The Pennsylvania State University

The Graduate School

INDIRECT MANIPULATION OF PLANT INDUCED DEFENSES BY PARASITOIDS OF CATERPILLARS

A Dissertation in

Entomology

by

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Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

August 2019

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Abstract

Almost all plant species are attacked by multiple herbivore species and have evolved various strategies to defend themselves. These plant defense strategies include inducible physical and chemical traits; for example, induced defensive proteins and secondary metabolites can impair herbivore growth and survival. These induced defenses rely on the recognition of herbivore presence. The oral secretions (regurgitant and saliva) of insect herbivores play a crucial role in providing cues that are recognized by plants, which then trigger plant defense responses. However, the interactions between plants and insects are considerably more complex in nature where other trophic levels are involved and can influence these interactions. Microorganisms are abundant in the environment and can impact interactions in many ways including altering the perception of herbivores by plants. Braconid parasitoids are small wasps which lay their eggs inside host caterpillars. These parasitoids possess obligate mutualistic viruses called polydnaviruses (PDVs). PDVs are injected by parasitoids with their eggs into host caterpillars. PDVs suppress caterpillar immune responses and metabolism, thus allowing parasitoid eggs to hatch and develop. In nature, 35-80% of caterpillars are parasitized, depending on locations and host plant species. However, it is not clear how parasitoid/PDVs influence plant and herbivore interactions. The main objective of this study were to: 1. Reveal the mechanism and impacts of the parasitoid and its PDV (*Microplitis croceipes*) on tomato plant defenses through its host caterpillar (*Helicoverpa zea*); 2. Evaluate the consequences of parasitoid suppression of induced plant defenses on plant fitness; and 3. Determine if the parasitoid effect on plant defenses are commonly present in other plant and insect systems. These objectives were approached by a series of biochemical,

physiological and molecular experiments and results provide solid evidence to support the hypothesis that plants can distinguish between feeding by parasitized and non-parasitized caterpillars, thus altering their defense responses accordingly. *Microplitis croceipes* parasitized *Helicoverpa zea* larvae produced lower elicitor activity in their saliva (i.e., glucose oxidase) compared with non-parasitized caterpillars, and significantly downregulated tomato defense-related gene expression and defense protein activities during feeding. The ultimate cause of downregulation of plant defense responses was due to the obligate mutualist PDVs of the parasitoid. PDVs suppressed GOX gene expression and activity in parasitized caterpillar salivary glands thereby downregulating plant defense responses. The lower induced plant defenses benefit the parasitoid by promoting parasitized caterpillar growth performance, producing heavier cocoon mass and overall higher parasitoid survival rate. Besides, tomato plants treated by parasitized caterpillar saliva had significantly *higher* fitness (increased flower number and fruit weight) compared to those treated by non-parasitized caterpillars. These results support the hypothesis that plants benefit from parasitoids indirectly. This is a previously unidentified benefit of parasitoids on plant productivity/fitness. Two other plant and insect systems were also tested and confirmed that parasitoids can indirectly influence plants perception of insect herbivores. These findings have revealed a novel aspect of microbe-mediated interactions between plants and insects. The symbiotic PDV virus not only alters the phenotype of its primary host (i.e., parasitoid) and secondary host (i.e., caterpillar), but also the host plant of the caterpillar. This is the most extreme example of the extended phenotype known: a virus phenotype that extends across three trophic levels. This work has important implications for the evolutionary ecology of plant-herbivore-parasitoid interactions and points out a new perspective of mutualism between plants and parasitoids.

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Acknowledgements

This dissertation would not be possible without all the support and love from my dear friends and family.

First, I would like to thank my advisor Dr. Felton, who I mat six years ago in Taiwan. Thank you for your trust and encouragement that allowed me to achieve my goals. I appreciate your hands-off style with full support and guidance these years. As the head of the department, you are busy all the time, but you always drop your work to listen and support me when I need advice. Thanks for sharing your love of science and being a role model who motivates me to be a better scientist. I would also like to thank my committee: Dr. Dawn S. Luthe, Dr. Jared G. Ali, Dr. Kelli Hoover, and Dr. Cristina Rosa, for advising and assisting from different perspectives. Your expertise in plant science, ecology, physiology and virology trained me to examine questions from multiple angles and broadened my mind.

Special thanks to Michelle Peiffer for all the help, guidance and patience that you provided to support me in science and your precious friendship. I would like to thank Dr. Seung Ho Chung, Dr. Flor Acevedo, Dr. Swayamjit Ray and Dr. Loren Rivera-Vega, who helped me went through the first few years in the program and gave valuable suggestions and assistances to my research. These amazing people are models for me in the program and in life. My thanks to Asher Jones, Po-An Lin, Sulav Paudel, Adam Damon, Shan Jin, Jie Wang and Xuwei Chen for your kindness friendship and positive collaborations that make our lab a wonderful place to work (especially on weekends when I'm not alone☺).

Thanks for the service from the administration staff in the Entomology Department: Ellen Johnson, Erika Stover, Marcia Kerschner, Dave Love and LaTrisha Hough. Special thanks to Nick Sloff for graphic and video assistance.

I appreciate my group of friends who make me feel State College is my second hometown. Thanks to Pei-Ying Yang, Shih-Jung Hsu, Ya-Fei Yang, Ming-Feng Liu, Yuan-Hsin Chen, Po-Yu-Chuang, Neil Huang and Bonita Chen for your sweet friendship and taking care of my wellbeing. And thanks to my little friends: Yu-Hsi, Leia and Aaron for filling my life with joy.

I would like to express my deepest appreciation to my significant other Ju-Che Lo. You help me to walk through the ups and downs of my life. You always fuel my soul and boost me to achieve my dreams. I wouldn't be able to make it without you by my side.

Finally, thanks to my family for supporting me on the path to achieving my dreams.

Chapter 1

Introduction

Plants are the most abundant organisms on earth in terms of biomass and include more than 374,000 species which provide energy to support higher trophic levels (Christenhusz and Byng 2016; Bar-On et al. 2018). Insecta are the most extraordinarily diversity taxa of all described living forms (ca. 1 million species) (Chapman 2009) and the majority of species are phytophagous (Howe and Jander 2008; Futuyma and Agrawal 2009). The long term co-occurrence of plants and herbivorous insects began ~400 million years ago (Early Devonian, Labandeira and Currano 2013) and their interactions have evolved to mutualisms such as pollination, protection and seed dispersal (Bronstein et al. 2006), as well as antagonisms through herbivore consumption of plants to carnivorous plants consuming insects (Suchan and Alvarez 2015; Ellison and Gotelli 2001; Fürstenberg-Hägg et al. 2013). However, plants and insects are not isolated in their natural environment and other trophic levels play key roles in mediating plant-insect interactions (Price et al. 1980). The goal of my research is to understand potential top-down effects from the higher trophic levels on plant defense responses and their consequences for plant and insect fitness.

Plant defense responses

Plants have developed diverse strategies and mechanisms to defend themselves from insect feeding which include direct and indirect defenses

(Fürstenberg-Hägg et al. 2013). Trichromes and spines on plants provide physical barriers to herbivores, wherease plant defense proteins and secondary metabolites are chemical traits used to deter, impair nutrition, or intoxicate herbivores. Both defensive traits of plants could directly interact with herbivores by reducing their food consumption, nutrient absorption, growth performance and reproduction (Zhu-Salzman et al. 2008; Mithöfer and Boland 2012). Also, plants use indirect defenses including volatile organic compounds (VOCs) and extrafloral nectar (EFN) released from damaged plants to recruit natural enemies (e.g. predators and parasitoids), which then reduce pest populations (Heil et al. 2001; Kessler and Baldwin 2001; Dalin et al. 2008; Dicke and Baldwin 2010).

Plant defense traits that are constantly presented at a basic level are known as constitutive defenses. Induced defense responses can be triggered by herbivores, which strengthen plant defenses by increasing concentration, activity and/or density of physical and chemical traits against herbivores (Agrawal 2000; Traw and Dawson 2002; Poelman et al. 2008; Zhu-Salzman et al. 2008). Herbivore induced plant defenses are regulated by phytohormones and mainly related to salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways. JA and ET are signaling compounds which trigger defense pathways against herbivores or necrotrophic pathogens (Berger 2002, De Vos et al. 2005, Thaler et al. 2012, Wasternack and Hause 2013). When biotrophic pathogens infest plants, the SA pathway is activated instead (De Vos et al. 2005, Leon-Reyes et al. 2010, Thaler et al. 2012). The activation of the SA signaling pathway can suppress the JA signaling pathway in plants and vice versa (De Vos et al. 2005, Leon-Reyes et al. 2010, Thaler et al. 2012). The inducible defense responses of plants and phytohormone crosstalk (antagonism) between SA and JA pathways against

intruders are assumed to reduce the cost of plant fitness (Thaler et al. 2012; Sugio et al. 2015).

Herbivore cues

Plant induced defense responses are considered costly and plants only activate these responses when receiving reliable cues that insect herbivores are present. There are several cues derived from insects that can be recognized and trigger induced defenses in plants, these include oral (Acevedo et al. 2015) and ovipositional secretions (Hilker and Meiners 2006; Reymond 2013), and feces (Ray et al. 2016). When insects feed on plants, insect oral secretions (regurgitant and saliva) are deposited on plant wounding sites and play a crucial role in providing cues (herbivore associated molecular patterns, HAMPs) that trigger plant defense responses (Bonaventure 2012; Maffei et al. 2012). Several classes of elicitors/effectors have been identified from insect oral secretions (Acevedo et al. 2015), e.g. fatty acid-amino acid conjugates such as volicitin in beet armyworm (*Spodoptera exigua*) oral regurgitant, which can trigger corn plants to release volatile compounds to attract its natural enemy (*Cotesia marginiventris*) (Alborn et al. 1997).

In caterpillar saliva, glucose oxidase (GOX) activity can mediate plant induced defense responses. Salivary GOX in *Helicoverpa zea* caterpillar functions differently among plant species; it can suppress or trigger plant induced defenses in tobacco and tomato, respectively (Musser et al. 2002; Tian et al. 2012). Most studies of HAMPs and plant defense responses have mainly focused on interactions between plants and insects. However, the interactions between plants

and insects are considerably more complex because other trophic levels (microbes, natural enemies) may affect these interactions (Price et al. 1980).

Microbes are involved in plant and insect interactions

Microorganisms are abundant in the environment and can impact the interactions between plants and herbivores in many ways, including altering the perception of herbivores by plants (Sugio et al. 2015; Shikano et al. 2017). For example, herbivore-associated microbes such as the oral microorganisms harbored by the Colorado potato beetle (*Leptinotarsa decemlineata*) and the false potato beetle (*Leptinotarsa juncta*), suppress tomato and horsenettle defense responses, therefore benefitting insect performance (Chung et al. 2013; Wang et al. 2016). A plant pathogenic virus (*Begomovirus*) carried by whitefly (*Bemisia tabaci*) reduces tobacco terpenoid-mediated plant defenses thus favoring its vector (Luan et al. 2013). Furthermore, plant-beneficial microbes and entomopathogenes may influence plant's perception of insect herbivores by changing the plant's (nutritional and/or phytochemical) and the insect herbivore's (immunity) physiological state (Shikano et al. 2017).

