPV-killed *T. ni* cadavers fed on tomato. Bacterial communities associated with freeze-killed and virus-killed cadavers from the same experiment were similar, although bacterial concentrations in virus-killed cadavers were higher. Virus-killed cadavers suppressed tomato plant defenses in mechanically wounded plants. However, I did not observe suppression of defenses in plants wounded by healthy or virus-infected caterpillars. Applications of cadavers to undamaged tomato plants did not alter *T. ni* oviposition behavior or larval feeding preference.

After caterpillars ingest viral OBs, the OVs penetrate the protective lining of the midgut (peritrophic matrix; PM) to access the midgut epithelial cells (Passarelli, 2011). The OVs replicate in the nucleus of these cells and produce the secondary BV virion phenotype. BVs move throughout the caterpillar body via tracheoles and haemocytes, eventually reaching the fat body which is a major site of BV replication. The midgut epithelial cells are often sloughed and regenerated after initial infection (Passarelli, 2011), allowing hosts to continue to feed and grow, leading to improved OB yields per cadaver (Slack & Arif, 2006). The effect of gut bacteria in the baculovirus infection process is not well established. Bacteria play an important role in the pathology of another insect pathogen, *Bacillus thuringiensis* (Bt). Bacterial toxins disrupt the caterpillar PM, allowing midgut bacteria to breach this barrier, enter the hemocoel, and cause septicemia (Broderick et al., 2006; Caccia et al., 2016). The reason for the higher bacterial abundances I observed in virus-killed cadavers compared with freeze-killed cadavers (Figure 3.1) could be due to several factors. It is possible that initial baculovirus infection in the midgut allows bacteria to enter and proliferate in the body cavity or other insect tissues, in a manner similar to Bt. Viral infection could also affect bacterial gut populations through modulation of immune responses. Sublethal infection by the baculovirus SeMNPV downregulated a number of immune-related genes in the *Spodoptera exigua* gut, and this was associated with increased gut bacterial titers (Jakubowska et al., 2013). SeMNPV also produced fewer OBs in antibiotic-treated larvae, suggesting that bacteria could play a role in baculovirus pathology (Jakubowska et al., 2013). More research is needed to understand how the host bacteriome affects baculoviruses at different stages of infection.

After caterpillars die from baculovirus infection, bacteria could exploit liquified cadaver tissues; the higher bacterial abundances that I observed in virus-killed cadavers could represent proliferation of bacteria after host death. In this study, I sampled bacterial communities from

virus-killed cadavers within 12 hours of death, whereas freeze-killed cadavers were sampled immediately after death. Thus, I could not determine whether bacterial concentrations increased in virus-killed cadavers prior to or after death. It is also likely that bacterial titers in freeze-killed cadavers would increase over time as bacteria break down corpse tissues (López-Riquelme and Fanjul-Moles, 2013). Studies that examine bacterial abundances over the course of bacterial infection and cadaver decomposition would help address these questions.

I used CFUs per mg tissue to measure cadaver bacterial abundance. Although this method is a commonly used to estimate bacterial concentrations, there are several limitations of this culture-dependent approach such as clumping of bacterial cells that affect counts and the presence of unculturable bacteria (Sutton, 2012). However, Illumina sequencing showed that cadaver bacterial communities were simple, being primarily composed of six OTUs representing *Enterococcus*, Enterobacteriaceae, *Pseudomonas*, *Pantoea*, and *Staphylococcus* (Figure 3.7). These highly abundant taxa represented 85–99% of sequencing reads across samples and are readily culturable, suggesting that the culture-based approach I used to estimate abundance is likely to be appropriate.

Over the process of host infection and cadaver decomposition, baculovirus-mediated changes to larval tissues and physiology could provide favorable conditions for proliferation of certain bacteria taxa over others. Interspecific competition among microbes could also shape the microbial composition of hosts (Wollenberg et al., 2016). Infection of grain beetles (*Tenebrio molitor*) with another pathogen, the tapeworm *Hymenolepis diminuta*, significantly altered the structure of bacterial and fungal gut communities (Fredensborg et al., 2020). In one of the few studies investigating bacterial communities associated with arthropod cadavers, waxworm cadavers killed by the entomopathogenic nematode *Heterorhabditis* contained bacterial communities that were influenced by the nematode's bacterial symbiont and changed over time (Wollenberg et al., 2016).

Contrary to my hypothesis, I did not find significant differences in the composition (Bray-Curtis) or membership (Jaccard) of bacterial communities between virus-killed (Virus) and freeze-killed cadavers (Freeze) from the same experiment (Figure 3.6; Tables 3.3 & 3.5). Virus- and freeze-killed cadaver bacterial communities also had similar richness and evenness (Figure 3.5; Table 3.1). When I analyzed abundance of individual OTUs identified by SIMPER

analysis as contributing most substantially to Bray-Curtis similarities, I found *Staphylococcus* OTU was more abundant in freeze-killed cadavers compared with virus-killed cadavers (Table 3.6). Abundance of this OTU was also low in virus-killed cadavers from the second experiment (Virus2), suggesting that virus infection could make cadavers unfavorable for *Staphylococcus* or inhibit growth of this bacteria somehow, although more studies are required to examine this. There were no differences in the abundance of other individual OTUs between Virus and Freeze cadavers (Table 3.6).

The bacterial community associated with virus-killed cadavers from the second experiment (Virus2) was markedly different from other treatments. This treatment had the highest alpha diversity (Figure 3.5), and dispersion analysis revealed that samples within this treatment were less variable compared with other treatments (Table 3.4). Virus and Virus2 samples were significantly different in terms of composition (Table 3.3) and membership (Table 3.5). In terms of individual OTUs, Virus and Virus2 had similar abundances of OTUs corresponding to *Enterococcus* and an unclassified Enterobacteriaceae, but Virus2 samples had higher levels of a *Pseudomonas* OTU and lower abundances of a *Staphylococcus* OTU compared to Virus samples (Table 3.6). This between-experiment variation in virus-killed cadaver bacterial communities may be driven by tomato phylloplane bacteria. Lepidopteran bacterial communities are influenced by host plant in other systems (Chaturvedi et al., 2017; Jones et al., 2019; Mason & Raffa, 2014; Priya et al., 2012). Although both sets of experimental caterpillars received tomato foliage of the same genotype grown in the same greenhouse, these plants were grown at different times and could have contained different foliar bacteria. Unfortunately, I did not obtain sufficient sequencing reads from foliage samples to analyze tomato bacterial communities, and culture-dependent plating of foliage also showed extremely low CFU abundance (data not shown). Although the food plant likely provides initial inoculum for *T. ni* bacterial communities, bacteria can proliferate in the lepidopteran midgut (Mason et al., 2020), and communities are ultimately the result of dynamic processes that are not well understood. Most insect microbiome studies do not present data from independent experimental repeats, but this study suggests that such replication could be an important factor to consider.

Unsurprisingly, bacterial communities associated with artificial diet-fed cadavers were the least rich and even (Figure 3.5) and were dominated by an *Enterococcus* OTU (Figure 3.7). These findings mirror other studies that show the influence of artificial diet on caterpillar gut

bacteria (Mason et al., 2020) and highlight the importance of using host plants when investigating bacterial communities of Lepidoptera. Bacterial communities associated with tomato-fed cadavers (Freeze, Virus, Virus2) had higher alpha diversity than those of diet-fed cadavers (Figure 3.5); these communities were composed primarily of bacteria belonging to Lactobacillales, Enterobacteriales, Pseudomonadales, Bacillales, and Actinomycetales (Figure 3.3), in agreement with a recent study of gut bacterial communities of *T. ni* fed on different host plants (Garcia et al., 2020).

Analysis of dominant OTUs in tomato-fed cadavers were identified as *Pseudomonas*, *Enterococcus*, *Pantoea, Staphylococcus, Paenibacillus, Kocuria, Stenotrophomonas,* unidentified Enterobacteriaceae, and unidentified Comamonadaceae (Figure 3.7). While many of these taxa (e.g. *Pantoea, Enterococcus*, *Pseudomonas*) were identified in both culture-independent and culture-dependent studies (Appendix B), other taxa (e.g. *Paracoccus*, *Stenotrophomonas*, *Kocuria*) were only identified from culture-independent sequencing (Appendix B). These discrepancies could be due to biases in culture-based methods (Shi et al., 2010) and/or represent variation in bacterial composition between experiments.

Many of the bacteria genera and families I identified in *T. ni* cadavers (Figure 3.4; Figure 3.7) are commonly associated with lepidopteran guts (Paniagua Voirol et al., 2018) and were identified from *T. ni* fed on cabbage (Lawrence et al., 2020). Some of these taxa (e.g. *Pantoea, Pseudomonas*, and Enterobacter sp.) have also been isolated from the oral secretions of other folivorous insects and are able to suppress plant defenses when applied to tomato leaves (Acevedo et al., 2017; Chung et al., 2013; Wang et al., 2016), suggesting that cadaver-associated bacteria could influence plant defenses.

To inform my experiments on the effects of cadavers on plant defenses, I first measured the location of virus-killed cadavers on plants. Specifically, I was interested in whether cadavers died on leaves damaged with feeding wounds, or if larvae moved to intact leaves prior to death. Approximately 45% of virus-killed cadavers were found on the ground, indicating that they had fallen from the plant prior to or at the point of death. Although other studies have reported infected larvae falling off plants prior to death, this “knock off” behavior was associated with a recombinant AcMNPV virus (Hails et al., 2002; Hoover et al., 1995). Of the cadavers that died

on plants, most died adjacent to feeding wounds, indicating that application of virus-killed cadavers to wounded plant tissue is an appropriate treatment.

I first examined the influence of cadavers on tomato defenses by applying water, freeze-killed cadavers, or virus-killed cadavers to mechanically wounded tomato plants. Virus-killed cadavers applied to wounds suppressed PPO to levels close to those of undamaged plants, whereas freeze-killed cadavers and water applied to mechanical wounds increased PPO levels compared with undamaged plants (Figure 3.8). However, in experiments where plants were wounded by healthy or AcMNPV-infected *T. ni* larvae, application of virus-killed cadavers did not affect PPO levels (Figure 3.9 and 3.10). Although mechanical wounding often activates similar plant responses compared to true herbivory, the expression of defenses often depends on plant recognition of herbivore-associated cues in saliva and other secretions (Erb et al., 2012; Waterman et al., 2019). My findings suggest that elicitors present in *T.* ni saliva may override the effects of virus-killed cadavers on downregulating tomato defenses. I also did not find differences in PPO or TPI levels in plants fed on by healthy or AcMNPV-infected larvae, in contrast to a recent study that observed higher TPI levels in tomato plants fed on by AcMNPV-infected *H. zea*, compared with healthy *H. zea* (Pan et al., 2019). The discrepancy between studies could be due to differences in caterpillar species, viral dose (lethal vs. sublethal), or infection duration.