Parasitoid and symbiotic polydnavirus

Another important player in phytobiome interactions are the endoparasitoid wasps of insect herbivores. Parasitism of caterpillars commonly occurs in nature,

depending on locations and host plant species. For example, larval parasitism rates of the large white butterfly (*Pieris brassicae*) can range over 35% on cabbage (Razmiet al. 2011) and more than 70% of the fall armyworm (*Spodoptera frugiperda*) were parasitized on corn (Ashley et al. 1983; Ashley 1986). Corn earworm (*Helicoverpa zea*) parasitism rates can range from 50% to 82% (Young and Price 1975; King and Coleman 1989; Tipping et al. 2005) in the field. In the Hymenoptera, Braconidae and Ichneumonidae, parasitoid wasps possess obligate mutualistic polydnaviruses (PDVs), that are transferred to their hosts when the parasitoid deposits their egg(s) (Burke and Strand 2012; Strand and Burke 2014, 2015). The PDV genomes are stably integrated into the genomes of parasitoid wasps (Strand and Burke 2014). The infection cycles of PDVs occur between two hosts: PDV particles replicate only in the wasps, but infect tissues (including salivary glands) of, and express viral genes in, their caterpillar hosts (Strand and Burke 2012; Herniou et al. 2013). PDVs use virulence factors to manipulate the immune systems of their caterpillar hosts to enable the survival of parasitoid eggs and larvae (Strand and Burke 2014; Beckage 1998; Kroemer and Webb 2004).

The effects of parasitism may also influence plant induced defenses responses by changing the composition of caterpillar oral secretions. Poelman et al. (2011) found that the color of oral regurgitant from *Pieris rapae* and *P. brasscae* caterpillars changed after parasitization (*Cotesia glomerate* and *Hyposoter ebeninus*). The regurgitant of parasitized caterpillars induced higher transcriptome levels of plant defense-related genes in cabbage, thus reducing diamondback moth ovipositional preference. Later on, they found that cabbage plants expressed unique transcriptional levels and produced different volatile compounds while being fed on by parasitized (*Pieris spp.*) caterpillars compared with non-parasitized

caterpillars (Zhu et al. 2015). These studies provide important information that parasitoids can cause top-down effects on plant defenses by manipulating its host caterpillars. However, the key mechanisms of how insect oral cues are changed by parasitism are not known. Moreover, the role of PDVs in mediating these possible changes in salivary components has not been reported.

This dissertation focused on the following objectives: 1. Revealing the mechanism and impacts of parasitoid/PDVs (*Microplitis croceipes*) on tomato plant defenses through its host caterpillar (*Helicoverpa zea*); 2. Evaluating the consequences of parasitoid suppression of induced plant defenses on plant fitness; and 3. Determining if the effects of parasitoids on plant defenses is commonly present in other plant and insect systems. The main hypotheses are that parasitized (P-) caterpillars induce lower plant defense responses than non-parasitized (NP-) caterpillars. Consequently, this suppression of defenses can benefit both the plant and the parasitoid by minimizing plant defense costs and providing a better host quality for the parasitoid. Furthermore, the manipulation of plant defense responses by parasitoids may commonly occur in different systems.

Experimental system

To address the first two objectives, I used the tomato plant, *Helicoverpa zea* larvae, and the specialist parasitoid *Microplitis croceipes* to reveal the mechanism and impacts of parasitoid/PDVs on plant defenses through its host caterpillar. In the third objective, I tested two more plant-insect systems: tomato-*Heliothis virescens*-*Microplitis croceipes* and tobacco-*Helicoverpa zea*-*Microplitis croceipes*

to determine if parasitoid effects on plant perception occurs in different systems.

Tomato (*Solanum lycopersicum*) is one of the most widely distributed plant in the world with a high economic value. Corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) (Lepidoptera, Noctuidae) are generalist herbivores that feed on several plant families and cause serious economic losses in agricultural systems (Harding 1976; Neunzig 1963). *Microplitis crocepies* (Hymenoptera, Braconidae) is a solitary endoparasitoid that specializes on noctuid larvae (Lewis 1970; Lewis and Snow 1971). In the field, *M. croceipes* is the most abundant parasitoid of *H. zea* caterpillars in some areas (Young and Price 1975; Tipping et al. 2005).

Chapters overview

Chapter 2 focuses on the mechanism and impacts of parasitoid/PDVs on plant defenses through its host caterpillar. There are three main questions addressed in this chapter: 1) How does the parasitoid affect host caterpillar oral cues, thereby influencing plant defenses? 2) What is the factor that influences salivary gland elicitors produced by parasitized caterpillars? 3) How does the lower level of induced plant defenses influence parasitoid performance in parasitized caterpillars? This chapter was published in the Proceeding of the National Academy of Science USA (Tan et al. 2018).

Chapter 3 evaluates the consequences of parasitoid suppression of induced plant defenses on plant fitness. From a plant's perspective, induced defenses can be

costly. The lower induced responses elicited by parasitized caterpillars may benefit the plant by reducing defense energy costs compared to those from responses to non-parasitized caterpillars. To date, no studies have tested the effects of parasitoid on plant fitness via mediation of different levels of plant defenses. Thus, the purpose of this study is to test the hypothesis that the lower salivary elicitor activities in parasitized caterpillars and subsequent reduced levels of induced defenses may enhance plant fitness relative to feeding by non-parasitized caterpillars. To test this hypothesis, a long-term (>4 month) plant reproduction experiment was conducted.

Chapter 4 investigates if the phenomenon of parasitoids affecting plant perception of insect herbivores is generalizable to other plant-insect systems. *Microplitis croceipes* is a solitary parasitoid that specializes on noctuid larvae, including *Helicoverpa zea* and *Heliothis virescens*, both of which are naturally distributed in the US (Lewis and Snow 1971; Hopper and King 1984). Two plant-insect systems, tomato-*Heliothis virescens*-*Microplitis croceipes* and tobacco-*Helicoverpa zea*-*Microplitis croceipes*, were used to test if parasitoids affect plant defenses in these two different systems.

Finally, Chapter 5 is a summary of the dissertation, which highlights the significant results of the research and suggestions for future directions.

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Chapter 2

Symbiotic polydnavirus of a parasite manipulates caterpillar and plant immunity

Tan C-W, Peiffer M, Hoover K, Rosa C, Acevedo FE, and Gary WF (2018) Symbiotic polydnavirus of a parasite manipulates caterpillar and plant immunity. Proc Natl Acad Sci USA 115:5199-5204.

Abstract

Obligate symbioses occur when organisms require symbiotic relationships to survive. Some parasitic wasps of caterpillars possess obligate mutualistic viruses called "polydnaviruses." Along with eggs, wasps inject polydnavirus inside their caterpillar hosts where the hatching larvae develop inside the caterpillar. Polydnaviruses suppress the immune systems of their caterpillar hosts, which enables egg hatch and wasp larval development. It is unknown whether polydnaviruses also manipulate the salivary proteins of the caterpillar, which may affect the elicitation of plant defenses during feeding by the caterpillar. Here, we show that apolydnavirus of the parasitoid *Microplitis croceipes*, and not the parasitoid larva itself, drives the regulation of salivary enzymes of the caterpillar *Helicoverpa zea* that are known to elicit tomato plant-defense responses to herbivores. The polydnavirus suppresses glucose oxidase, which is a primary plant-defense elicitor in the saliva of the H. zea caterpillar. By suppressing plant defenses, the polydnavirus allows the caterpillar to grow at a faster rate, thus improving the host suitability for the parasitoid. Remarkably, polydnaviruses

manipulate the phenotypes of the wasp, caterpillar, and host plant, demonstrating that polydnaviruses play far more prominent roles in shaping plant–herbivore interactions than ever considered.

Introduction

Plant–herbivore interactions do not occur in isolation, but are part of a complex, multitrophic network of associated microscopic and macroscopic organisms termed "the phytobiome" (Leach et al. 2017). Microbes associated with herbivores are one component of the phytobiome, and they may perform important functions in facilitating host use by aiding in digesting plant tissues, detoxifying plant toxins, directly supplying nutrients, or facilitating protection from natural enemies (Douglas 2016). Microbial mediation of plant–herbivore interactions may also occur when microbes directly interfere with the perception of herbivores by plants. Plant-defense induction depends upon the plant's ability to detect cues associated with herbivory (Acevedo et al. 2015, Howe and Jander 2008). Plants are able to recognize herbivore cues such as touch, wounding, oviposition, and the feeding cues from oral secretions (e.g., saliva and/or regurgitant) (Acevedo et al. 2015, Bonaventure 2012). However, microbes present in the herbivore's gut can alter the composition of oral secretions and thus may trigger or suppress plant defensive responses (Wang et al. 2017, Chung et al. 2013).

Another important player in phytobiome interactions is the endoparasitoid wasp of insect herbivores. Some parasitoid species possess obligate mutualistic polydnaviruses (PDVs), which are transferred to their caterpillar hosts when the parasite deposits their egg(s) within their hosts (Strand and Burke 2014). PDV

genomes are stably integrated in the genomes of parasitoid wasps (Strand and Burke 2014). The infection cycles of PDVs occur between two hosts: PDV particles replicate only in the wasps, but infect tissues (including salivary glands) of, and express viral genes in, their caterpillar hosts (Herniou et al. 2013). PDVs use virulence factors to manipulate the immune systems of their caterpillar hosts to enable the survival of parasitoid eggs and larvae (Strand and Burke 2014, Beckage 1998, Kroemer and Webb 2004). PDVs are associated with parasitic wasps belonging to the Braconidae and Ichneumonidae families, respectively (Strand and Burke 2014).

The ability of PDVs to interfere with the expression of plant defenses has not been reported, but a few investigations indicate that parasitoids can alter plant responses to herbivores. Poelman et al. (2011) showed that several parasitoid species of pierid caterpillars differentially elicited defense responses in the host plant Brassica oleracea. It was striking that the species of parasitoid had a stronger effect on the induced plant responses than the identity of the caterpillar host. The parasitoids directly affected the caterpillar's oral secretions and its subsequent ability to elicit defense responses, but the role of PDVs in mediating possible changes in salivary components was not reported (Poleman et al. 2011). In another study with *B. oleracea* using the caterpillar *Trichoplusia ni* and its parasitoid *Copidosoma floridanum*, parasitized caterpillars induced 1.5 times higher levels of indole glucosinolate defenses in the plant compared with the nonparasitized caterpillars (Ode et al. 2016). The differential induction in this case was attributed to increased feeding in the parasitized caterpillars (Ode et al. 2016). In contrast to parasitoids that carry polydnavirus symbionts, this particular parasitic wasp maintains and even enhances the host immune system (Nishikawa et al. 2013).

This wasp species is in the Encyrtidae family, members of which do not possess polydnavirus symbionts. Here we report on the multitrophic role of a symbiotic PDV in mediating the phenotypes of the caterpillar and its host plant using the braconid parasitoid *Microplitis croceipes*, the host noctuid caterpillar Helicoverpa zea, and the host plant tomato as an experimental system. The parasitoid uses a PDV, the Bracovirus (*Mc*BV), to immunosuppress its caterpillar host *H. zea*, which allows eggs to hatch and the larvae to feed on the hemolymph of the caterpillar (Kadash et al. 2003). In contrast to the study with parasitism in *T. ni*, this parasitoid does not cause hosts to spend more time feeding (Hopper 1984). An earlier study found that *M. croceipes* strongly suppressed protein synthesis in the salivary glands of its host *Heliothis virescens* (Rana et al. 2002), although effects of caterpillar saliva on host plants were not investigated. Salivary glands are the major source of oral secretions in many caterpillars including *H. zea* (Peiffer and Felton 2009), thus the ability of PDVs to suppress salivary proteins seems likely based upon previous findings with *M. croceipes*. Notably, glands of *H. virescens* and *H. zea* contain a highly abundant immune-related protein, glucose oxidase (GOX), which also acts as an elicitor of plant defenses during feeding (Musser et al. 2002, Tian et al. 2012). The enzymatic products of GOX are D-glucono-δ-lactone and H_2O_2; the latter product possesses antimicrobial activity and acts as a second messenger for the induction of defense proteins such as polyphenol oxidase and proteinase inhibitors in tomato plants (Orozco-Cárdenas et al. 2001).

Materials and Methods

Insect colonies

H. zea eggs were purchased from Frontier Agricultural Sciences, and the colony was maintained in our laboratory for multiple generations. Larvae were fed an artificial diet (Peiffer and Felton 2005) and reared individually until pupation. Pupa were collected and placed in a plastic container [15 (diameter) cm × 28 (height) cm] through adult emergence, and sugar solution (10%) was provided as food for adults.

The *M. croceipes* colony was obtained from Henry Fadamiro, Auburn University, Auburn, AL, and maintained in our laboratory according to established protocols (Lewis and Burton 1970). At the last day of the second instar stage (head capsule slippage stage), *H. zea* larvae were offered to one female parasitoid (>1 d old). Caterpillars were removed immediately following a single oviposition by the parasitoid to avoid multiple attacks by the parasitoid. Parasitized caterpillars were fed an artificial diet until parasitoid larval egression. Both insect species were reared in a growth incubator (25 ± 2 °C, 16 h light:8 h dark). Parasitoid larvae typically require 10 d to fully develop, egress, and spin the pupal cocoon (on the seventh day of the fourth instar stage *H. zea*). Adult wasps were sexed using the length of antenna and presence of an ovipositor (Lewis and Burton 1970). Following pupal eclosion, each female was maintained with several males in a container [9.5 (diameter) cm × 6 (height) cm] and fed on cotton saturated with 30% honey solution. Parasitized caterpillars in all experiments were used 6 d after parasitism (the third day of fourth instar stage), and nonparasitized caterpillars were used on the second day of the fourth instar stage (there are 3 d in the fourth

instar stage) unless otherwise noted.

Plants

Tomato (*Solanum lycopersicum* cv. Betterboy) seeds were germinated in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) and grown in a greenhouse (16 h light:8 h dark) at Pennsylvania State University. Seedlings were transferred to pots (10 × 10 × 9 cm) 2 wk after germination and watered daily. Three grams of fertilizer (Osmocote, 15–9-12) was applied on the top of the soil to promote plant growth. Plants with five to six fully expanded leaves (4–5 wk old) were used in all experiments.

Caterpillar feeding and plant-defense responses

To determine how parasitized caterpillars affect plant-defense responses, plant-defense–related gene expression and protein activities were examined in this study. Plants were separated into three treatment groups: P-caterpillars feeding, NP-caterpillars feeding, and control plants (C) without caterpillars. One P- or NP-caterpillar was placed in a clip cage on the third (counting from the bottom) terminal leaflet of each tomato plant. This method restricts caterpillars to consuming a similar amount of leaf tissues (3.15 cm2) at a particular site during a prescribed time period. In the control treatment, an empty cage was placed on the tomato plant. Leaf cages were removed when the caterpillar consumed the entire leaf tissue inside the cage within 10 h.

Twenty-four hours after placing the caterpillars on the plants, 100 mg of the third terminal leaflet was collected for RNA extraction. Leaf samples were ground

(Geno/Grinder 2000, Spex Sample Prep) with liquid nitrogen. RNA was extracted with TRizol reagent (Ambion). One microgram of RNA was used to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a PCR thermal cycler (GeneAmp PCR System 9700) (Chung et al. 2013). Gene primers of defense genes used for quantitative real-time PCR (qRT-PCR) analysis were designed by Primer Quest Software (Applied Biosystems) and listed (Table 2-1). qRT-PCR analysis was performed by the 7500 Fast Real-Time PCR System (Applied Biosystems) with the FastStart Universal SYBR Green PCR Probe Master (Roche). Actin (ACT) and ubiquitin (UBI) genes were used as reference genes in tomato plants. Target genes' relative expressions were compared with intact control (C) by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

Table 2-1. Primer Pairs used for tomato, *H. zea*, and *Mc*B*V* gene expression

Gene name	Description	Species	Forward	Reverse	Accession No.
AspPI	Aspartic proteinase inhibitor	Tomato	AGCCAGTCCTTGAC ACAAGTGGTA	AGGTACACATCACC ACCTAACGCA	SGN-U143342[a]
CysPI	Cysteine proteinase inhibitor	Tomato	GGTGAAGGAATGGG AGGACTTCAA	GGAGGTTTGGGAA TGGAACATTGG	AF198390
PPOB	Polyphenol oxidase B	Tomato	TTCGCGAGTGGGAAT ACCTCGTTT	AGTCAGGGACTGT TTGGACACGAA	Z12834
PPOE	Polyphenol oxidase E	Tomato	TAACTCCTTGCAAAC TCCGCCTTA	AACCCTTACCGTGT GAAAGTCCGA	Z12837
PPOF	Polyphenol oxidase F	Tomato	ATGTGGACAGGATGT GGAACGAGT	ACTTTCACGCGGTA AGGGTTACGA	Z12838
TD2	Threonine deaminase 2	Tomato	CCCTGGGAGGTGAT GTAGTTCT	TCGAATGGTGGGAT GTATTTGAG	M61915
PIN2	Wound-induced proteinase inhibitor 2	Tomato	GGATTTAGCGGACTT CCTTCTG	ATGCCAAGGCTTGT ACTAGAGAATG	K03291
MTS1	Linalool synthase	Tomato	GTAACATAGGGATGA	CTGAACGCCTTGT	NM001246876

	(TPS5)		TGGTGGTCACCTT	GGTGGAAAT	
GAME4	Glycoalkaloid metabolism 4	Tomato	TCCTTCGAGATGGGA TGATATTG	GCTTGGCCAAATTG GATCCT	SGN-U588897[a]
PAL5	Phenylalanine ammonia-lyase 5	Tomato	AAGATTGTAGCTTTC GAGGACGAA	GGAATTGCAGGGT TTCCACTT	NM001320040
UBI	Ubiquitin	Tomato	GCCAAGATCCAGGA CAAGGA	GCTGCTTTCCGGCG AAA	X58253
ACT	Actin-7	Tomato	AGGTGTTATGGTCGG AATGG	TCATCCCAATTGCT GACTATACC	AB199316
GOX	Glucose oxidase	*H. zea*	ATTGCGGCTGCCAAA CA	CGCTACATGCTGTG GAGT TCA	FJ460711
ACT	Actin	*H. zea*	GATGCCGTTGGCTTC CAT	CGTTGCCCTGAGG CTCTCT	XM_021337743
OrphM 5	*Microplitis demolitor* bracovirus segment M	*Mc*BV	TTCAGACAGAAACG ATGCAGA	GATGCGTTTCTTCG TACGTG	AY875688

[a] Solanaceous Gene Network (SGN) Unigene id

Forty-eight hours after treatments, 50 mg of the third terminal leaflet was collected for PPO and TI activity assays. Leaf samples were frozen with liquid nitrogen and groundwith the Genogrinder. Immediately, 1.25mLof phosphate buffer (0.1 M, pH 7) with 5% polyvinylpyrrolidone (PVP) (Alfa Aesar 41631) was added, and samples were vortexed and incubated on ice for 5 min. Supernatant was collected after centrifugation (4 °C, 11,000 × g, 10 min). To measure PPO activity, 5 μL of each sample was added to 200 μL caffeic acid (3 mM; Sigma C0625), and the change in absorbance at λ450 was recorded in the Spectramax 190 plate reader (Molecular Devices) (Felton et al. 1989). For trypsin inhibition analysis, samples were powdered by the Genogrinder, and then 1.25 mL of extraction buffer (0.046 M Tris and 0.0115 M CaCl2; pH 8.1) with 5% PVP was added. The supernatant (4 °C, 11,000 × g, 10 min) was collected for TI activity measurement. Ten microliters

of sample was mixed with 10 μL of trypsin (20 μg/mL; Sigma T1426) and 80 μL of extraction buffer. Ten minutes later, 100 μL of p-toluene-sulfonyl-L-arginine methyl ester (0.002 M; Sigma T4626) was added, and absorbance values were read at 247 nm (Chung et al. 2013). Percentage inhibition was calculated by comparing the activity of trypsin and extraction buffer alone.

Caterpillar Saliva and Plant-Defense Responses

To determine if saliva is responsible for the differences that we observed in plant-defense responses from feeding caterpillars, caterpillars were parasitized as described above. Labial salivary glands were collected from P- and NP-caterpillars. Glands were homogenized with phosphate buffer (0.1 M, pH 7), and then supernatant was collected after centrifugation (4 °C, 7,500 × g, 10 min). Protein in the supernatant was quantified by Bradford assay (Bradford 1976), and all samples were diluted to 1 μg/μL. The third terminal leaflets of tomato plants were treated with mechanical wounding and application of 15 μL of salivary gland supernatant from parasitized and nonparasitized caterpillars, respectively. The third group was the intact control plant without any treatment. For gene expression experiments, samples of the wounded leaf were collected 24 h after treatment. PPO was analyzed 48 h after treatment.

Caterpillar Salivary Glucose Oxidase Transcript Levels and Enzyme Activities

To determine how parasitism affects GOX gene expression and enzyme activity, time-course experiments were conducted for P- and NP-caterpillars. Due to the very small size of younger caterpillars and feasibility of dissections, third

instar stage *H. zea* caterpillars were used for these two experiments. For GOX gene expression, on the last day of the third instar stage, *H. zea* larvae were parasitized by *M. croceipes*. Salivary glands were collected from P-caterpillars 1, 2, and 6 d after parasitism. Because NP-caterpillars develop faster, NP-caterpillar salivary glands were collected at the same developmental stage as the P-caterpillar treatment. Caterpillar salivary gland RNA extraction and cDNA synthesis were as described above. *H. zea* glucose oxidase (GOX) gene expression was tested by qRT-PCR analysis, and actin (ACT) was used as the reference gene. Gene relative expression was calculated as described above. For GOX enzyme activity, on the last day of the third instar stage, *H. zea* larvae were parasitized by *M. croceipes*. P-caterpillar salivary glands were collected at 0, 2, 4, and 6 d after parasitism. NP-caterpillar salivary glands were collected at the same developmental stage as caterpillars. GOX enzyme activity was analyzed as described (Eichenseer et al. 1999).

GOX Dosage Effect on Plant-Induced Defense Response

To determine the effect of GOX dosage on plant-defense response, tomato plants were treated with varying concentrations of GOX from Aspergillus niger (Sigma-Aldrich) based upon a previous estimate of levels of GOX secretion by *H. zea* (Peiffer and Felton 2005). The youngest fully expanded terminal leaf was mechanically wounded, and immediately 20 μL of GOX was diluted in 0.1 M phosphate buffer (pH 7.2). After 48 h, 50-mg samples of tissue were collected from the wounded leaf, frozen in liquid nitrogen, and then stored at −80 °C until further analysis. Leaf tissue was analyzed for trypsin inhibitor activity as previously described.

Caterpillar Host Performance

To evaluate the effect of plant-defense responses on P-caterpillar performance, we performed a larval relative growth rate experiment. There were three groups in the experiment: caterpillars feeding on plants treated with saliva from P-caterpillars, NP-caterpillars, and intact control plants (C). Plants were treated with labial salivary gland homogenate as described above. Forty-eight hours after treatment, the treated leaf was collected for bioassay. Third instar stage *H. zea* of similar body size were selected and parasitized by *M. croceipes*. Caterpillars were weighed and then fed on the treated tomato leaves in plastic cups lined with 2% agar to keep leaves moist. Twenty-four hours later, caterpillars were reweighed and relative growth rate was calculated as follows: (final weight − initial weight)/(average weight × no. of days).