Although cadaver applications did not influence induction of tomato defenses in response to herbivory, my finding that virus-killed cadavers suppressed PPO in mechanically damaged plants provides the first evidence that baculoviruses can modulate plant defenses via host cadavers. PPOs are important inducible defense enzymes present in many plants. The induction of PPO is regulated by the phytohormone jasmonic acid (JA), and expression of this enzyme is often used as a marker for JA-induced defenses in tomato (Bosch et al., 2014). Baculoviruses could benefit from the suppression of plant defenses because these compounds can reduce the infectivity of OBs (Cory and Hoover, 2006). Oxidation of phenolic compounds by PPO produces quinones which can inhibit baculovirus infection by binding to OBs and preventing release of virions (Felton and Duffey, 1990). However, cadavers represent highly concentrated levels of OBs, hundreds to thousands of times higher than the LD_{50} doses reported for AcMNPV (Haas-Stapleton et al., 2005; Kunimi et al., 1997; Wilson et al., 2000); extremely high doses of OBs are likely to override the influence of phytochemicals on viral infectivity (Shikano, 2017). PPOs also

play important roles in plant defense against chewing herbivores because quinones can interfere with insect digestion (Constabel and Barbehenn, 2008), and PPO activity is often negatively correlated with caterpillar growth (Castañera et al., 1996; Felton et al., 1989). Suppression of PPO by virus-killed cadavers could enhance insect preference for feeding on these plants and increase the likelihood of hosts ingesting OBs. JA also regulates production of tomato volatiles such as terpenoids (Degenhardt et al., 2010; van Schie et al., 2007), which could influence attraction or egg-laying behavior of hosts.

The suppression of plant defenses by baculovirus cadavers that I observed in mechanically wounded plants could be mediated by viral OBs or cadaver-associated bacteria. Plants use receptor proteins to recognize microbes through conserved microbial-associated molecular patterns (MAMPs), triggering plant responses that predominantly involve the SA signalling pathway (Dodds and Rathjen, 2010). Induction of the SA pathway by cadaver-associated microbes could suppress JA-mediated defenses through signalling crosstalk (Li et al., 2019). Baculoviruses and cadaver bacteria may possess MAMPs similar to those described from plant pathogenic bacteria and viruses (Calil and Fontes, 2017; Newman et al., 2013). Additionally, baculoviruses produce occlusions, crystalline lattices of polyhedron protein that surround virions, enclosed by a polyhedral envelope (Slack & Arif, 2006). Carbohydrates (e.g. hexoses, pentoses, uronic acids, and hexosamines) and proteins (e.g. polyhedral envelope protein) that comprise the OB envelope (Slack & Arif, 2006) could provide further cues for plant recognition of baculoviruses, a hypothesis that warrants further attention.

In addition to plant-mediated effects, virus-killed cadavers could directly affect insect behavior or performance. Because I did not find evidence that virus-killed cadavers influenced plant defenses induced by herbivory, I only examined *T. ni* behavior in response to cadavers applied to intact plants. Surprisingly, I found no evidence that virus-killed cadavers or freeze-killed cadavers affected *T. ni* larval feeding preferences or leaf consumption (Figure 3.10). In contrast, other studies have reported that gypsy moth (*Lymantria dispar*) avoided foliage contaminated with baculovirus-killed cadavers (Capinera et al., 1976; Parker et al., 2010). In another study, healthy *Spodoptera exigua* larvae preferred feeding on leaf discs treated with virus-killed cadavers compared to control (water-applied) leaf discs, but there was no difference in preference between virus-killed cadavers and control cadavers (Rebolledo et al., 2015). However, on plants, *S. exigua* encountered and fed on baculovirus-killed cadavers more

frequently than non-infected cadavers. The authors attributed this to virus-killed larvae tending to die on the upper portion of plants, corresponding to the area of the plant on which larvae preferentially fed (Rebolledo et al., 2015).

I also examined how cadavers influenced *T. ni* oviposition behavior. I am not aware of other studies that have examined whether presence of cadavers influences oviposition preference of host insects. Given the patchy nature of viral OBs on a plant (Goulson, 1997), and limited mobility of neonate caterpillars (Soler et al., 2012), the location of egg deposition by female moths in relation to cadavers could play an important role in virus infection and transmission rates. In contrast with my hypothesis, I found no evidence that *T. ni* oviposition behavior was affected by the presence of cadavers. Females laid equal numbers of eggs on tomato plants treated with virus-killed cadavers, freeze-killed cadavers, or water (Figure 3.11).

Given that baculoviruses are expected to impose intense selection pressure on insects (Parker et al., 2010), it may be surprising that *T. ni* caterpillars and moths do not avoid virus-killed cadavers. Herbivorous insects face a wide variety of predators, parasitoids, and pathogens, and cues associated with these phylogenetically distant threats are likely highly diverse. Insects have small brains and may face neural constraints that limit the amount of sensory information that they can process (Bernays, 2001). Because generalist insects, such as *T. ni*, must process sensory information associated with multiple host plants, they may be less able to discriminate threats than specialist insects (Bernays, 2001). Yet, gypsy moth (*Lymantria dispar)*, another generalist lepidopteran, was able to avoid virus-killed cadavers and this behavior was heritable (Parker et al., 2010). A previous study found that gypsy moth also avoided uninfected cadavers and that this deterrence effect was due to larval body components, rather than OBs (Capinera et al., 1976). Feeding on conspecifics is likely to be a risky behavior due to the potential for transmission of a range of pathogens and parasites (Rebolledo et al., 2015), so a more general avoidance of cadavers by larvae could be adaptive. In my study, the presence of freeze-killed control cadavers also did not affect *T. ni* oviposition or larval choice. It is possible that the ability to detect and avoid cadavers has been lost over many generations in the lab.

One of the factors limiting more widespread adoption of baculovirus-based biopesticides is the stability of formulations (Popham et al., 2016). Purified OBs are rapidly inactivated by UV light, whereas OBs in cadavers can persist longer on plant surfaces, likely due to the protective

nature of host body parts (Dolinski et al., 2015; Fuller et al., 2012; Shapiro-Ilan et al., 2001). It is also possible that bacteria could play a role in OB persistence through by producing metabolites or modifying cadaver pH (Ratzke and Gore, 2018). Although use of ground-up cadavers in commercial formulations may not be feasible due to product regulations (Fuller et al., 2012), better understanding of cadaver factors that protect OBs could improve baculovirus biopesticides, particularly if larvae and moths do not avoid these components.

Baculoviruses also interact with other trophic levels besides herbivores. Parasitoids have been shown to mechanically transmit baculoviruses from moribund larvae or cadavers to healthy larvae, and baculovirus infection can negatively impact developing endoparasitoids (Cossentine, 2009). Parasitoids may also avoid ovipositing in virus-infected hosts (Cossentine, 2009). However, once baculoviruses cause host death and liquification, there have been few studies investigating how cadavers interact with other trophic levels and influence viral transmission. Arthropod cadavers can be food resources for ants and other predators (Baur et al., 1998; Seastedt et al., 1981) and presence of non-infected cadavers on plants has been shown to affect predator behavior. Arthropod carrion entrapped in tobacco trichomes enhanced the retention of the predator *Jalysus wickhami* on plants, reduced cannibalism, and increased oviposition (Nelson et al., 2020). In another study, compounds produced by the symbiotic bacteria associated with some entomopathogenic nematodes were found to deter ants from scavenging on cadavers, thus protecting EPNs from consumption by non-hosts (Foltan and Puza, 2009; Zhou et al., 2002). Although baculoviruses could benefit from repelling predators to limit OB consumption by non-permissive hosts, studies have found that viable OBs can be excreted by predators (Fuxa et al., 1993; Vasconcelos et al., 1996), suggesting predators could spread OBs through the environment. More research is needed to investigate how other natural enemies interact with virus-killed cadavers.

Although many questions remain about the ecological impacts of virus-killed cadavers on plants and insects, I hope these initial insights provide a starting point for future studies. In addition to characterizing the bacterial communities associated with AcMNPV-killed cadavers, I found limited evidence that these cadavers could suppress plant defenses. *T. ni* did not discriminate between plants treated with water or cadavers, despite the inherent risks of contact with cadavers containing high concentrations of infectious OBs. *T. ni* is highly polyphagous, feeding on more than 160 plant species, and host plant species strongly affects the transcriptome

of *T.* ni salivary glands (Rivera-Vega et al., 2017). Given that gut bacterial communities of larvae = also likely differ according to host plant (Garcia et al., 2020), I expect that the effects of insect feeding and application of virus-killed cadavers on plant defenses are highly system-specific. Future research aimed at understanding evolution of baculovirus–insect–plant interactions would likely benefit from using a natural system (rather than a domesticated plant such as tomato) and more highly coevolved organisms (i.e., specialist insects and baculoviruses).

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Chapter 4

Potential impacts of translocation of neonicotinoid insecticides to cotton (*Gossypium hirsutum*) extrafloral nectar on parasitoids

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Abstract

Neonicotinoid seed treatments are frequently used in cotton (*Gossypium hirsutum*) production to provide protection against early-season herbivory. However, there is little known about how these applications affect extrafloral nectar (EFN), an important food resource for arthropod natural enemies. Using enzyme-linked immunosorbent assays, I found that neonicotinoids were translocated to the EFN of clothianidin- and imidacloprid-treated, greenhouse-grown cotton plants at concentrations of 77.3 ± 17.3 and 122.6 ± 11.5 ppb, respectively. I did not find differences in the quantity of EFN produced by neonicotinoid-treated cotton plants compared to untreated controls, either constitutively or after mechanical damage. Metabolomic analysis of sugars and amino acids from treated and untreated plants did not detect differences in overall composition of EFN. In bioassays, female *Cotesia marginiventris* (Hymenoptera: Braconidae) parasitoid wasps that fed on EFN from untreated, clothianidin-treated, or imidacloprid-treated plants demonstrated no difference in mortality or parasitization success. I also conducted acute toxicity assays for *C. marginiventris* fed on honey spiked with clothianidin and imidacloprid and established LC_{50} values for male and female wasps. Although LC_{50} values were substantially higher than neonicotinoid concentrations detected in EFN, caution should be used when translating these results to the field where other stressors could alter the effects of neonicotinoids. Moreover, there are a wide range of possible sublethal impacts of neonicotinoids that were not explored here. These results suggest that EFN is a potential route of exposure of neonicotinoids to beneficial insects and that further field-based studies are warranted.

Introduction

Use of insecticidal seed treatments in production of field crops, such as corn (*Zea mays*), soybean (*Glycine max*), and cotton (*Gossypium hirsutum*), is widespread in the United States and worldwide (Douglas and Tooker, 2015; Jeschke et al., 2011; Stewart and Baute, 2013). Seeds are commonly coated with neonicotinoid insecticides, including thiamethoxam, clothianidin, and imidacloprid. Although these systemic chemicals can provide protection from herbivorous insect pests during early stages of plant growth (Goulson, 2013; Jeschke et al., 2011), it has been suggested that the prophylactic use of neonicotinoid seed treatments (NSTs) violates principles of integrated pest management (IPM) because insecticides are used indiscriminately without regard for pest pressure (Tooker et al., 2017). Although overall insecticide application rates across the United States have decreased, recent analyses show that total land area receiving insecticides and the potency of insecticides have increased, leading to a greater risk of toxicity for beneficial insects (DiBartolomeis et al., 2019; Douglas et al., 2020). These trends are primarily due to an increase in adoption of NSTs (Douglas et al., 2020). Despite being applied to seeds, neonicotinoids can reach beneficial insects via a variety of routes, including soil (Atwood et al., 2018; Zaller et al., 2016), insecticidal dust associated with planting of coated seeds (Krupke et al., 2017; Nuyttens et al., 2013), translocation to guttation droplets (Girolami et al., 2009) and floral resources (Botías et al., 2015; Krupke et al., 2012), and by feeding on tainted prey (Douglas et al., 2015) or honeydew excreted by hemipterans (Calvo-Agudo et al., 2019).

Exposure to neonicotinoid residues can have both lethal and sublethal effects on non-target beneficial arthropods including parasitoids and predators of herbivorous insects (Douglas and Tooker, 2016; Hopwood et al., 2013). Sublethal doses of neonicotinoids can affect insect movement and orientation (Baines et al., 2017; Tappert et al., 2017), foraging behavior (Schneider et al., 2012), communication (Tappert et al., 2017), learning (Piiroinen and Goulson, 2016; Tan et al., 2015), immunity (Brandt et al., 2017; Di Prisco et al., 2013), and reproduction (Straub et al., 2016; Whitehorn et al., 2015). Such negative impacts could affect the role natural enemies play in biological control of plant pests and as components of IPM programs (De Bach and Rosen, 1991; Orr, 2009).