Parasitoid Performance

To determine if different levels of induced plant defense responses caused by parasitized and nonparasitized caterpillars influence parasitoid development, we conducted a parasitoid performance experiment. Plants were treated with labial salivary gland homogenate as described above. Forty-eight hours after treatment, the treated leaf was collected and placed in a plastic cup lined with 2% agar to keep leaves moist. Third instar stage *H. zea* larvae of similar body size were parasitized by *M. croceipes* and fed in one of two treatments: P-caterpillar–treated plants or NP-caterpillar–treated plants (total n = 54; six replicates of nine individuals per treatment). Leaves were changed every other day to keep food fresh until parasitoid larvae spin the pupa cocoon. Larval duration, cocoon weight, pupal

duration, larval mortality, cocoon formation failure rate, adult emergence rate, and survival rate were recorded. Cocoon weight was measured 2 d after cocoon formation, and adult emergence rate was calculated 30 d after cocoon formation. For the percentage of larval mortality, percentage of cocoon formation failure rate, percentage of adult emergence, and percentage of total survival, data were calculated from six replicates with n = 9 individuals per treatment.

Experiments with *M. croceipes* Polydnavirus (*Mc*BV)

*Mc*BV was purified from the calyx region (Fig. 2-1) of the ovaries of *M. croceipes* following an established protocol (Fathpour and Dahlman 1995) with slight modifications. Briefly, female *M. croceipes* were chilled on ice for 15 min and rinsed in autoclaved water before dissection. Ovaries were collected from 30 females into a 1.7-mL microtube and then homogenized with 100 μL of Tris buffer (0.5 M, pH 6.8). Crude calyx extract was collected after centrifugation (4 °C, 800 × g, 15 min). One hundred microliters of Tris buffer was added to the pellet for a second extraction. The two extracts were pooled and centrifuged (4 °C, 12,000 × g, 5 min). The supernatant was applied to a sucrose gradient (40–70% sucrose) and centrifuged in a swinging bucket rotor (4 °C, 24,000 × g, 30 min). After centrifugation, the PDVs were visible as a blue band in the sucrose gradient. The band was collected with a micropipette, and PDVs were pelleted by centrifugation (4 °C, 49,000 × g, 45 min). The resulting pellet was resuspended in Pringle's saline (pH 7.39) and adjusted to a suitable concentration
for injection. Virus collected from each female wasp is defined as one female equivalent (FE). For injection, 5 μL of Pringle's saline (S) or 5 μL of 0.02 FE of PDV (0.1 FE) was injected into last-day third instar stage *H. zea* caterpillars using

a microinjector (Burkard PAX 100) with a 32-gauge hypodermic needle. All processes, including PDV collection, purification, and injection were finished within 4.5 h. The same stage of *H. zea* caterpillars was offered to *M. croceipes* females for parasitism (P) treatment. Two days after injection or parasitism, labial glands were collected from a subset of each treatment.

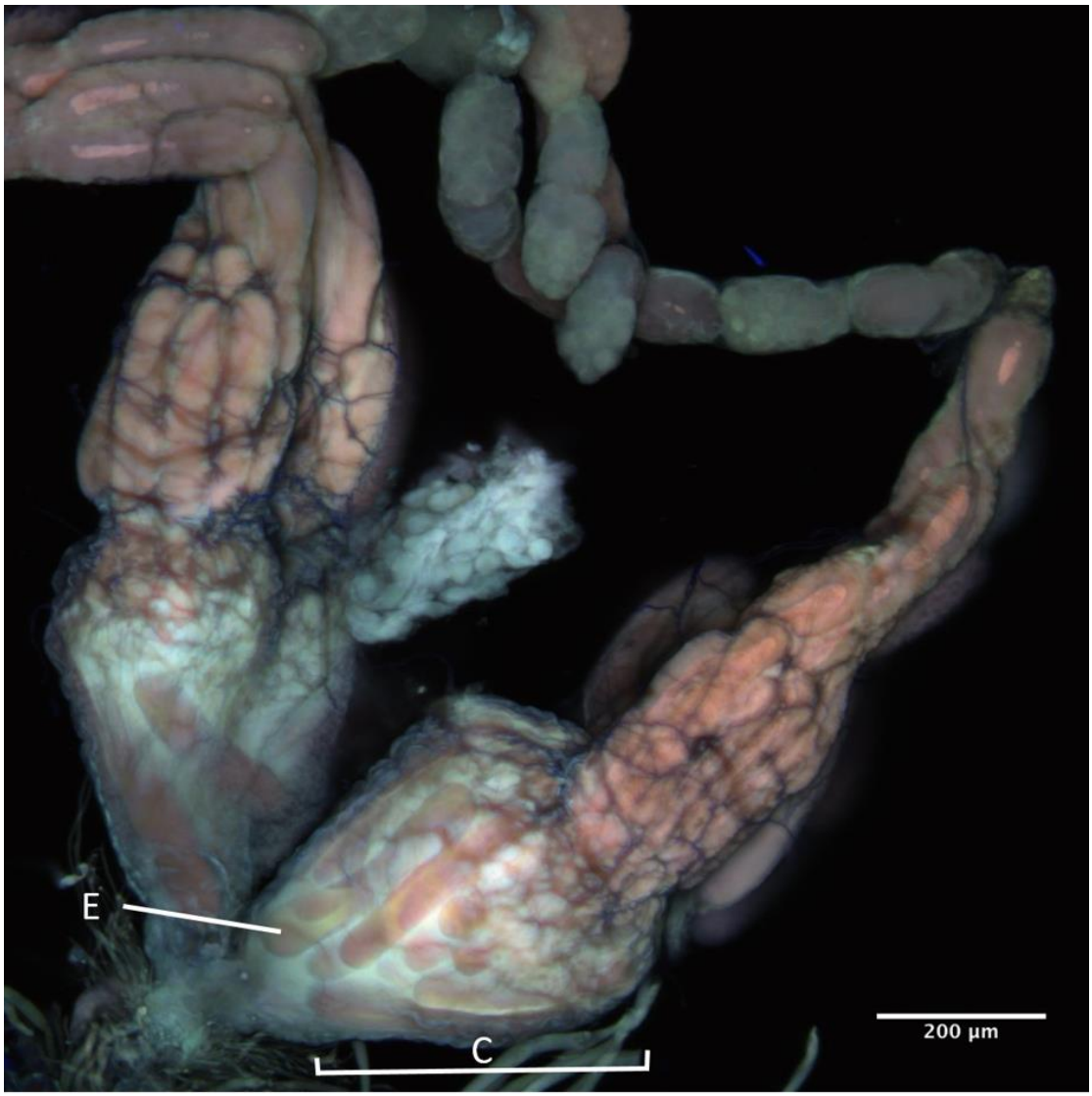

Fig. 2-1. Confocal Laser Scanning Microscopy of female M. croceipes reproductive organs. The light blue autoflorescence is light scattering by polydnavirus particles in the calyx fluid (Tyndall effect). C: calyx; E: eggs.

To confirm virus expression in the labial glands of injected caterpillars, the PDV gene ORPHM5 was amplified after RNA extraction and cDNA synthesis (as described above). The primers used to amplify the putative region of the *Mc*BV segment are shown in Table 2-1. All caterpillars (n = 6) that were injected with *Mc*BV showed viral gene expression in the labial glands (Fig. 2-2). Six days after parasitism (third day of the fifth instar stage and second day of the fifth instar stage of S- or *Mc*BV-treated *H. zea* caterpillars) were caged on plants for measuring plant-defense gene expression and PPO and TI activities as described above.

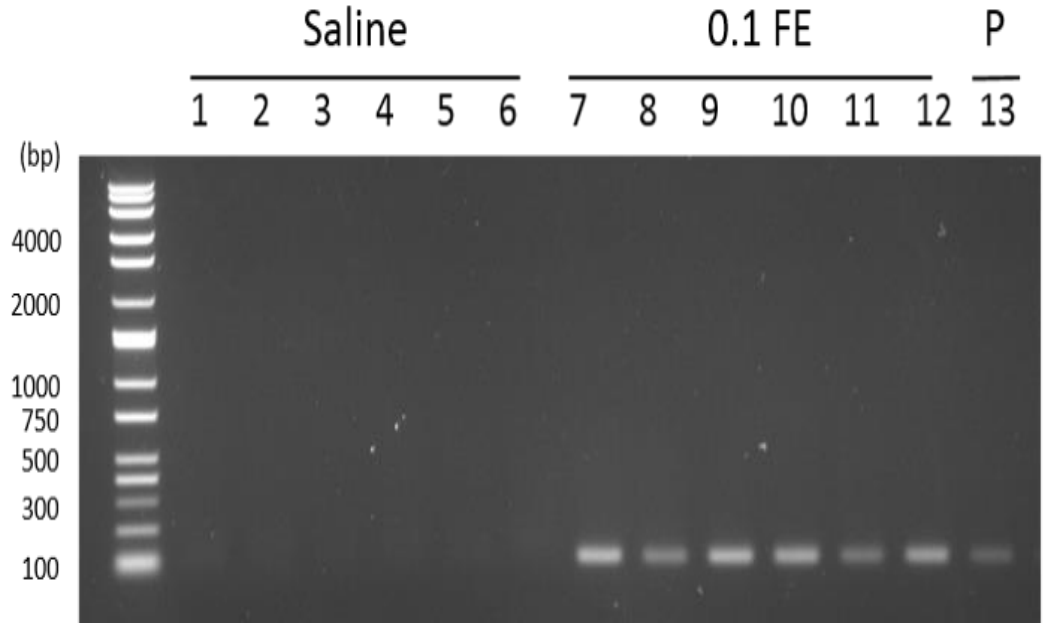

Fig. 2-2. Presence of McBV in Helicoverpa zea larvae. Caterpillars were treated with saline injection, 0.1FE *Mc*BV injection or parasitization treatments. Salivary glands were collected 48 h after treatment and tested by Orph M5 primer. 1-6, saline injected caterpillars; 7-12, 0.1 female equivalent *Mc*BV injected caterpillars; 13, parasitized caterpillars.

To determine the effect of virus on salivary enzyme activity, labial glands were collected from injected caterpillars 6 d post injection, and GOX activity was assayed as described above. To determine if *Mc*BV is the factor reducing *H. zea* salivary gland GOX gene expression, a GOX relative expression experiment was conducted. *Mc*BV were collected and purified as described above. There were three groups treated: S-injected, 0.1 FE *Mc*BV-injected, and parasitized caterpillars. Caterpillar salivary glands were collected 2 d after treatment. Salivary gland RNA extraction, cDNA synthesis, and qRT-PCR analysis were as described above.

Confocal Laser-Scanning Microscopy of Female *M. croceipes* Reproductive Organs

Female adult wasps were chilled on ice and dissected in 0.1 M monobasic phosphate buffer. After removing the female ovipositor, the attached reproductive organs were fixed (2.5% glutaraldehyde, 0.1 M phosphate buffer, and 0.05 g/mL sucrose) in room temperature. The tissues were placed on microscope slides with glycerol and then examined with an Olympus FV10i Confocal Laser Scanning Microscope (CLSM) using the following excitation wavelengths: 405, 473, and 559 nm with emission wavelengths 430, 520, and 600 nm, respectively. Images were processed using FIJI–Image-J (Fig. 2-1).

Statistical Analyses

Data were transformed as needed to obtain a normal distribution and to address residuals with heterogeneity of variance; SAS 9.4 (SAS Institute) was used for all analyses. Plant-defense responses (gene expression and TI and PPO

activities) and caterpillar performance bioassays were analyzed using one-way ANOVA (Proc GLM), followed by means comparisons using Tukey's Least Significant Difference (LSD) test (significance level, $P < 0.05$). Insect salivary gland (GOX) gene expression and GOX enzyme activity (time course) were compared using Student's t test. GOX concentration on TI activity was analyzed by one-way ANOVA (Proc GLM), followed by means comparisons using Tukey's LSD test (significance level, $P < 0.05$). Parasitoid performance (larval duration, cocoon weight, pupal duration, and percentage of larval mortality, cocoon formation adult emergence, and total survival) were compared using Student's t test. *Mc*BV injection experiments (plant defense responses, insect GOX gene expression, and enzyme activity) were analyzed using one-way ANOVA (Proc GLM), followed by means comparisons using Tukey's LSD test (significance level, $P < 0.05$).

Results and Discussion

To determine if parasitism by *M. croceipes* affects the ability of *H. zea* to induce plant defenses, both parasitized (P) caterpillars and nonparasitized (NP) caterpillars were allowed to feed on tomato leaves for 10 h while the total feeding damage between treatments was kept consistent by restricting caterpillars to feed within a cage. Forty-eight hours later the activities of plant defense proteins [i.e., trypsin inhibitor (TI) and polyphenol oxidase (PPO)] were assayed on the damaged leaves. P-caterpillars induced significantly lower levels of trypsin inhibitor and PPO activities (Fig. 2-3) than did the NP-caterpillars. Both protease inhibitors and PPO are known to reduce the growth rate of caterpillars, and of *H. zea* in particular (22–24). We then examined the transcript levels of defense genes encoding PPO, protease inhibitors, and other defense proteins (at 24 h post feeding) known to be induced by *H. zea* saliva (20, 25) and found that P-caterpillars consistently induced lower levels of transcripts encoding plant defensive proteins such as PPO (*PPOB, PPOE, PPOF*), threonine deaminase (*TD2*), and proteinase inhibitors (*AspPI, CysPI*) than did NP-caterpillars (Fig. 2-4). Threonine deaminase degrades the essential amino acid threonine in the guts of caterpillars, thus impairing their growth (26). Transcripts encoding proteins for biosynthesis of toxic secondary metabolites including terpenes (*MTS1*), phenolics (*PAL5*), and glycoalkaloids (*GAME4*) were also attenuated in plants fed on P-caterpillars compared with their nonparasitized counterparts (Fig. 2-4).

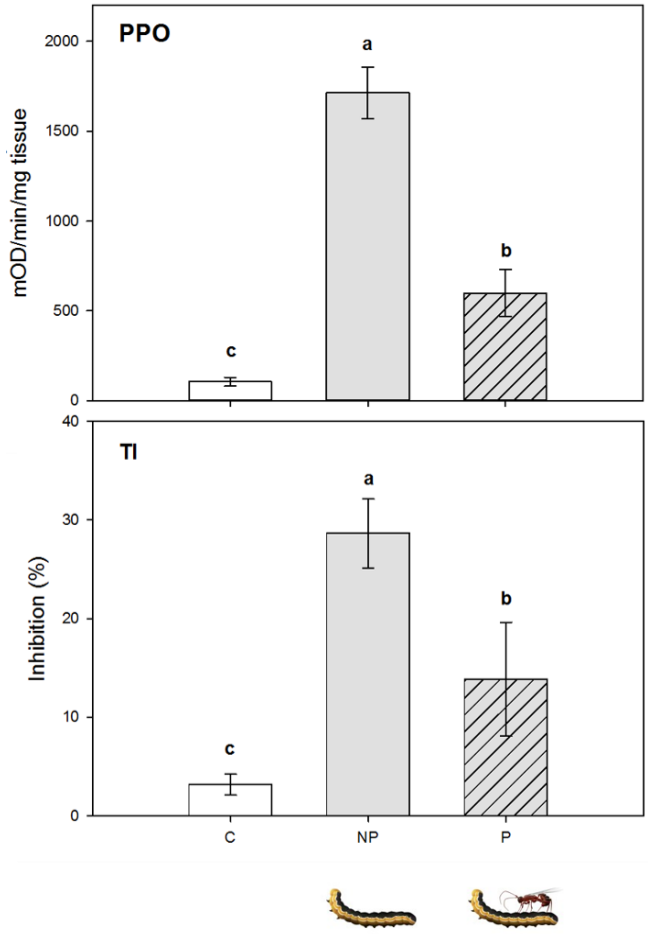

Fig. 2-3. Effect of caterpillar parasitism on induction of plant defensive proteins. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; PPO, n = 7–9, $F_{(2, 21)} = 58.5$, $P < 0.0001$; TI, n = 6–8, $F_{(2, 17)} = 79.0$, $P < 0.0001$. C, intact control plant; NP, plant treated with nonparasitized caterpillar; P, plant treated with parasitized caterpillar; PPO, polyphenol oxidase; TI, trypsin inhibitor.

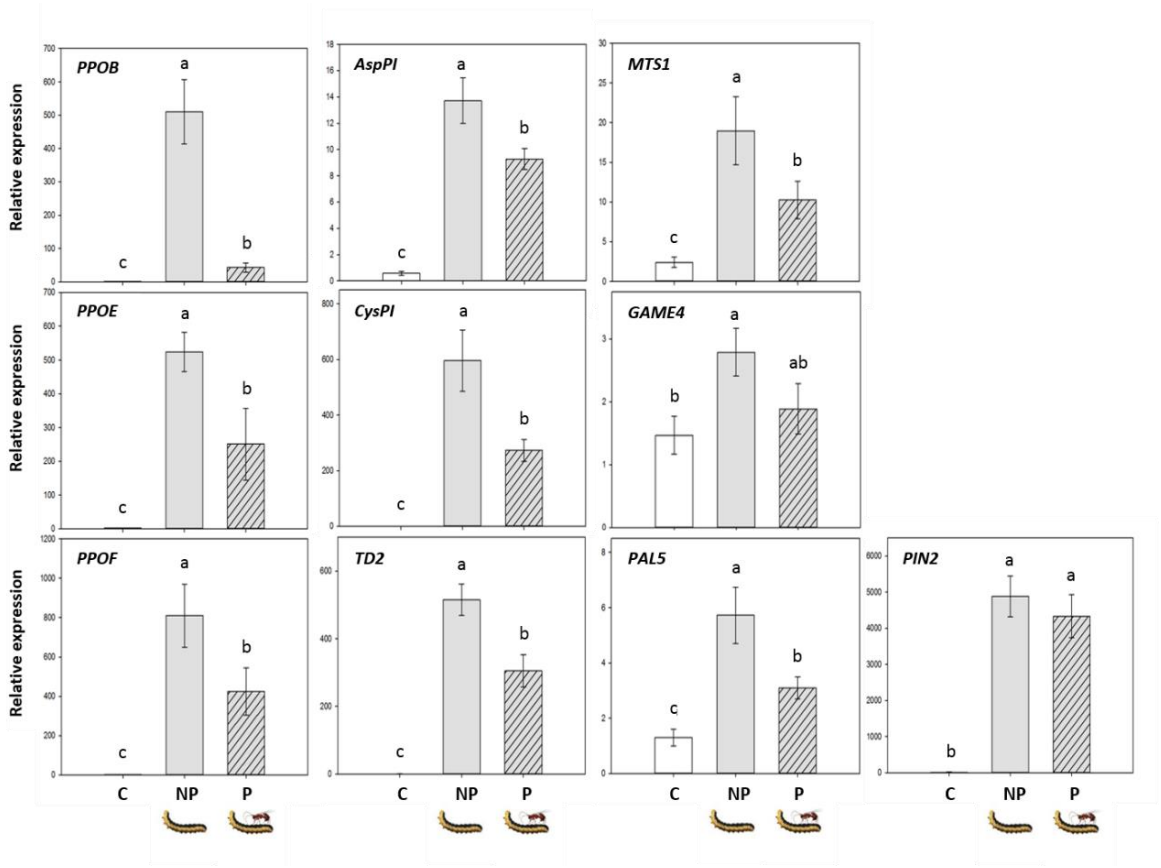

Fig. 2-4. Expression levels of defense protein and secondary metabolite synthesis genes in plants treated with non-parasitized caterpillars (NP), parasitized caterpillars (P) or unwounded control plants (C). All gene expressions were measured 24 h after insect wounding. Values are untransformed means ± SEM (n=4-8). Different letters indicate significant differences between treatments (ANOVA followed by LSD test, a=0.05: *AspPI*, $F_{(2, 20)} = 143$, $P < 0.0001$; *CysPI*, $F_{(2, 12)} = 306$, $P < 0.0001$; *PPOB*, $F_{(2, 16)} = 74.5$, $P < 0.0001$; *PPOE*, $F_{(2, 17)} = 95.3$, $P < 0.0001$; *PPOF*, $F_{(2, 20)} = 62.07$, $P < 0.0001$; *TD2*, $F_{(2, 18)} = 675$, $P < 0.0001$; *PIN2*, $F_{(2, 21)} = 209$, $P < 0.0001$; *MTS1*, $F_{(2, 17)} = 22.0$, $P < 0.0001$; *GAME4*, $F_{(2, 20)} = 3.68$, $P = 0.04$; *PAL5*, $F_{(2, 19)} = 23.7$, $P < 0.0001$). C, control plant; P, plant treated with parasitized caterpillar; NP, plant treated with non-parasitized caterpillar. *AspPI*, aspartic proteinase inhibitor; *CysPI*, cysteine proteinase inhibitor, *PPOB/E/F*, polyphenol oxidase B/E/F; *TD2*, threonine deaminase; *PIN2*, proteinase inhibitor 2; *MTS1* (*TPS5*), linalool synthase; *GAME4*, glycoalkaloid metabolism 4; *PAL5*, phenylalanine ammonia-lyase 5.

To determine if caterpillar saliva was responsible for the observed differential plant responses, we applied saliva from P- and NP-caterpillars to plant wounds and then assayed for a subset of the plant-defense genes including *PIN2*, *TD2*, and *AspPI* and the defense protein PPO. Saliva from P-caterpillars induced lower levels of PPO and the three defense genes compared with saliva from NP-caterpillars, indicating that parasitism alters salivary components responsible for eliciting defenses (Fig. 2-5). We then measured the effect of parasitism on GOX, which is a highly abundant immunity-related protein in the saliva of *H. zea*, that elicits antiherbivore defenses in tomato, including the aforementioned plant-defense genes (Tian et al. 2012). P-caterpillars had dramatically lower GOX transcript levels than did NP-caterpillars at 2 d post parasitism (Fig. 2-6A), and GOX enzymatic activity was significantly lower at 4 and 6 d following parasitism (Fig. 2-6B). The response of tomato to GOX is dose dependent: leaves treated with increasing levels of GOX triggered higher levels of protease inhibitors (Fig. 2-7).