Extrafloral nectar (EFN) is another potential source of neonicotinoid exposure for beneficial insects (Bredeson and Lundgren, 2018; Moscardini et al., 2014; Stapel et al., 2000).

This sugary substance is secreted from nectaries located on non-floral parts of many plant species, including crops such as castor (*Ricinus communis*), bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), and cotton. In contrast to floral nectar that serves to attract pollinators, the primary function of EFN is thought to be a reward for predators and parasitoids (Lundgren, 2009; Röse et al., 2006) that can deliver top-down control of herbivore pests (Heil, 2008), possibly improving plant fitness (Cuautle and Rico-Gray, 2003; Kost and Heil, 2005, 2008). Although concern over pollinator declines has generated a multitude of studies on neonicotinoid translocation into floral nectar (Pisa et al., 2017), less attention has been given to the effects of neonicotinoid use on EFN (Bredeson and Lundgren, 2018; Moscardini et al., 2014; But see Stapel et al., 2000). Recently, it has been shown that neonicotinoid active ingredients can be translocated to sunflower EFN from seed dressings (Bredeson and Lundgren, 2018), indicating that EFN could be a route of exposure to natural enemies feeding on this resource.

Damage to plants from herbivory or mechanical wounding can lead to greater production of EFN (Heil, 2015) and enhanced attraction of natural enemies (Ness, 2003). EFN production is thought to be primarily regulated by the plant hormone jasmonic acid (JA) (Heil et al., 2001; Schmitt et al., 2018). On the other hand, neonicotinoids have been shown to upregulate the salicylic acid (SA) pathway in some plants (Ford et al., 2010). Because the JA and SA signaling pathways often exhibit negative cross-talk (Thaler et al., 2012), application of neonicotinoids could affect plant regulation of EFN and alter the quality or quantity of this resource for natural enemies.

NSTs are commonly used in cotton growing operations in the United States. Between 2010 and 2013, approximately 52–77% of cotton produced in the United States was grown from neonicotinoid-treated seeds (Douglas and Tooker, 2015). Although seed treatments can protect cotton yield (North et al., 2017), there is concern about non-target effects (Hladik et al., 2018; Tooker et al., 2017) and plant pests developing resistance to neonicotinoids (Herron and Wilson, 2011; Huseth et al., 2018). Parasitoids and predators including wasps, flies, ants, coccinellids, and spiders are often found in cotton fields and can provide biological control of plant pests (Ali et al., 2016; Luo et al., 2014; Wu and Guo, 2004). NSTs can reduce the abundance of natural enemies associated with cotton, particularly when applied at higher than the recommended dose (Saeed et al., 2016), although the contribution of EFN to such declines has not been addressed in cotton.

In this study, I examined the hypotheses that NSTs alter the quantity or composition of EFN in cotton and negatively influence natural enemies that consume EFN. Using cotton grown from seeds treated with either clothianidin or imidacloprid, I measured the amount of EFN produced constitutively and in response to mechanical damage. I also used untargeted metabolomic analysis of sugars and amino acids to compare the composition of cotton EFN grown from treated and untreated seeds. I ran enzyme-linked immunosorbent assays (ELISAs) to determine the concentration of neonicotinoid residues present in the EFN. To investigate potential impacts on natural enemies feeding on this resource, I used laboratory bioassays to examine lethal and sublethal responses of the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) feeding on EFN. I also conducted acute oral toxicity assays with *C. marginiventris* to establish LC_{50} values for both clothianidin and imidacloprid.

Methods

Plants and insects

Cotton (Malvales: Malvaceae) 'UA222' (Bourland and Jones, 2012) seeds were left untreated or were treated with the neonicotinoids clothianidin or imidacloprid at a rate of 0.375 mg of active ingredient per seed. Treatments were provided by Bayer Crop Science (Durham, NC). No fungicides were applied to the seeds. I planted seeds in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) and added 2.5 g of Osmocote (ICL Specialty Fertilizers, Dublin, OH) fertilizer when two true leaves were visible. I first grew plants in a temperature-controlled growth chamber (16:8 h L:D, 27 °C, 50–60% humidity) to minimize damage by greenhouse pests. Three days prior to experiments, I relocated plants to a greenhouse at Pennsylvania State University (University Park, PA) with natural sunlight augmented with high-pressure sodium and metal halide lights (400 W).

For bioassays, I used the solitary endoparasitoid wasp *C. marginiventris*, a generalist parasitoid of noctuid larvae. This wasp species is often collected in cotton fields (Carpenter and Jewett, 2003) and is known to feed on cotton EFN (Röse et al., 2006). The colony was established in 2016 with individuals obtained from Ted Turlings (University of Neuchâtel) and has since been maintained in the laboratory on fall armyworm (*Spodoptera frugiperda* (J. E. Smith); Lepidoptera: Noctuidae). To maintain the colony, I offered healthy second-instar fall armyworm larvae to mated female wasps for 3 h to allow parasitization. I reared parasitized

caterpillars on artificial diet in a growth chamber (26 ± 1 °C, 16 h light: 8 h dark) until parasitoid larval egression and cocoon formation. After parasitoid cocoons formed and hardened, I transferred them to glass test tubes closed with cotton wool. Upon adult eclosion, I transferred wasps to large plastic containers at a female: male ratio of 1:2. Wasps had *ad libitum* access to water and a 20% honey solution.

Extrafloral nectar quantification

To characterize the influence of neonicotinoid seed treatments on production of EFN, I quantified the volume of EFN produced by undamaged and damaged plants grown from untreated seeds or those treated with clothianidin or imidacloprid. For these experiments, I used cotton plants that were approximately 24 d old with four fully expanded leaves. I used four to eight plants for each treatment combination and repeated the experiment on two separate dates. I washed extrafloral nectaries with Milli-Q (Millipore, Bedford, MA) water prior to the experiment to remove residual EFN that had accumulated. Using a custom tool that creates many small holes over a circular area (1-cm diameter), I produced the damage treatment by mechanically wounding leaves along the midvein of the second, third, and fourth leaves. For the undamaged treatment, I did not damage leaves. I excluded the first leaf of each plant in these experiments because in this variety of cotton the first leaf is highly variable in size and shape, with some leaves that are particularly small and have very large nectaries, which could skew the results. Despite some variation in the first leaf, the remaining leaves of all plants were similar in size and shape.

To prevent water stress, which can affect EFN secretion (Newman and Wagner, 2013), I watered plants twice daily once they were moved to the greenhouse. To determine volume per nectary, I collected and measured nectar for each individual leaf using 5-µl microcapillary tubes approximately 48 h after damage. I applied 1–2 µl of Milli-Q water to the nectary and collected it into the same microcapillary tube to obtain the total volume of EFN and water. Water was added for three reasons: to ensure that all sugars were collected from the nectary, to dilute the nectar so that it fell within the limits of the refractometer (0–50% Brix), and to increase the total volume of some samples up to ≥1 µl to meet the minimum volume requirements of the refractometer. I measured the Brix value of the nectar–water solution using an Eclipse 0–50% Brix low-volume refractometer (Bellingham & Stanley, Suwanee, GA) and adjusted this value based on ambient temperature of the greenhouse, according to manufacturer instructions. Using

the total volume of the nectar–water solution and adjusted Brix value, I then calculated the total soluble sugars for each leaf.

Extrafloral nectar metabolomics

To compare EFN metabolites between untreated and neonicotinoid-treated cotton plants, I obtained seven samples from each plant treatment for metabolomics analysis. For each sample, I pooled EFN from 4–6 plants over several days to meet the minimum volume requirement of 60 µl. On each individual plant, I damaged all fully-expanded leaves along the midvein using the wounding tool described above; 48 h later I collected nectar using microcapillary tubes and froze immediately at –80 °C. After collection, I wounded the leaf again and collected nectar 48 h later. From each plant within a batch, I collected EFN 2–4 times.

I expelled thawed nectar from the capillary tubes into Eppendorf tubes and weighed with a microbalance (Metter Toledo, Columbus, OH) to the nearest 0.001 mg. For each sample, I diluted the nectar 1:2 with HPLC-grade water, vortexed to mix, transferred 60 µl to a 2 mL Eppendorf tube, and refroze at –80 ˚C. I sent frozen samples for analysis at the West Coast Metabolomics Center (University of California; Davis, CA). Metabolites included in the analysis were carbohydrates and sugar phosphates, amino acids, hydroxyl acids, free fatty acids, purines, pyrimidines, and aromatics.

Metabolite samples were analyzed following published methods (Fiehn et al., 2008). In short, metabolites were separated and identified using gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF MS) with an Agilent 6890 gas chromatograph. The GC was equipped with an Rtx-5Sil MS column (30 m long x 0.25 mm internal diameter, 0.25 µm film, 95% dimethyl / 5% diphenylpolysiloxane; Restek Corp, Bellefonte, PA). Helium was used as the mobile phase at a flow rate of 1 mL per min. A 0.5 µl aliquot of each sample was injected into a multi-baffled glass liner using splitless injector mode with 25 second purge time. Injection temperature was 50°C ramped to 250°C at a rate of 12°C per second. The oven temperature program was 50°C for 1 min, then ramped at 20°C per min to 330°C, then held constant for 5 min. The GC was coupled to a Leco Pegasus IV mass spectrometer used with unit mass resolution at 17 spectra per second from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source.

Analytes were identified using retention index and mass spectral similarity. ChromaTOF version 2.32 was used for data pre-processing using previously described criteria (Fiehn et al., 2008). Absolute spectra intensities were exported and further processed using the BinBase algorithm (Fiehn et al., 2008). Quantification of analytes is reported as peak height for the quantification ion (mz value) at the specific retention index. Peak height is reported to be more precise than peak area for low-abundant metabolites due to the larger influence of baseline determinations on areas compared to heights. Furthermore, it is more difficult to deconvolute co-eluting ions on peak areas.

Neonicotinoid residue quantification in EFN using ELISA

To quantify neonicotinoid residues present in EFN, I used ELISAs for imidacloprid and clothianidin (Abraxis, Warminster, PA). I damaged cotton plants on each leaf as previously described and after 48 h collected EFN using 5-μl microcapillary tubes. Each sample comprised the nectar pooled from all leaves on an individual plant. I collected 4–6 samples for untreated plants and 10–12 samples for clothianidin- and imidacloprid-treated plants. I diluted neonicotinoid-treated samples 1:150 using the sample diluent provided in the kit so that samples fell within the detection limits of the assay (0.06 to 1.2 ng/mL); I diluted untreated samples 1:30, which was the minimum dilution to achieve the necessary volume for the test. I performed the ELISA according to manufacturer's instructions and calculated neonicotinoid concentrations via a standard curve. I then multiplied test values by the dilution factor to determine concentration in ng/mL (ppb). Due to variation in cross-reactivity between compounds, the ELISA recognizes clothianidin and imidacloprid at 121% and 100%, respectively. I adjusted values for clothianidin to account for this cross-reactivity. The experiment was repeated twice with a separate set of plants. Using honey spiked with 0.5 ppb imidacloprid, I measured the ELISA kit recovery as 98.8%.

Parasitoid bioassay

To examine the effects of feeding on neonicotinoid-treated EFN, I conducted a bioassay using parasitoid females. I transferred 40 newly eclosed parasitoids to individual 473 ml inverted plastic cups (Global Supply Store, Inc., Pomona, CA) and randomly assigned each wasp to one of five treatments: untreated EFN, clothianidin-treated EFN, imidacloprid-treated EFN, honey, or water (n = 8 per treatment). I conducted the bioassays in a growth chamber (same conditions as above).

I provided wasps with water and a 4 µl droplet of EFN. For positive and negative control treatments, I provided wasps with droplets of honey solution or distilled water, respectively. To ensure wasps could feed *ad libitum*, I replenished droplets of EFN, honey, or water every three days. I collected EFN from plants as described above and stored it at –20 °C until a sufficient volume had been collected for the experiment. I diluted EFN and honey approximately 1:2 with distilled water to achieve Brix values of 32–33 % as measured using a refractometer. I diluted EFN because the high sugar concentration meant that evaporation of the droplets would rapidly lead to crystallization of sugars and impede wasp feeding (Lange et al., 2017). Although dilution of EFN and subsequent evaporation would affect neonicotinoid concentration, EFN sugar concentration varies temporally with evaporation and stomatal closure (Lange et al., 2017), indicating that insects would likely be exposed to this variation in the field. I recorded mortality twice daily until all wasps had died. As a proxy for wasp size, I also measured tibia lengths using a stereomicroscope (Olympus SZX10, Tokyo, Japan) with the measuring tool included in cellSens Standard 1.6 software.

To examine potential sublethal effects of neonicotinoid-treated EFN, I measured parasitism success for the EFN and honey treatments (n = 8 wasp per treatment). Because wasps from the water treatment died after just 72 h (Figure 4.3), they could not be included in this experiment. On the sixth day of the experiment, I introduced one male wasp into each container for 24 h to allow mating. I then removed males and added 20 second instar fall armyworm for 3.5 h to allow females the opportunity to parasitize. I transferred fall armyworm larvae to individual cups with artificial diet and monitored until parasitoid egression, caterpillar pupation (indicating unsuccessful parasitization), or death. I also recorded parasitization rate, development time, cocoon weight (to the nearest 0.001 mg) and sex of the parasitoid offspring.

Acute toxicity assays

To determine acute oral toxicity of clothianidin and imidacloprid to *C. marginiventris*, I conducted feeding assays with spiked honey for both male and female wasps. I dissolved technical grade clothianidin and imidacloprid (Chem Service Inc, West Chester PA) in acetone to achieve a concentration of 10^6 ppb (1 mg/ml) and serially diluted these stock solutions in acetone to concentrations of 10^5 ppb, 10^4 ppb, and 10^3 ppb. To make spiked honey, I combined 9 parts honey solution (33.3%) and 1 part neonicotinoid solution to produce 30% honey solutions with the appropriate neonicotinoid concentration. I tested the following concentrations of each

neonicotinoid: 10^2 ppb, 10^3 ppb, 10^4 ppb, and 10^5 ppb. A 9:1 honey to acetone solution served as a control. I stored all solutions at –20˚C in the dark and prepared spiked honey freshly before use.

To assess toxicity of the neonicotinoid-spiked honey solution, I set up containers similar to those used for the bioassays described above with a water wick and a 5 µl droplet of spiked or control honey solution. I replenished droplets after 48 h. I transferred five newly eclosed male or female wasps into each container and recorded the time. At each concentration, I set up between 5–7 containers for males and females, giving a total of between 20–30 individuals per control dose, and 30–35 for most other doses. Based on the initial 30 wasps I tested, female wasps had a considerably higher tolerance for imidacloprid than males. Thus, for the two highest doses of 10^4 ppb and 10^5 ppb, I tested an additional 25 wasps, giving a total of 55 individuals for each of these doses.

I monitored wasps at 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, and 96 h to determine time of death. To confirm death, I gently prodded wasps with forceps and carefully observed them for movement of limbs or antennae.

Statistical analyses

I analyzed EFN quantity as total soluble sugars per plant using a two-way ANOVA with seed treatment and damage as factors and experimental run as the blocking variable. I used SAS 9.4 (SAS Institute, Cary NC) to perform these analyses.

To analyze metabolomics data, I removed two compounds that were likely contaminants prior to further analysis: triethanolamine and phthalic acid. For the remaining compounds, I first normalized the data to reduce the effect of instrument sensitivity drift caused by machine maintenance, aging and tuning parameters. The total average peak height (APH) sum of all identified (genuine) metabolites (APH_{total}) across treatments was used for normalization. To normalize each compound, I used the following formula for each metabolite *i* of sample *j*:

$$\mathrm{metabolite}_{\mathrm{ij,normalized}} = \left(\frac{\mathrm{metabolite}_{\mathrm{ij,raw}}}{\mathrm{APH}_{\mathrm{j}}}\right) * \mathrm{APH}_{\mathrm{total}}$$

I scaled the normalized data using the Pareto method (van den Berg et al., 2006; Yang et al., 2015) and analyzed global multivariate differences in metabolite composition by seed

treatment using ANOVA-simultaneous component analysis (ASCA) with 1000 permutations (Smilde et al., 2005) in R 3.4.3 (R Core Team, 2017). I also conducted one-way ANOVA to test for differences in abundance of each individual metabolite between seed treatments using MetaboAnalyst 4.0 with Bonferroni corrections for multiple comparisons (Chong et al., 2018).

To determine differences in parasitization success between treatments, I used a one-way ANOVA after logit transformation to fulfill ANOVA assumptions of normality and homogeneity of variance. I conducted this analysis in R 3.4.3 (R Core Team, 2017).

To examine differences in longevity of female wasps feeding on different food sources, I used Kaplan–Meier analysis. I further analyzed the EFN and honey treatments using a Cox-Proportional Hazards model including hind tibia length as a proxy for parasitoid size and quality (Sagarra et al., 2001; Visser, 1994) as a covariate. I conducted survival analyses in R. 3.4.3 (R Core Team 2017) using the 'survival' and 'survminer' packages.

I calculated LC_{50} values for *C. marginiventris* wasps by probit analysis using the LC_probit function of the 'ecotox' package in R 3.4.3 (R Core Team 2017).

Results

Mechanically-damaged cotton plants produced significantly more EFN than undamaged plants ($F = 161.34$; df = 2, 56; $P < 0.0001$). There was no effect of seed treatment ($F = 0.65$; df = 2, 56; $P = 0.525$) or its interaction with damage ($F = 0.19$; df = 2, 56; $P = 0.831$) on the amount of EFN produced by cotton plants (Figure 4.1).

Untargeted GC-TOF-MS analysis of the extrafloral nectar metabolome of cotton detected 196 compounds, of which 49 could be identified. Identified analytes included sugars, sugar alcohols, fatty acids, organic acids, and esters.

Multivariate ASCA analysis detected no differences in overall metabolite composition between EFN from different seed treatments ($P = 0.158$). These data were visualized using a principal components analysis (Figure 4.2), although the first two principal components explained just 22.3% and 20.0% of the variation. I also found no differences in the amount of any individual metabolite between seed treatments ($P > 0.05$; data not shown).

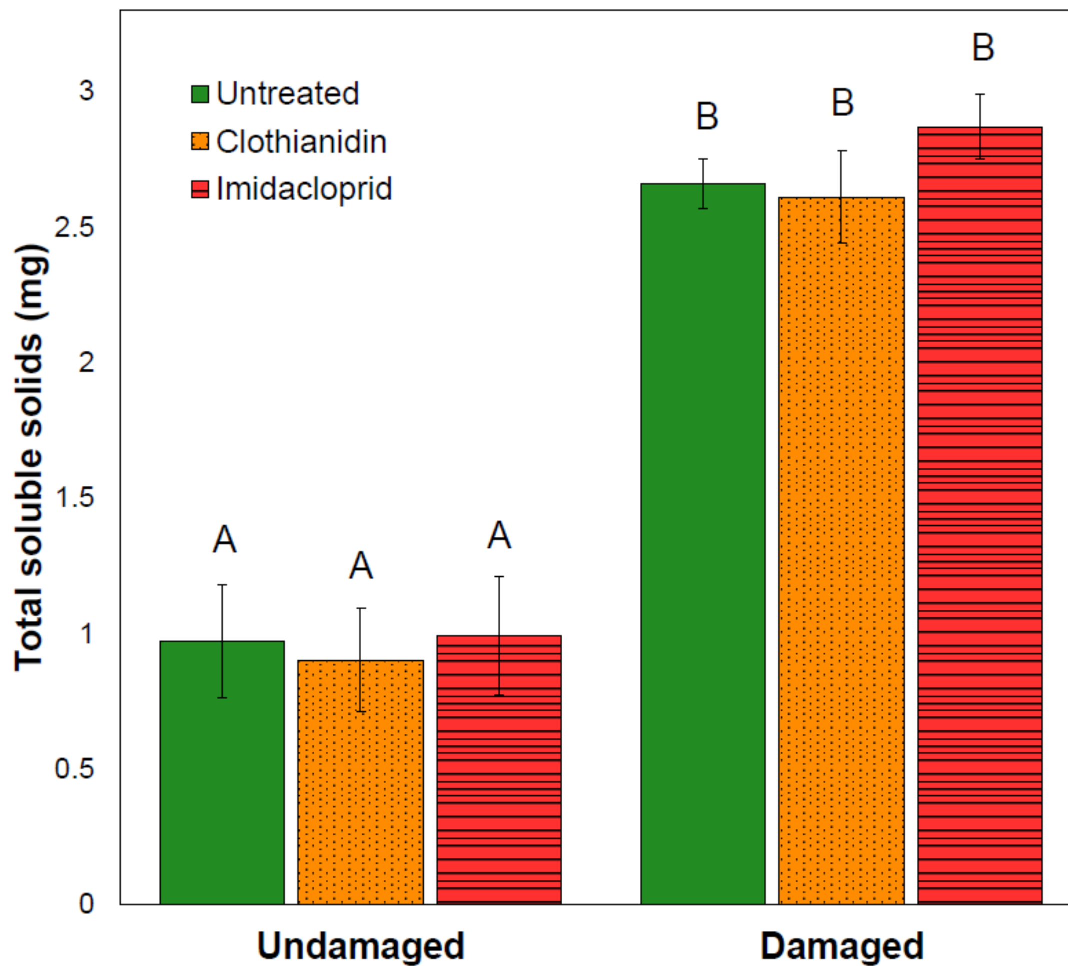

Figure 4.1: Total extrafloral nectar produced by cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Plants were left undamaged, or damaged mechanically. I quantified extrafloral nectar 48 h after damage. Bars represent means with standard errors. Letters above the bars denote significant differences at $P < 0.05$.

ELISA assays indicated that neonicotinoids were present in EFN of treated plants. In clothianidin- and imidacloprid-treated plants, the active ingredients were detected at concentrations of 77.3 ± 17.3 and 122.6 ± 11.5 ppb, respectively. In untreated plants, neonicotinoid concentrations were below the limits of detection for the test.