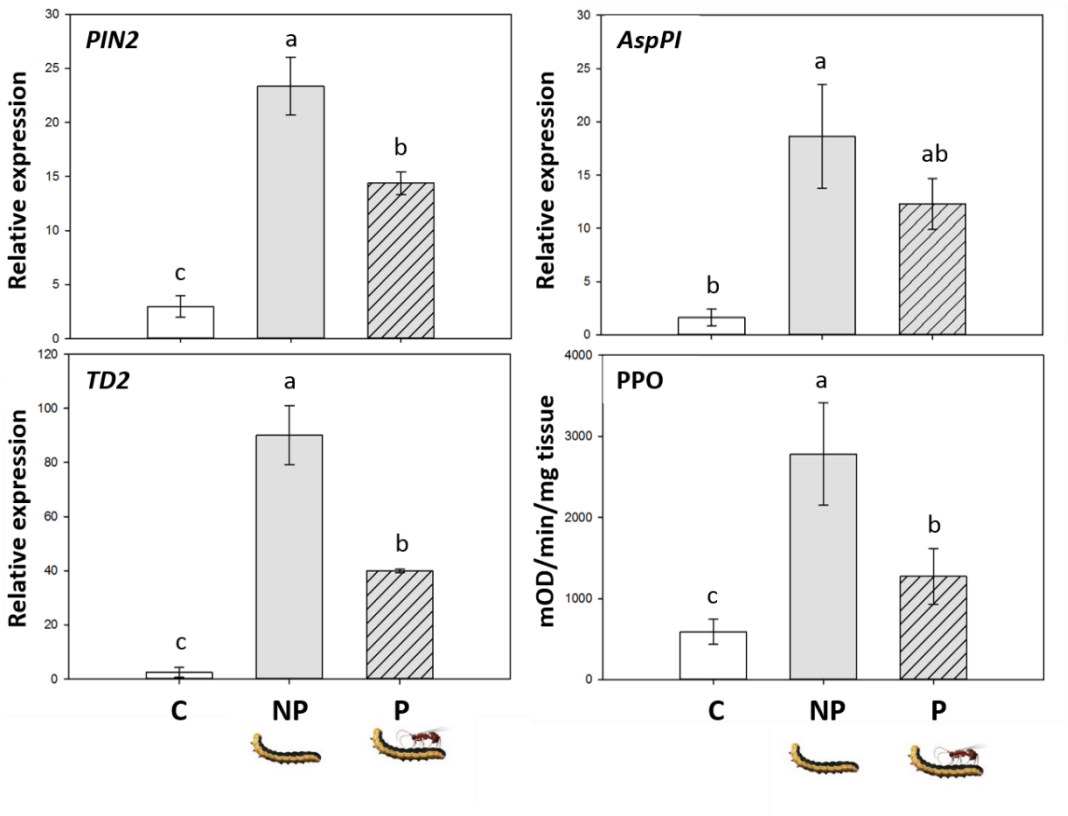

Fig. 2-5. Expression levels of defense protein genes and polyphenol oxidase (PPO) activity in plants treated with saliva of non-parasitized caterpillars (NP), parasitized caterpillars (P) and unwounded control (C). Gene expression was measured 24 h and PPO 48 h after saliva application. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, a=0.05: *PIN2*, $F_{(2, 7)} = 29.0$, $P = 0.0004$; *TD2*, $F_{(2, 7)} = 63.9$, $P < 0.0001$; *AspPI*, $F_{(2, 8)} = 5.61$, $P = 0.03$). PPO, $F_{(2, 12)} = 5.56$, $P = 0.0195$). C, intact control plant; P, plant treated with parasitized caterpillar saliva (15 µl (1 µg/µl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 µl (1 µg/µl protein)). *PIN2*, proteinase inhibitor 2; *TD2*, threonine deaminase; *AspPI*, aspartic proteinase inhibitor.

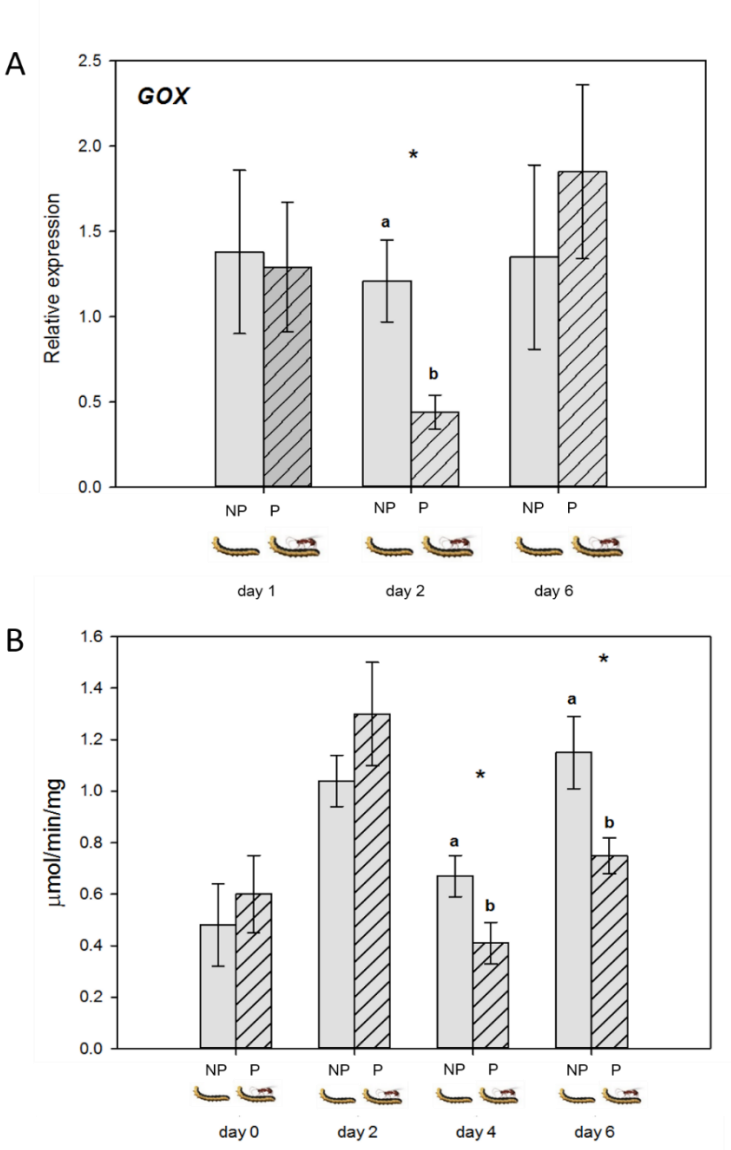

Fig. 2-6. GOX transcript levels and enzyme activity at different time points after parasitism. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments. Treatments include P and NP-caterpillars. Relative expression of GOX (A) was measured 1, 2, and 6 d after parasitism: ANOVA, $\alpha = 0.05$; followed by Student's t test; day 1, n = 5–8, $F_{(1, 11)} = 0.0225$, $P = 0.882$; day 2, n = 11–14, $F_{(1, 23)} = 11.29$, $P = 0.003$; day 6, n = 6, $F_{(1, 10)} = 0.8836$, $P = 0.37$. GOX activity (B) was measured at 0, 2, 4, and 6 d after parasitism: ANOVA, $\alpha = 0.05$; followed by Student's t test; day 0, n = 12–15, $F_{(1, 25)} = 0.69$, $P = 0.42$; day 2, n = 15, $F_{(1, 28)} = 0.74$, $P = 0.4$; day 4, n = 9–11, $F_{(1, 18)} = 4.75$, $P = 0.04$; day 6, n = 15, $F_{(1, 28)} = 5.57$, $P = 0.027$.

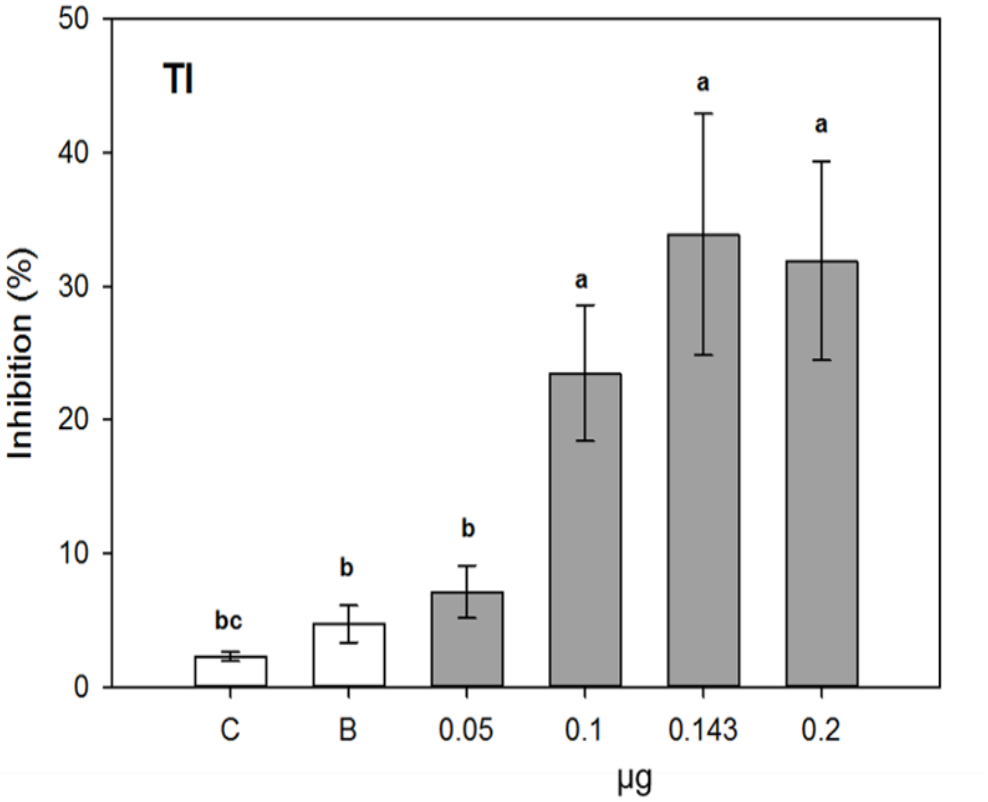

Fig. 2-7. Plant induced defense response in different glucose oxidase (GOX) concentrations. Trypsin inhibitor (TI) activity was measured 2 days after treatment. Treatments include untreated control (C), buffer (B), 0.05 µg GOX (0.05), 0.1µg GOX (0.1), 0.143 µg GOX (0.143) and 0.2 µg GOX (0.2) treatments. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, α=0.05; TI, n=4-10, $F_{(5, 38)} = 7.54$, $P < 0.0001$).

To determine if *M. croceipes* polydnavirus is directly responsible for suppressing GOX activity, we purified *Mc*BV from the calyx region of the wasp ovaries, injected the virus into caterpillars, and then measured GOX activity and expression after verifying *Mc*BV infection by PCR. *Mc*BV markedly suppressed GOX enzyme and transcript levels in a similar fashion as parasitism by *M. croceipes* wasps (Fig. 2-8 and Fig. 2-9). Feeding by caterpillars that were injected with PDV induced significantly lower defense gene expression (e.g., *PPOB*, *CysPI*, *AspPI*, *TD2*, *PIN2*) and defense protein activity (TI, PPO) than did the

saline-injected caterpillars (Fig. 2-10), which indicates that *Mc*BV is the primary driver in mediating these multitrophic interactions.