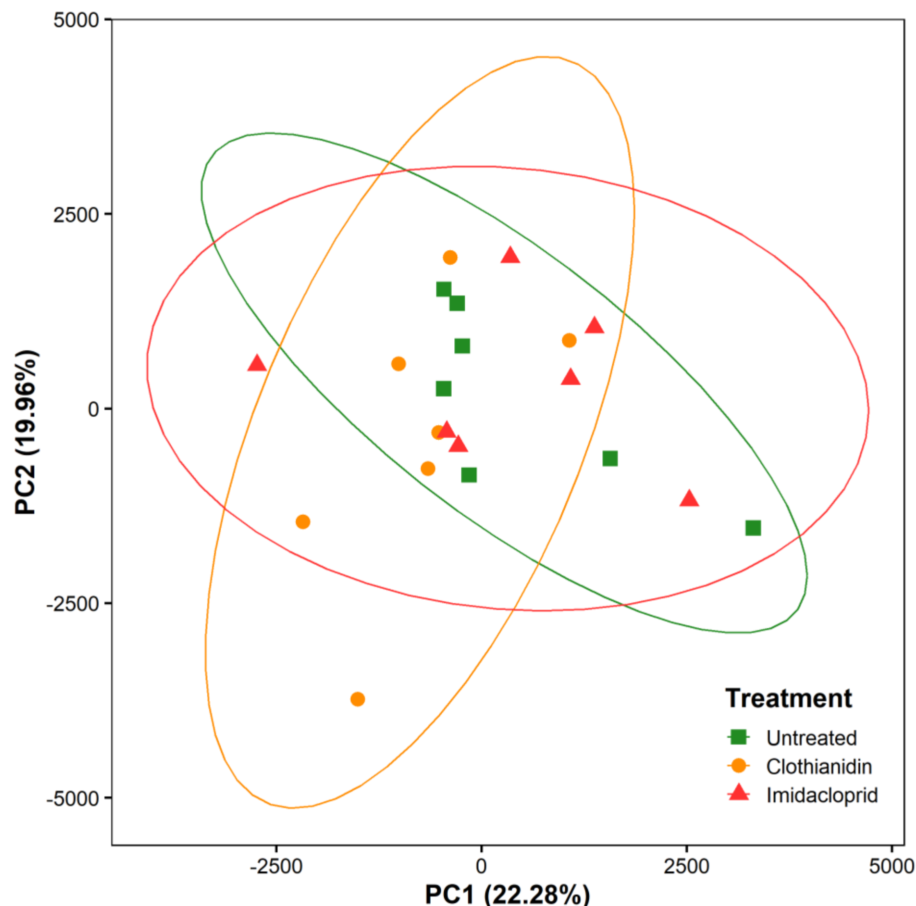

Figure 4.2: Principal components analysis of metabolites detected from extrafloral nectar of cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Multivariate analysis via ANOVA-simultaneous component analysis (ASCA) with 1000 permutations indicated that there was no significant difference in metabolite composition between treatments ($P = 0.158$). Ellipses indicate 95% confidence intervals.

When analyzed using the Kaplan–Meier method, the longevity bioassay with *C. marginiventris* female wasps indicated that there was an overall significant difference in survival times between treatments groups that received different food types ($P < 0.001$; Figure 4.3).

Pairwise comparisons using a log-rank test revealed that only the water treatment was significantly different from the honey and EFN treatments ($P < 0.05$). Further analysis of the honey and EFN treatments using Cox proportional hazards model, including hind tibia length as a covariate, found no difference in survival times between treatments (p= 0.162).

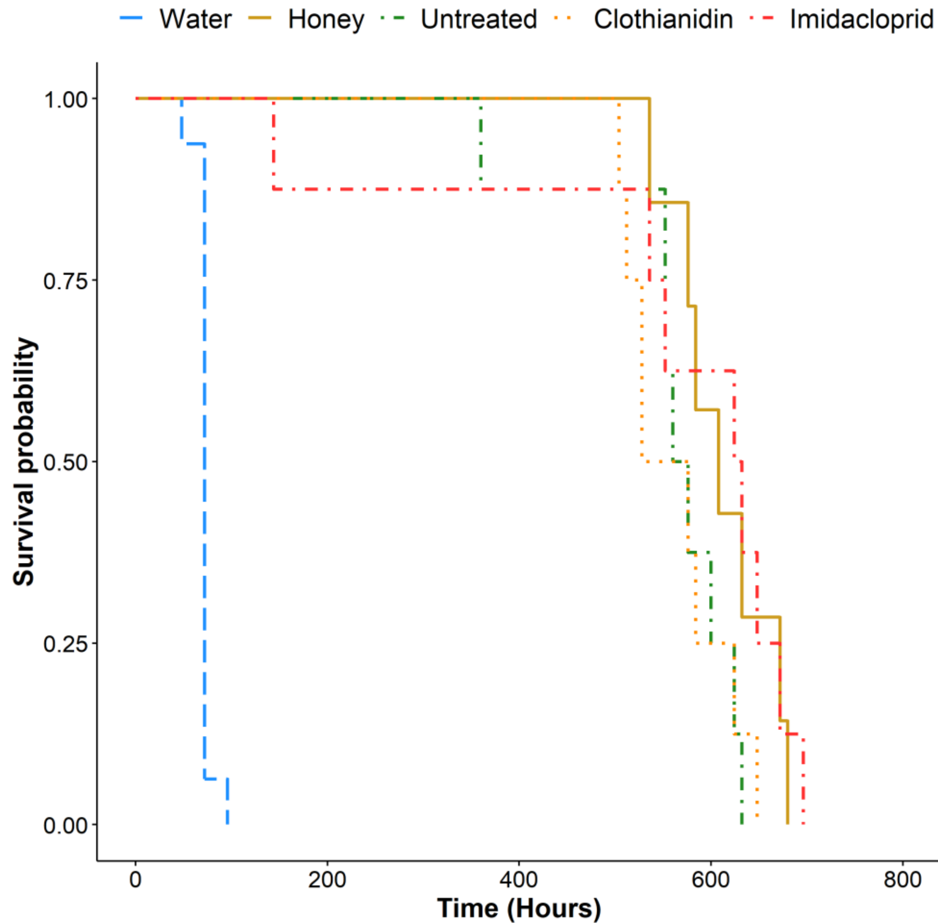

Figure 4.3: Kaplan–Meier survival curve depicting longevity of *Cotesia marginiventris* female wasps provided with different food sources: water, honey, or extrafloral nectar collected from cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Overall there was a significant difference in survival between treatment groups ($P < 0.001$); pairwise comparisions via log-rank test indicate that only the water treatment was different from other treatments ($P < 0.05$).

I found no difference in parasitization success between females fed honey or different EFN treatments (Table 4.1; $F = 0.99$; df = 3, 26; $P = 0.411$). The offspring of these females exhibited no difference in development time (Table 4.1; $F = 1.78$; df = 3, 26; $P = 0.179$) or cocoon weight (Table 4.1; $F = 1.75$; df = 3, 26; $P = 0.184$) between treatments. Across all treatments, only male offspring were produced.

Table 4.1: Performance of offspring from *Cotesia marginiventris* females fed on honey or extrafloral nectar (EFN) from untreated, clothianidin-treated, or imidacloprid-treated cotton plants. Wasps developed in *Spodoptera frugiperda* larvae. Development time indicates the number of days from egg to adult eclosion.

Treatment	% Larvae parasitized	Development time (d)	Cocoon weight (mg)
Honey	64.1 ± 13.3	12.42 ± 0.09	2.10 ± 0.02
Untreated EFN	81 ± 6.8	12.32 ± 0.08	2.14 ± 0.01
Clothianidin EFN	68.8 ± 7.3	12.55 ± 0.08	2.15 ± 0.01
Imidacloprid EFN	54.9 ± 15.3	12.30 ± 0.09	2.13 ± 0.02

For clothianidin, 48-h LC_{50} values for males and females were similar (8,267 and 7,827 ppb, respectively; Table 4.2; Figure 4.4). For imidacloprid, the 48-h LC_{50} for males was 7,291 ppb, substantially lower than the 48-h LC_{50} of 49,800 ppb calculated for females. Non-significant ($P > 0.05$) Chi-square values for all models indicate that the data fitted well to the probit analysis model (Table 4.2). At 72 h and 96 h, the LC_{50} decreased for both neonicotinoids in male and female wasps (Tables 4.3 and 4.4). However, significant Chi-square values for probit analysis of females receiving imidacloprid and males receiving clothianidin at 96 h indicate a poor fit of the data and confidence intervals were unable to be calculated for these two LC_{50} values (Table 4.4). Mortality in the control treatments was never above 4%.

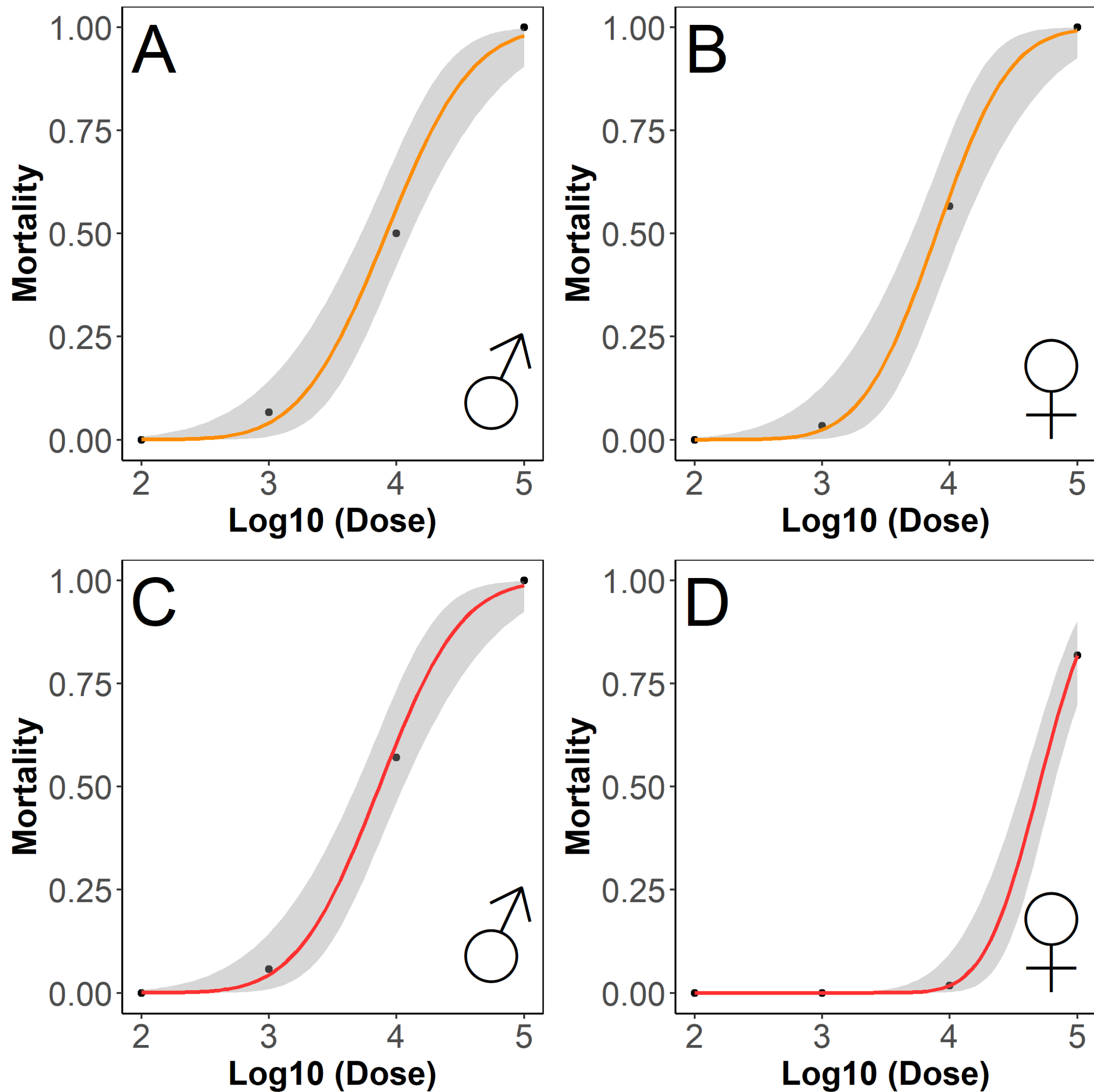

Figure 4.4: Survivorship curves of *Cotesia marginiventris* wasps receiving neonicotinoids via oral feeding assays at 48 h: A) males receiving clothianidin B) females receiving clothianidin C) males receiving imidacloprid D) females receiving imidacloprid. For clothianidin, the LD_{50} was similar between males and females at 8.3 mg/L (8300 ppb) and 7.8 mg/L, respectively. For imidacloprid the LD_{50} values between males and females were disparate at 7.3 mg/L (7300 ppb) and 49.8 mg/L (49800 ppb) for males and females, respectively. Grey shaded areas on plots represent the 95% CI.