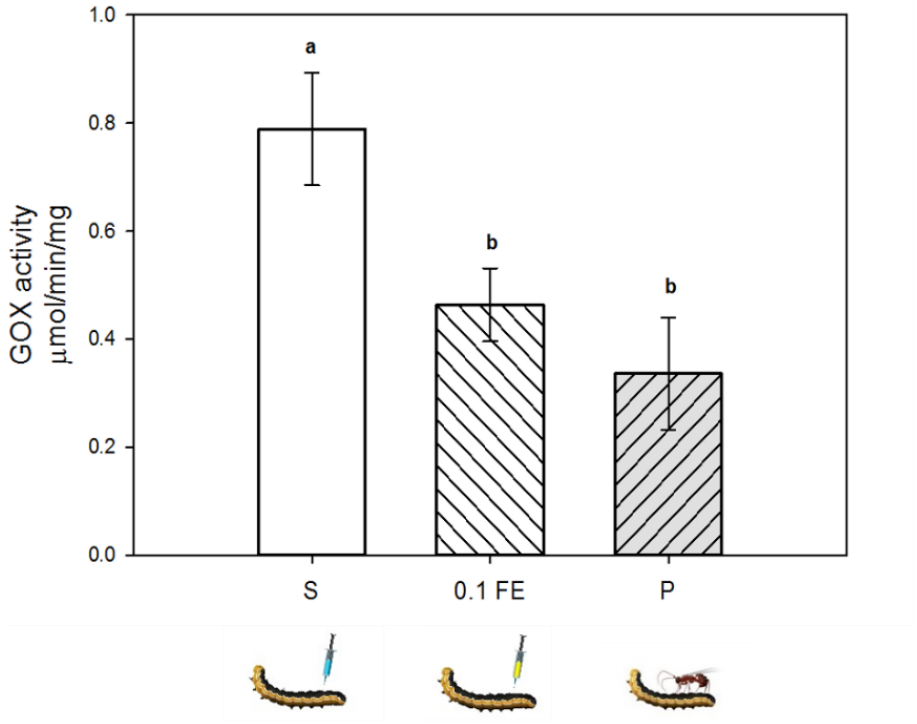

Fig. 2-8. Effects of parasitism and polydnavirus on salivary GOX activity of caterpillars. Treatments include saline (S), 0.1 FE and P-treated caterpillars. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$: n = 13–16, $F_{(2, 41)} = 6.33$, $P = 0.004$. P, parasitized caterpillars; S, Pringle's saline injected caterpillars; 0.1 FE, caterpillars injected with 0.1 FE purified *Mc*BV.

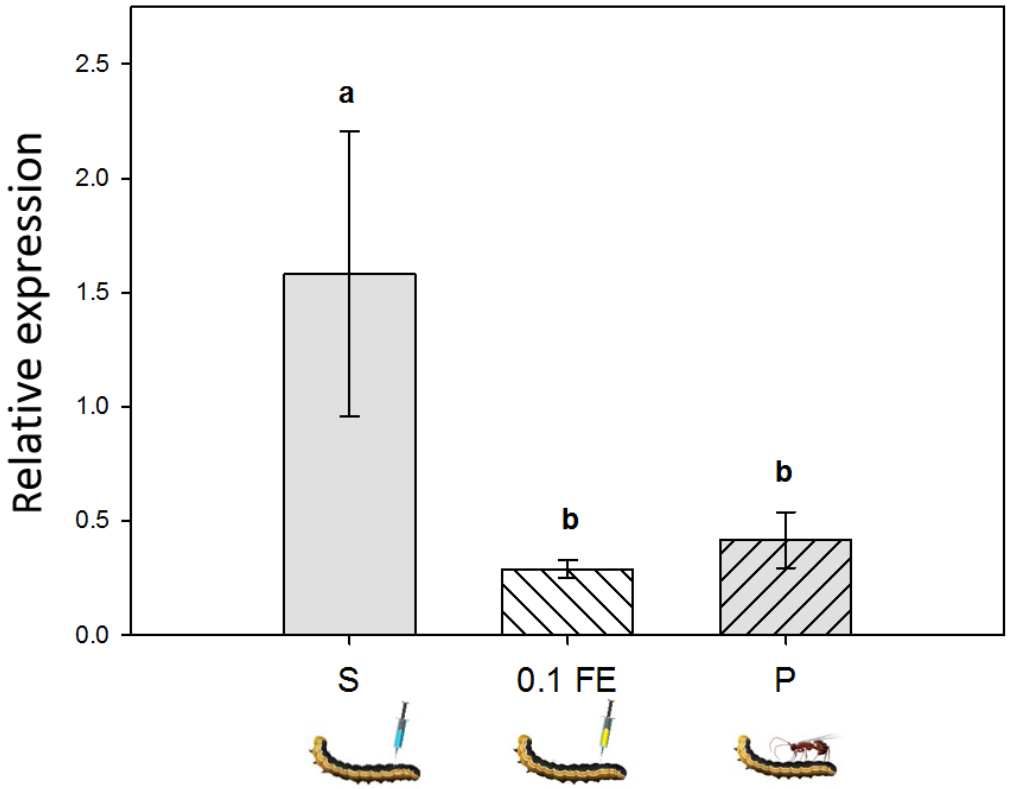

Fig. 2-9. Effects of parasitism and polydnavirus on salivary glucose oxidase (GOX) gene expression. Relative expression of *GOX* was measured 2 days after treatment. Treatments include saline injected (S), 0.1 FE (female equivalent) *Mc*BV and parasitized (P) caterpillars. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA, α = 0.05; followed by T-test; day 1, n = 4-5, $F_{(2, 11)} = 5.08$, $P = 0.0274$).

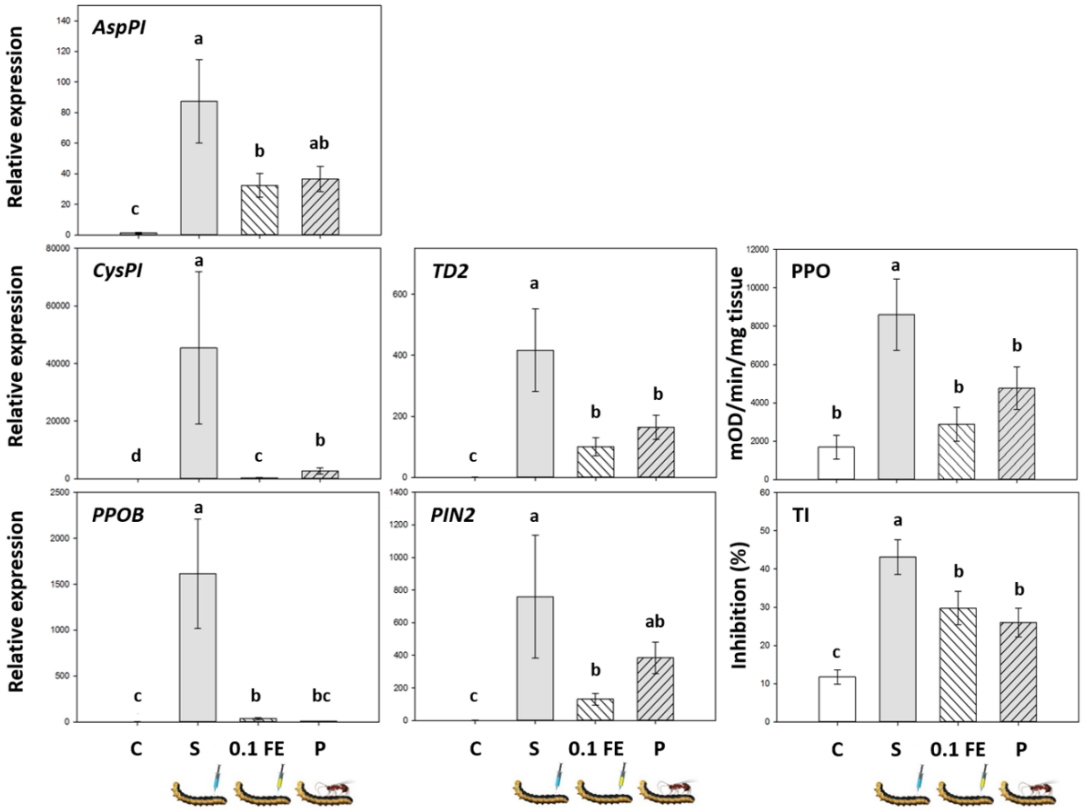

Fig. 2-10. Gene expression levels and activities of defense proteins in plants fed on by saline-injected (S), 0.1 FE *Mc*BV-injected, parasitized (P)-treated caterpillars, and intact control plants (C). Relative expression of all genes was measured 24 h and protein activities 48 h after insect feeding. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; AspPI, $F_{(3, 23)} = 30.3$, $P < 0.0001$; CysPI, $F_{(3, 22)} = 25.8$, $P < 0.0001$; PPOB, $F_{(3, 22)} = 11.5$, $P < 0.0001$; TD2, $F_{(3, 22)} = 65.1$, $P < 0.0001$; PIN2, $F_{(3, 23)} = 41.6$, $P < 0.0001$; PPO, $F_{(3, 35)} = 5.42$, $P = 0.0036$; TI, $F_{(3, 34)} = 11.8$, $P < 0.0001$. AspPI, aspartic proteinase inhibitor; CysPI, cysteine proteinase inhibitor; PIN2, proteinase inhibitor 2; PPO, polyphenol oxidase; PPOB, polyphenol oxidase B; TD2, threonine deaminase; TI, trypsin inhibitor.

To determine if attenuation of plant defenses benefited caterpillar growth, we fed P-caterpillars leaves from plants that were previously treated with saliva from NP- and P-caterpillars. The growth rate of P-caterpillars was higher when fed on leaves treated with saliva from P-caterpillars compared with leaves treated with saliva from NP-caterpillars (Fig. 2-11). These results suggest that the improved growth of the P caterpillars would also benefit parasitoid growth by increasing their host resources/suitability (Vinson and Iwantsch 1980). In *H. zea* it has been shown that diets that stress the caterpillar host to the greatest extent have the greatest negative effects on parasitoids (Bloem and Duffey 1980).

Parasitoid performance was shown to be reduced when caterpillar hosts are reared on induced tomato foliage compared with unwounded foliage (Thaler 1999). Indeed, the performance of the parasitoids (e.g., successful pupation, pupal weights, total percentage of survival) was improved when their hosts were fed plants treated with saliva from P-caterpillars compared with hosts fed plants treated with saliva from NP-caterpillars (Table 2-2). Parasitoid survival through the adult stage was nearly twofold higher when hosts fed on the plants treated with saliva from P-caterpillars compared with the NP saliva treatment. Plant secondary metabolites such as alkaloids may be responsible for the adverse effects on parasitoid larvae (Barbosa et al. 1986). In tomato, the glycoalkaloid α-tomatine has been shown to be toxic to an endoparasitoid larva when ingested by its *H. zea* host (Campbell and Duffey 1979) and to cause disruption of pupation (Campbell and Duffey 1981), which is similar to our findings reported here. Notably, the regulation of glycoalkaloid biosynthesis is likely to be down-regulated by the parasitoid/virus as GLYCOALKALOID METABOLISM 4 (GAME4) transcript levels in plants fed on by parasitized caterpillars were not significantly higher than

the unwounded controls (Fig. 2-4). GAME4 plays a key regulatory role in glycoalkaloid biosynthesis in tomato (Itkin et al. 2013)