Table 4.2: Oral toxicity of clothianidin and imidacloprid (mg/L) at 48 h to *Cotesia marginiventris* males and females calculated using probit analysis.

		n	Slope + SE	48-h LC_{50} (95% CI)	Chi-square	*P*-value
Clothianidin	**Male**	128	1.90 + 0.31	8.27 (5.25–12.88)	1.737	0.420
	Female	120	2.20 + 0.42	7.83 (5.00–12.20)	0.404	0.817
Imidacloprid	**Male**	131	1.98 + 0.33	7.29 (4.76–11.24)	0.718	0.698
	Female	160	3.00 + 0.45	49.8 (36.39–65.00)	< 0.001	1.000

Table 4.3: Oral toxicity of clothianidin and imidacloprid (mg/L) at 72 h to *Cotesia marginiventris* males and females calculated using probit analysis.

		n	Slope + SE	72-h LC_{50} (95% CI)	Chi-square	*P*-value
Clothianidin	**Male**	128	2.08 + 0.36	5.69 (3.67–8.70)	0.258	0.879
	Female	120	2.24 + 0.43	7.31 (4.68–11.33)	0.268	0.875
Imidacloprid	**Male**	131	2.44 + 0.41	4.48 (3.02–6.56)	0.018	0.991
	Female	160	3.15 + 0.45	46.2 (33.70–60.20)	< 0.001	1.000

Table 4.4: Oral toxicity of clothianidin and imidacloprid (mg/L) at 96 h to *Cotesia marginiventris* males and females calculated using probit analysis.

		n	Slope + SE	96-h LC_{50} (95% CI)	Chi-square	*P*-value
Clothianidin	**Male**	128	1.79 + 0.29	3.53 (NaN*)	12.67	0.002
	Female	120	2.41 + 0.47	5.95 (3.83–9.03)	0.059	0.971
Imidacloprid	**Male**	131	2.54 + 0.42	4.21 (2.85–6.11)	0.008	0.996
	Female	160	2.19 + 0.29	26.6 (NaN*)	37.02	<0.001

*Confidence intervals were unable to be calculated due to a poor fit of the data to the probit model

Discussion

EFN is an important plant resource for predators, parasitoids, and other beneficial arthropods. Yet the widespread use of neonicotinoid seed treatments can alter this resource and potentially affect natural enemies. Exposure to neonicotinoids can have both lethal and sublethal effects on non-target insects (Müller, 2018). The majority of published studies on risks of neonicotinoids have focused on bees (Decourtye and Devillers, 2010), with impacts to natural enemies less well understood (Wood and Goulson, 2017). In this study, I sought to address possible impacts of neonicotinoid seed treatments on natural enemies via extrafloral nectar.

EFN can contribute to biological control of plants by attracting and sustaining natural enemies (Jones et al., 2017). Plants with reduced production of EFN are often associated with fewer natural enemies, leading to declines in plant fitness and fruit production (Mathews et al., 2007). Contrary to my hypothesis, I did not find evidence that cotton plants treated with imidacloprid or clothianidin produced different amounts of EFN, either constitutively or in response to damage. Available evidence suggests that neonicotinoids can upregulate the SA pathway in several plant species (Ford et al., 2010; Szczepaniec et al., 2013), which can be antagonistic to JA-mediated defenses such as EFN (Heil et al., 2001; Schmitt et al., 2018). I did not measure SA and JA of cotton plants in this study, so it is unclear if the neonicotinoid seed treatments affected plant defense pathways. Although NSTs did not appear to suppress EFN production in cotton plants, I found that plants translocated these insecticides to the EFN, and thus treated plants produce similar amounts of a potentially toxic resource.

Metabolomics analysis of cotton EFN revealed that this resource predominantly comprised sugars, namely fructose, glucose, sucrose, 1-kestose and raffinose. Cotton EFN also had small amounts of fatty acids, sugar alcohols, and esters. Interestingly, the analysis detected only one amino acid, glycine. A previous study identified 24 amino acids from cotton extrafloral nectar (Hanny and Elmore, 1974). The high number of unidentified compounds in this study may reflect that untargeted metabolomics was insufficient to accurately identify all amino acids present in cotton EFN. Nevertheless, I did not find differences in overall metabolite composition or individual compounds between EFN collected from untreated and treated plants. This result mirrors the finding that NSTs did not affect the quantity of EFN produced by cotton nectaries

and suggests that these seed treatments did not appreciably alter metabolic pathways regulating EFN production in cotton.

I detected neonicotinoids in EFN of cotton plants grown from seeds treated with two commonly applied neonicotinoids, clothianidin and imidacloprid, at concentrations of 77 and 123 ppb, respectively. Neonicotinoids have been detected in floral nectar of plants applied with NSTs at concentrations ranging from <1 to 16 ppb (EFSA, 2012; Rundlof et al., 2015). My finding that neonicotinoids are translocated from seed treatments to EFN corroborates results from a recent study that found thiamethoxam occurred at concentrations of 1–5 ppb in EFN of greenhouse-grown sunflowers (Bredeson and Lundgren, 2018). Neonicotinoids are highly water soluble, and the discrepancy between studies could be due to a number of factors, including application rate, plant species, plant age (Alford and Krupke, 2017), watering regime, and soil type. Future studies should examine neonicotinoid concentrations in EFN from field-grown cotton plants.

Extrafloral nectar is a food resource for many natural enemies such as ants, parasitoid wasps, lacewings, beetles, and spiders (Hespenheide, 1985; Klein et al., 2016; Limburg and Rosenheim, 2001; Taylor and Foster, 1996; Taylor and Pfannenstiel, 2008). Honey bees (*Apis mellifera*) have also been observed foraging at extrafloral nectaries (Cuautle and Rico-Gray, 2003; Koptur, 1992). Honey bees are often used as an indicator species for insecticide toxicity (Medrzycki et al., 2013) and have reported oral LD_{50} values of 0.0079 and 0.0037 µg/bee for clothianidin and imidacloprid, respectively (DiBartolomeis et al., 2019). On the basis of these values, bees would need to consume approximately 0.103 mL and 0.03 mL of imidacloprid- and clothianidin-containing EFN to experience 50% mortality. Honey bee foragers consume up to 321 mg (approximately 0.321 mL) of nectar per day (Rortais et al., 2005). These calculations suggest that bees using EFN as a significant food resource could ingest quantities of neonicotinoids that approach or exceed their oral LD_{50}. In contrast with floral nectar that is only produced by mature, flowering plants, cotton EFN is produced by all leaves including the cotyledons, and is available as early as one to two weeks after planting (personal observation). Thus, in certain environments, extrafloral nectar from neonicotinoid-treated plants may represent a consistent and ongoing route of insecticide exposure to beneficial insects.

I conducted a bioassay with the parasitoid wasp *C. marginiventris* to explore effects to natural enemies from feeding on EFN from neonicotinoid-treated cotton plants. I did not find significant differences in mortality between females feeding on untreated and treated plants. Similarly, the lady beetle *Coleomegilla maculata* did not experience differences in mortality rates from feeding on artificial nectar spiked with thiamethoxam and clothianidin at concentrations up to 100 ppb (Bredeson and Lundgren, 2018). Although *Microplitis croceipes* parasitoids displayed reduced longevity and foraging ability after feeding on EFN from cotton plants sprayed with imidacloprid (Stapel et al., 2000), insecticide concentrations in the nectar were not measured, making it difficult to compare directly with my results. Honeydew, another sugary food resource, contaminated with thiamethoxam at concentrations of approximately 18 ppb, was highly toxic to the hoverfly pollinator *Sphaerophoria rueppellii* and moderately toxic to the parasitoid wasp *Anagyrus pseudococci* (Calvo-Agudo et al., 2019). Honeydew with imidacloprid concentrations of 15–68 ppb was moderately toxic to *S. rueppellii* but did not alter mortality of *A. pseudococci* (Calvo-Agudo et al., 2019). These findings suggest that the concentrations of neonicotinoids I found in cotton EFN could be toxic to some beneficial insect species.

To examine acute oral toxicity of clothianidin and imidacloprid to *C. marginiventris*, I conducted feeding assays with spiked honey. I determined 48 h LC_{50} concentrations for clothianidin as 8.3 mg/L (8300 ppb) and 7.8 mg/L (7,800 ppb) for males and females, respectively, and for imidacloprid as 7.3 mg/L (7,300 ppb) and 49.8 mg/L (49,800 ppb) for males and females, respectively. It is unclear why the LC_{50} value for females exposed to imidacloprid was considerably higher than for males, although sex-specific responses to neonicotinoids have been reported (Mobley and Gegear, 2018; Nielsen et al., 2008). For clothianidin, the LC_{50} for males and females is approximately 100 times greater than the concentration I detected in cotton EFN. For imidacloprid the LC_{50} is approximately 60 and 400 times greater than the concentration I measured in cotton EFN for males and females, respectively. This suggests that *C. marginiventris* wasps are unlikely to experience direct mortality from feeding on neonicotinoid-treated cotton EFN and corroborates the bioassay results of no difference in mortality among treatments.

Other studies investigating acute toxicity from neonicotinoids in natural enemies have found highly variable results depending on the species and chemical tested. LC_{50} values for the

parasitoid *Trichogramma confusum* in response to topical application of acetamiprid, imidacloprid, thiacloprid, and thiamethoxam were 93.21 mg/L (93,210 ppb), 754.2 mg/L (75,4200 ppb), 176.5 mg/L (176,500 ppb), and 0.24 mg/L (240 ppb), respectively (Wang et al., 2013). Likewise, LC_{50} values from contact exposure to acetamiprid for the four parasitoid wasps *Aphytis melinus*, *Gonatocerus ashmeadi*, *Eretmocerus eremicus*, and *Encarsia formosa* were 0.005 mg/L (5 ppb), 0.134 mg/L (134 ppb), 12.02 mg/L (12,020 ppb), and 108.27 mg/L (108,270 ppb), respectively (Prabhaker et al., 2007). However, because the chemicals in these studies were administered topically, they are not directly comparable to the oral feeding assays I presented in this study. Neonicotinoids are usually more toxic when ingested compared with contact exposure, likely due to low penetration of the cuticle (Decourtye and Devillers, 2010).

Although lethal thresholds are informative, they do not give the complete picture of the impact of an insecticide on an insect species (Müller, 2018). Insecticides can have a wide range of sublethal effects on insect behavior (e.g. courtship, locomotion, foraging), physiology (e.g. immunity, sex ratio, detoxification) and communication (e.g. chemical communication, host location). However, it can be difficult to assess the concentration at which these effects occur (Müller, 2018). I examined whether exposure to field-realistic rates of neonicotinoid insecticides via EFN can influence female parasitization rate and development rate. Although I did not find significant differences among treatments, it is unknown whether higher concentrations would have altered parasitization and offspring development rates. If neonicotinoid application rates on cotton seeds continue to increase as they have on maize seeds (Tooker et al., 2017), it is likely that field-realistic concentrations of neonicotinoids in EFN will rise, potentially having a greater chance of influencing parasitoid fitness. The parasitoid *Anagrus nilaparvata* experienced reduced parasitization rates from ingesting honey containing imidacloprid at concentrations corresponding to LC_{10} (10 mg/L; 10,000 ppb) and LC_{20} (20 mg/L; 20,000 ppb) values (Liu et al., 2010), although these concentrations are about 100–200 times higher than neonicotinoid concentrations I detected in cotton EFN.

I did not explore parasitoid preference for EFN from treated and untreated plants, but given that there was no difference in *C. marginiventris* mortality between EFN treatments, it seems unlikely that wasps would avoid neonicotinoid-containing nectar in a no-choice scenario. A previous study found that gustatory neurons of bumble bees and honey bees did not respond to stimulation by imidacloprid, thiamethoxam, and clothianidin, indicating that bees cannot taste

neonicotinoids (Kessler et al., 2015). Moreover, bees did not avoid sugar resources containing these neonicotinoids, despite negative effects of thiamethoxam and clothianidin on bee survival (Kessler et al., 2015).

Crops grown from neonicotinoid-treated seeds are ubiquitous throughout the United States, yet there is still much to learn about how these insecticides affect plants and beneficial insects in agricultural systems. I found that cotton plants translocated clothianidin and imidacloprid to EFN, indicating that insects consuming this resource would be exposed to neonicotinoids. Although I did not detect differences in mortality in *C. marginiventris* females feeding on treated EFN in the laboratory, I recognize the limitation of testing just one parasitoid species from one lab colony. Toxicity of insecticides can vary between related species (Prabhaker et al., 2007; Wang et al., 2013), and between different populations of the same species (Huseth et al., 2016).

Furthermore, lab-based studies may not fully reflect how insects respond to neonicotinoids in the field. The effect of insecticides on insects is a complex interaction between susceptibility and exposure, with the latter dictated by the insect's behavior in an agroecosystem (Stark et al., 1995). Moreover, because insects are exposed to pathogens, other pesticides, and other stressors (e.g., limited food, temperature extremes) in the field, interactions between these factors could affect survival and fitness (Cresswell, 2011; Doublet et al., 2015; Grassl et al., 2018; Poquet et al., 2016). Further investigation of how NSTs affect EFN of field-grown crops is warranted, including examination of lethal and sublethal impacts on arthropods using this food source.

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Chapter 5

Conclusions and future directions

Because herbivores link primary production to wider food webs, a major goal of ecology is understanding plant–herbivore interactions (Burkepile and Parker, 2017). Plants are also associated with a wide variety of other organisms that collectively comprise the phytobiome (Leach et al., 2017), and the complex interactions among plants, herbivores, microbes, and higher trophic levels shape ecological communities (Figure 1.1). These multitrophic associations are important for understanding the ecology and evolution of organisms in natural systems and for applications to improve management of agroecosystems (Leach et al., 2017; Silva et al., 2018).

Insect herbivores are a major cause of annual crop losses in agricultural plant production (Deutsch et al., 2018). To combat insect herbivores and protect plants, integrated pest management (IPM) is a multipronged approach that employs tactics such as host plant resistance, biological control, and judicious use of insecticides (Panda and Khush, 1995; Stern et al., 1959; Figure 5.1). These components of IPM can interact and influence each other in various ways, with synergistic or disruptive effects (Peterson et al., 2016; Stenberg, 2017). Therefore, better understanding of these interactions has the potential to develop and improve IPM strategies towards the goal of sustainable pest management (Peterson et al., 2016). This dissertation improves basic knowledge of interactions among plant defenses, natural enemies, and insecticides in crop systems. In Chapter 1, I reviewed studies on natural enemy modulation of plant defenses. In Chapter 2–4, I used model crop systems to investigate multitrophic interactions among different trophic levels and in response to insecticides (Figure 5.1).

Despite Lepidoptera being one of largest insect orders and containing many economically important pest species (Cunningham and Zalucki, 2014; Kfir et al., 2002; Zalucki et al., 2012), relatively little is known about factors that shape larval gut microbiomes and the physiological and ecological roles of these bacterial associates (Paniagua Voirol et al., 2018). In Chapter 2 and 3, I showed that host plant species is an important factor driving composition of bacterial communities in *Spodoptera frugiperda* and *Helicoverpa zea*, a finding consistent with other

lepidopteran studies (Mason and Raffa, 2014; Priya et al., 2012), although other factors may also be involved (Chaturvedi et al., 2017; Hannula et al., 2019; Tang et al., 2012; Xiang et al., 2006).

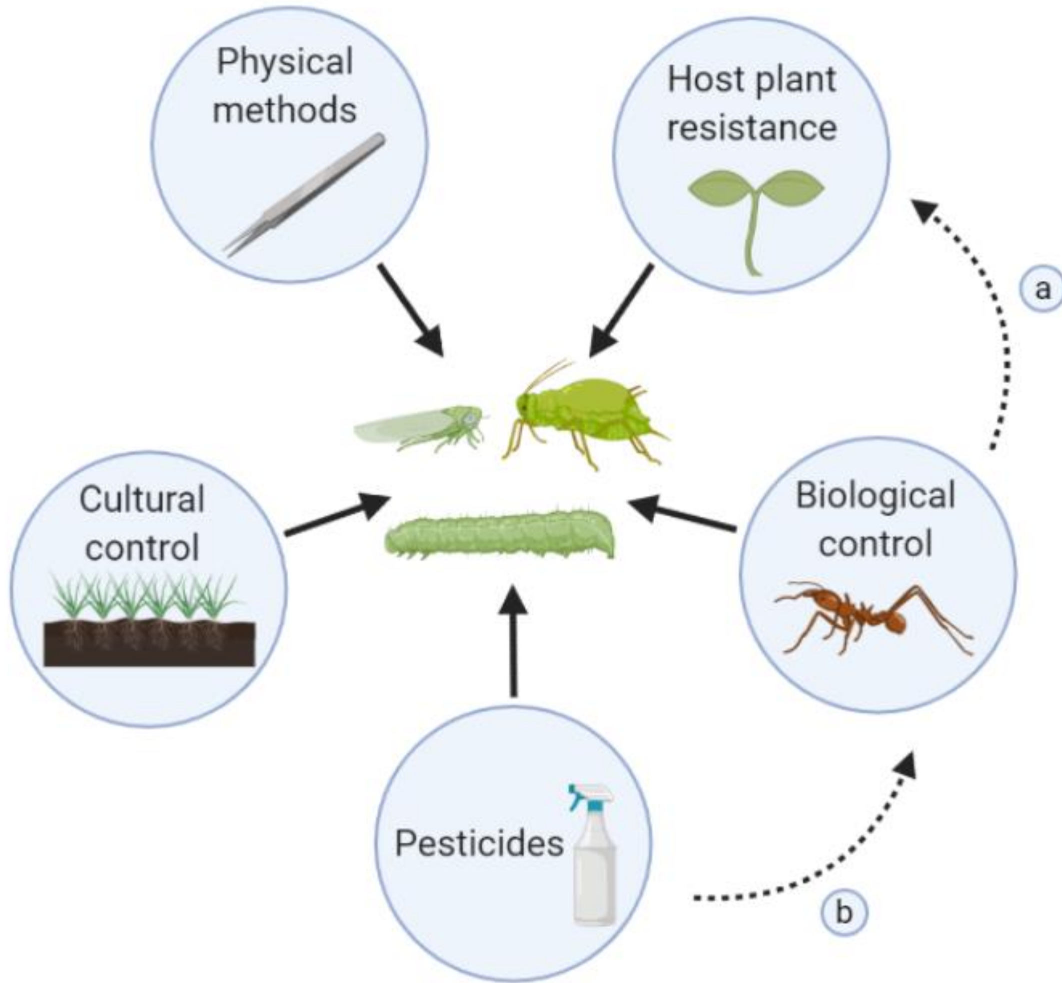

Figure 5.1: Integrated pest management (IPM) techniques to control herbivore pests. Solid lines indicate components of IPM programs that target herbivores. Dotted lines indicate areas of interactions between components that this dissertation focused on: a) The effects of natural enemies of plant defenses (Chapter 1 and 3), b) The effects of neonicotinoid seed treatments on extrafloral nectar and parasitoid natural enemies (Chapter 4).

Caterpillar bacterial communities often exhibit high intraspecific variability, and many lepidopteran species appear to lack resident gut associates (Priya et al., 2012; Staudacher et al., 2016; Tang et al., 2012). My work supports the notion that lepidopteran bacterial communities are indeed variable and dynamic. However, these features have led to suggestions that lepidopteran gut bacteria are merely transient associates and are unimportant for caterpillar biology (Hammer et al., 2017; Staudacher et al., 2016). In a follow-up study to Chapter 2, Mason et al. (2020) used axenic and gnotobiotic rearing methods to show that bacterial isolates established and proliferated in *S. frugiperda* and *H. zea* guts when fed sterile (gamma-irradiated) corn leaves, but not artificial diet. This finding challenges the view that caterpillar gut bacterial communities are simply a reflection of diet-associated bacteria. The finding that gut bacteria did not proliferate in caterpillars fed artificial diet (Mason et al. 2020), combined with the low diversity of bacterial communities in diet-fed larvae (Chapter 2; Chapter 3), underlines the importance of using plants in future insect microbiome studies.

My work on *S. frugiperda* also paved the way for additional studies examining how lepidopteran gut bacteria can influence plant–insect interactions. Introduction of bacterial isolates (Chapter 2) to axenic larvae reduced larval growth and increased mortality when fed leaves from resistant maize genotypes (Mason et al., 2019). By disrupting the insect's protective gut lining (peritrophic matrix), physical and chemical defenses of resistant maize genotypes created opportunities for gut microbes to penetrate the PM, upregulating insect immune responses and inducing septicemia (Mason et al., 2019). This research shows that gut bacteria can act as natural enemies of herbivores in some contexts, and suggests that plant–insect interactions may not be fully understood without considering plant- and insect-associated microbes.

Baculoviruses are important natural enemies of lepidopteran larvae in natural systems and are used as biopesticides in agricultural systems (Lacey et al., 2015). Transmission of these viruses primarily occurs via host ingestion of viral occlusion bodies (OBs) that are released from virus-killed cadavers on plant surfaces. Yet, little is known about the ecology of these cadavers, and whether cadaver-associated bacteria or OBs could mediate interactions between plants and healthy insects. In Chapter 3, I provided the first characterization of bacterial communities associated with insect cadavers killed by a baculovirus pathogen. Although bacterial

communities were similar between *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)-killed and uninfected freeze-killed *Trichoplusia ni* cadavers, bacterial titers were significantly higher in virus-killed cadavers. Moreover, I found the first evidence that baculovirus-killed cadavers suppress plant defenses, contributing to a growing body of literature showing that natural enemies can modulate plant defenses (Chapter 1). Future research should examine whether bacteria, viral OBs, or a combination of these microbes were responsible for defense modulation.

Although I did not find evidence that cadavers suppressed defenses in response to *T. ni* herbivory in tomato plants, different results could be observed in different tritrophic systems. Given the risk of larval contact with virus-killed cadavers, it was surprising that *T. ni* behavior was not affected by presence of virus-killed or freeze-killed cadavers. However, it is noteworthy that both *T. ni* and AcMNPV are generalists in terms of host plant and host insect, respectively. In a more highly coevolved system, specialist insects may be more discriminating of cues associated with specialist pathogens (Bernays, 2001). Moreover, because the process of domestication often decreases plant defenses and alters tritrophic interactions (Chen et al., 2015), it would be interesting to measure plant and insect responses to baculovirus-killed cadavers in a natural system.

Because plant defensive compounds can affect baculovirus–host interactions in a variety of ways (Cory and Hoover, 2006; Shikano, 2017), baculovirus-mediated modulation of plant defenses could enhance or diminish virus success. Evaluating how these interactions affect virus transmission and plant fitness will be important for understanding the evolution of cadaver-mediated interactions (Chapter 1). From an applied standpoint, understanding the prevalence and importance of baculovirus impacts on plant defenses could offer opportunities to integrate biological control by baculoviruses with host plant resistance.

Control of insect herbivores in agroecosystems is often achieved with use of insecticide applications. In IPM, insecticides should be applied minimally and only when needed (Stern et al., 1959). Yet, the prophylactic use of neonicotinoids as seed treatments in US crop production is extensive (Douglas and Tooker, 2015), thus violating IPM principles (Tooker et al., 2017). Neonicotinoid seed treatments can also affect other components of IPM as these systemic

chemicals are absorbed by seeds and translocated throughout plant tissues (Alford and Krupke, 2017). Neonicotinoids have been shown to alter host plant resistance through modulation of plant defense signalling pathways (Ford et al., 2010; Szczepaniec et al., 2013), and these treatments can negatively affect biological control services through contamination of prey or honeydew resources (Calvo-Agudo et al., 2019; Douglas et al., 2015).

In Chapter 4, I examined the effects of neonicotinoid seed treatments on extrafloral nectar (EFN), a plant-produced sugar resource that provides nutrition for natural enemies that indirectly defend plants from herbivores (Heil, 2015; Jones et al., 2017). Seed treatments did not alter the quantity or metabolite composition of EFN secreted by cotton plants. However, I found that both clothianidin and imidacloprid neonicotinoids were translocated from seed coatings to the EFN of cotton (*Gossypium hirsutum*). Using oral feeding assays with neonicotinoid-spiked honey, I established LC_{50} values for the parasitoid wasp *Cotesia marginiventris*. Although the concentrations of neonicotinoids detected in EFN were several orders of magnitude lower than LC_{50} values for this wasp species, other natural enemies and beneficial insects that use EFN could have lower tolerance to these insecticides. It is also important to note that these lab-based studies are unlikely to directly translate to the field where other stressors could alter the effects of neonicotinoids. More studies that examine how neonicotinoid seed treatments affect populations of natural enemies in the field are needed, including assessments of sublethal effects (Müller, 2018).

Overall, this dissertation contributes to fundamental knowledge of interactions between caterpillars, their natural enemies, and microbes in crop plants. While many unanswered questions remain, these studies provide groundwork for future research in multitrophic systems and identify potential opportunities and challenges for integration of plant resistance, biological control, and pesticide components of IPM.

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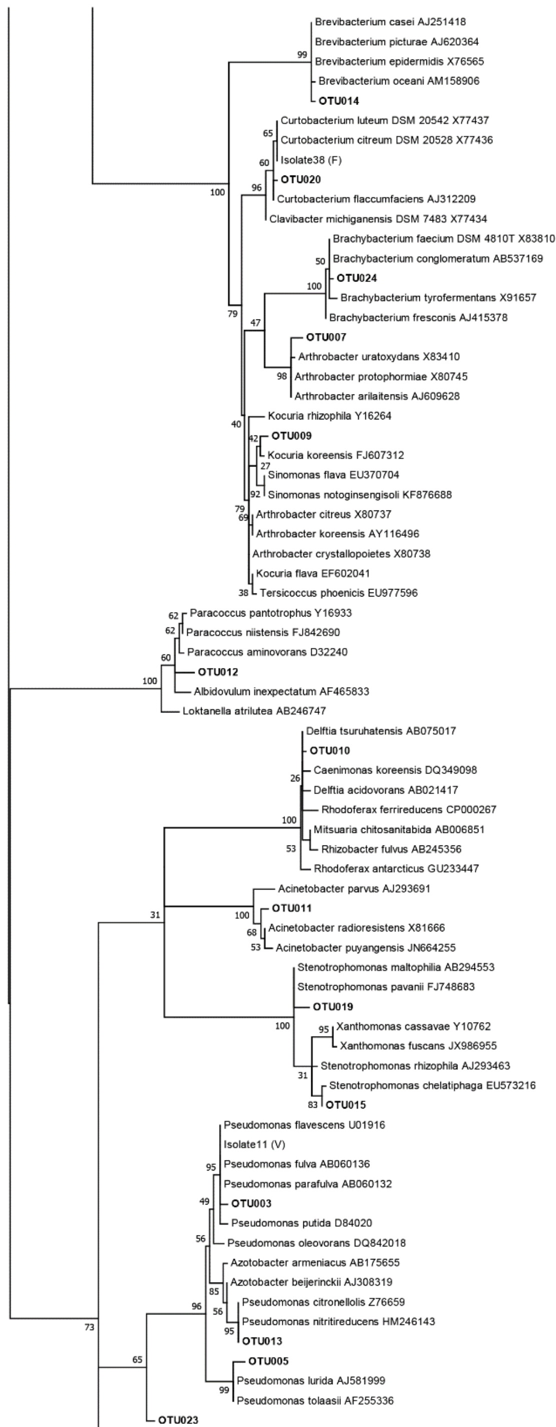
Appendices

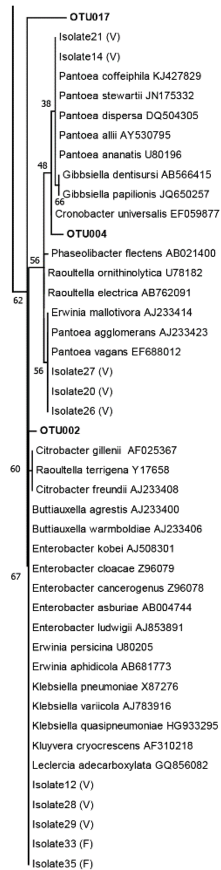
Appendix A: Site descriptions from Chapter 2

Appendix A: Site descriptions and locations of field-collected plant and insect material

Site	Location	Coordinates	Crops grown	Material collected	Date collected
HF	Centre County, PA	40.7601319 -77.8787313	Sweet corn	*H. zea* larvae *S. frugiperda* larvae	3-7 Sep 2016
RS	Centre County, PA	40.7106224 -77.9644764	Soybean Sweet corn	*H. zea* larvae Soybean foliage Corn silk	7 Sep 2016
PRL	Lajas, PR	18.03161111 -67.07361111	Field corn	*S. frugiperda* larvae	14-15 Feb 2017
PRS	Salinas, PR	17.96122222 -66.26805556	Sorghum	*S. frugiperda* larvae	14-15 Feb 2017
PRJ	Juan Diez, PR	18.02763889 -66.52722222	Field corn	*S. frugiperda* larvae	14-15 Feb 2017

Appendix B: Phylogenetic tree of bacteria from Chapter 4


Brevibacterium casei AJ251418
Brevibacterium picturae AJ620364
Brevibacterium epidermidis X76565
Brevibacterium oceani AM158906
OTU014
Curtobacterium luteum DSM 20542 X77437
Curtobacterium citreum DSM 20528 X77436
Isolate38 (F)
OTU020
Curtobacterium flaccumfaciens AJ312209
Clavibacter michiganensis DSM 7483 X77434
Brachybacterium faecium DSM 4810T X83810
Brachybacterium conglomeratum AB537169
OTU024
Brachybacterium tyrofermentans X91657
Brachybacterium fresconis AJ415378
OTU007
Arthrobacter uratoxydans X83410
Arthrobacter protophormiae X80745
Arthrobacter arilaitensis AJ609628
Kocuria rhizophila Y16264
OTU009
Kocuria koreensis FJ607312
Sinomonas flava EU370704
Sinomonas notoginsengisoli KF876688
Arthrobacter citreus X80737
Arthrobacter koreensis AY116496
Arthrobacter crystallopoietes X80738
Kocuria flava EF602041
Tersicoccus phoenicis EU977596
Paracoccus pantotrophus Y16933
Paracoccus niistensis FJ842690
Paracoccus aminovorans D32240
OTU012
Albidovulum inexpectatum AF465833
Loktanella atrilutea AB246747
Delftia tsuruhatensis AB075017
OTU010
Caenimonas koreensis DQ349098
Delftia acidovorans AB021417
Rhodoferax ferrireducens CP000267
Mitsuaria chitosanitabida AB006851
Rhizobacter fulvus AB245356
Rhodoferax antarcticus GU233447
Acinetobacter parvus AJ293691
OTU011
Acinetobacter radioresistens X81666
Acinetobacter puyangensis JN664255
Stenotrophomonas maltophilia AB294553
Stenotrophomonas pavanii FJ748683
OTU019
Xanthomonas cassavae Y10762
Xanthomonas fuscans JX986955
Stenotrophomonas rhizophila AJ293463
Stenotrophomonas chelatiphaga EU573216
OTU015
Pseudomonas flavescens U01916
Isolate11 (V)
Pseudomonas fulva AB060136
Pseudomonas parafulva AB060132
OTU003
Pseudomonas putida D84020
Pseudomonas oleovorans DQ842018
Azotobacter armeniacus AB175655
Azotobacter beijerinckii AJ308319
Pseudomonas citronellolis Z76659
Pseudomonas nitritireducens HM246143
OTU013
OTU005
Pseudomonas lurida AJ581999
Pseudomonas tolaasii AF255336
OTU023



Appendix B: Phylogenetic tree based on V4 region of the bacterial 16S rRNA gene. Samples include bacteria isolated from virus-killed (V) and freeze-killed cadavers (F), the 25 most abundant bacterial OTUs identified from Illumina sequencing of cadavers (shown in bold), and type strains obtained from the Ribosomal Database Project. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-3051.63) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.5126)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 23.05% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 192 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 243 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

VITA

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Education

2015–2020	PhD in Entomology Penn State University, University Park, PA, USA
2010–2011	Master of Science in Biological Sciences (First Class Honours) University of Auckland, New Zealand
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Selected publications

- Mason, C.J., St. Clair, A., Peiffer, M., Gomez, E., **Jones, A.G.**, Felton, G.W., & Hoover, K. (2020). Diet influences proliferation and stability of gut bacterial populations in herbivorous lepidopteran larvae. *PLOS ONE*, 15(3): e0229848.
- **Jones, A.G.**, Hoover, K., Tooker, J., Pearsons, K. & Felton, G.W. (2020). Potential impacts of translocation of neonicotinoid insecticides to cotton (*Gossypium hirsutum* [Malvales: Malvaceae]) extrafloral nectar on parasitoids. *Environmental Entomology,* 49(1):159–168.
- Mason, C.J., Ray, S. Shikano, I., Peiffer, M. **Jones, A.G.**, Luthe, D.S., Hoover, K. & Felton, G.W. (2019). Plant defenses interact with insect enteric bacteria by initiating a leaky gut syndrome. *Proceedings of the National Academy of Sciences,116* (32): 15991–15996.
- **Jones, A.G.**, Mason, C.J., Felton, G.W. & Hoover, K. (2019). Host plant and population source drive diversity of microbial gut communities in two polyphagous insects. *Scientific Reports, 9*: 2792.
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Selected grants and awards

- AAAS Mass Media Science & Engineering Fellowship (2020)
- Michael E. Duke Memorial Award in Entomology (2019)
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- Penn State University Graduate Fellowship (2015)

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- Teaching assistant: ENT432 Insect Biodiversity and Evolution (Fall 2016)
- Teaching assistant: ENT202 The Insect Connection (Spring 2016)