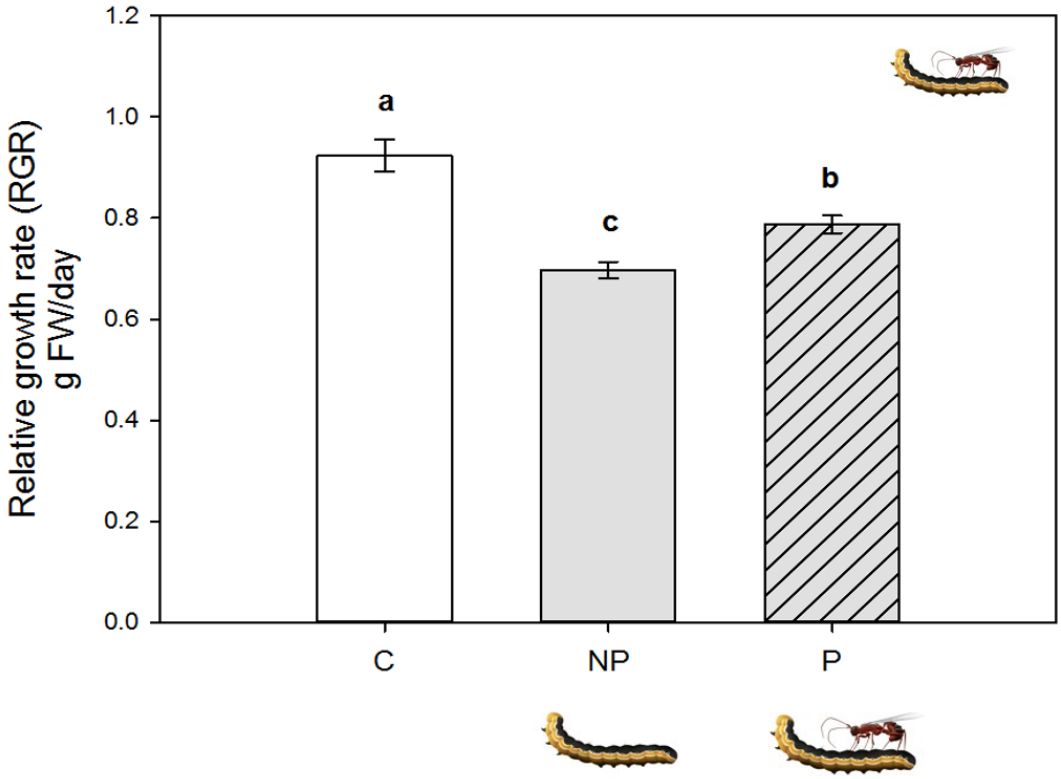

Fig. 2-11. Relative growth rate of parasitized caterpillars feeding on plants previously treated with saliva from parasitized caterpillars (P), saliva from non-parasitized caterpillars (NP) or untreated control plants (C) for 24 h. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, $\alpha = 0.05$: n = 28-33, $F_{(2, 88)} = 23.1$, $P < 0.0001$. C, intact control plant; NP, plant treated with nonparasitized caterpillar; P, plant treated with parasitized caterpillar.

Table 2-2. Parasitoid performance with host feeding on different plant treatment

	Larval duration (day)	% Larval mortality	% Cocoon formation failure	Cocoon weight (mg)	Pupal duration (day)	% Adult emergence	% Total survival
P	13.78 ± 0.20 (51)	5.56 ± 2.48 (6)	15.51 ± 6.64 (6) [b]	15.52 ± 0.27 (43) [a]	12.32 ± 0.21 (25)	57.36 ± 6.18 (6)	46.30 ± 6.68 (6) [a]
NP	14.17 ± 0.24 (47)	12.96 ± 5.30 (6)	34.41 ± 4.28 (6) [a]	14.48 ± 0.27 (31) [b]	12.31 ± 0.26 (13)	42.30 ± 10.4 (6)	24.07 ± 5.30 (6) [b]
	p=0.281	*p*=0.246	*p*=0.044	*p*=0.004	*p*=0.254	*p*=0.246	*p*=0.028
	F=1.188	F=1.588	F=5.712	F=9.181	F=1.323	F=1.563	F=6.812

P: saliva from parasitized caterpillar treated plant; NP: saliva from non-parasitized caterpillar treated plant. Values are untransformed mean ± SEM. Different letters indicate significant differences between treatments (Student's t-test, (n)).

In nature, a large percentage of *H. zea* caterpillars may be parasitized (Tipping et al. 2005), indicating that PDVs may be important and ubiquitous mediators of the interactions of *H. zea* with its host plants. Remarkably, *Mc*BV is able to manipulate phenotypes across multiple trophic levels including the phenotypes of a parasitoid, herbivore, and host plant. This finding reveals an important dimension of the symbiotic role and ecological benefits of the PDVs to their parasitoid hosts; by indirectly manipulating the host plant, PDVs potentially benefit the parasitoid larva growing inside the caterpillar host and ultimately their own fitness. The importance of PDVs as mediators of interactions within the phytobiome may be of very broad occurrence due to the estimated 50,000 braconid and 14,000 ichneumonid species harboring PDVs, which in turn infect thousands of insect herbivore species (Strand and Burke 2014). In certain contexts, there may be costs associated with PDV manipulation of host plant defenses. The benefits to parasitoid fitness provided by PDVs could be mitigated in circumstances where hyperparasitoids may more easily locate their parasitoid hosts through changes in herbivoreinduced plant volatiles (Zhu et al. 2018). Nevertheless, these studies reveal that PDVs play far more prominent roles in shaping plant–herbivore

interactions across multiple trophic levels than previously understood. Our findings indicate a unique aspect of how PDVs benefit their parasitoid hosts through suppression of both the caterpillar and the host plant defense or immune systems.

Acknowledgements

We thank Dr. Henry Fadamiro (Auburn University) for providing the *M. croceipes* colony; Ju-Che Lo for experimental assistance and colony maintenance; Kaixi Zhao for guidance on virus purification; and István Mikó for assisting with the CLSM image. The comments by two anonymous reviewers were very helpful. This research was supported by National Science Foundation Grant IOS-1645548 and Hatch Project Grant PEN04576. Data are archived in Dryad Digital Repository database, datadryad.org/ (doi: 10.5061/dryad.5tg4sv6).

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Chapter 3

Parasitoid indirectly increases plant fitness and subsequent performance of plant offspring

Abstract

Plants face many environmental factors that can impact their survival, development, and eventually reproductive success. Herbivorous insects are the most diverse and abundant organisms that consume plant tissues in nature. Plants produce direct chemical and physical defenses that reduce herbivory. They also release herbivore-induced plant volatiles (HIPVs) to recruit natural enemies that serve as indirect defenses to herbivory. The recruitment of parasitic wasps (parasitoids) can benefit plant fitness because parasitoids ultimately kill their insect hosts. In addition to the benefits of reducing herbivore survival, we tested if the reduction in induced defenses by parasitized caterpillars compared to non-parasitized caterpillars may also benefit plant fitness by reducing costs associated with defense expression. We provide evidence that parasitized caterpillar-treated tomato plants had significantly higher fitness, more flower numbers (16.3%) and heavier fruit weight (13.5%), compared to the non-parasitized caterpillar treated plants. The seed germination speed and rate were higher when the maternal plants were previously exposed to parasitized herbivore treatments compared to control plants. In this case, the higher plant fitness in parasitized caterpillar treated plants was due to lower induced defense costs rather than reduced herbivory or plant consumption.

Introduction

Many biotic and abiotic environmental factors can influence plant development and reproduction. Water (Aragón et al. 2008), pathogens (Tian et al. 2003), herbivores (Agrawal 1999; Mothershead and Marouis 2000; Pashalidou et al. 2015), plant genetic diversity (Johnson et al. 2006), plant population size (Kolb 2008), and soil microbial communities (Lau and Lennon 2012) were all shown to severely impact plant fitness (fruit and seed production). Herbivorous insects are the most abundant and diverse herbivores that consume plant tissues (Howe and Jander 2008) and can both negatively and positively affect plant fitness.

The negative effects of herbivores on plant fitness can be caused by loss of leaf tissues that reduce photosynthesis, changes in flower morphology that negatively affect pollinator preference, damage to reproductive organs (bud, flower and fruit) that limit seed production, and/or increases in plant mortality (Mothershead and Marouis 2000; Maron 1998; Kessler and Baldwin 2004; Strauss et al. 1996; Juenger and Bergelson 1998).

Induced plant defenses may protect plants from herbivores and a subsequent loss in plant fitness. For example, wild radish produces higher trichome densities in newly developed leaves after *Pieris rapae* larval feeding and reduces herbivory, which subsequently enhanced plant fitness as measured by higher fruit and seed production (Agrawal 1999). McArt et al. (2013) showed plants (*Oenothera biennis*) fed on by Japanese beetle (*Popillia japonica*) had higher ellagitannins concentrations in flower buds, which suppressed seed feeder ovipositional

preference and feeding amount, thus allowing plants to have better seed yield.

Besides direct effects, herbivore-induced plant volatiles (HIPVs) released by damaged plants can indirectly reduce herbivore populations by recruiting natural enemies (parasitoids and predators) (Kessler and Baldwin 2001; Mumm and Dicke 2010), which may facilitate plant reproduction and fitness (Hoballah and Turlings 2001; van Loon et al. 2003; Gómez and Zamora 1994). The effects of direct and indirect plant defenses on plant fitness can occur simultaneously. Wild tobacco (*Nicotiana attenuata*) plants damaged by mirid bug (*Tupiocoris notatus*) produced defense compounds and VOCs, which directly and indirectly suppressed hornworm (*Manduca quinquemaculata*) growth rates, reduced ovipositional preference, and increased egg predation rate. The mirid bug damaged plants had higher seed capsule numbers (Kessler and Baldwin 2004). Oviposition and feeding by *Pieris brassicae* on black mustard (*Brassica nigra*) induced plant defenses that ultimately caused increased in seed production due to direct negative effects on herbivore performance and increased in parasitism rate of the caterpillars (Pashalidou et al. 2015).

Recruitment of parasitoids by plants has been documented in many systems (Mumm and Dicke 2010) and may benefit plant fitness due to their specialization on specific host herbivore species, reduced impacts to beneficial insects (e.g., pollinators and seed dispersers), and reduction of plant tissue damage by reducing herbivore populations (Romero and Koricheva 2011; Gómez and Zamora 1994; Turlings and Erb 2018; Gols et al. 2015).

Recent studies revealed that plants show lower defense responses when fed on

by parasitized compared to non-parasitized caterpillars. *Microplitis croceipes* parasitized corn earworm (*Helicoverpa zea*) had lower elicitor activity in their saliva (i.e., glucose oxidase) and significantly downregulated tomato defense-related gene expression and defense protein activities, which benefitted parasitized caterpillar growth performance and parasitoid fitness (Tan et al. 2018). The downregulation of plant defense responses was caused by the obligate mutualist polydnavirus (PDVs) of the parasitoid. PDVs are injected into caterpillars by the female parasitoid along with eggs and venom during parasitism. PDVs suppress GOX gene expression and activity in the parasitized caterpillar salivary glands, which downregulates plant defense responses. The downregulation of insect saliva elicitors was also found in *Cotesia glomerata* parasitized *Pieris rapae* larvae (Cusumano et al. 2018): the PDVs and parasitoid venom suppressed elicitor gene (glucose dehydrogenase, GDH) expression in the caterpillar's salivary glands.

From a plant's perspective, induced defenses can be costly. The lower induced responses elicited by parasitized caterpillars may benefit the plant by reducing defense energy costs compared to responses to non-parasitized caterpillars. To date, no studies have examined the effects of different levels of plant defenses mediated by parasitized caterpillars on plant fitness. In this research, I hypothesize that the lower salivary elicitor activities of parasitized caterpillars and subsequent reduced levels of induced plant defenses will enhance plant fitness relative to treating by non-parasitized caterpillars. Second, I determined how parasitoids indirectly influence plant seed germination and induced defense responses in the plants from the next generation.

Materials and methods

Insects

Helicoverpa zea eggs were purchased from Benzon Research (Carlisle, PA). Larvae were fed on artificial diet (Peiffer & Felton 2005) and reared individually until pupal formation. Pupae were placed in a container [15 (diameter) cm x 28 (height)] and 10% sugar solution was provided as food for adult moths. Eggs were collected daily for the experiments. The colony has been kept in our lab for several generations.

Microplitis croceipes pupae were kindly provided by Dr. Henry Fadamiro (Auburn University, Auburn, AL) and a colony was established and maintained in our lab. Briefly, 10 caterpillars (second and/or third instars) were exposed to one female parasitoid for 1 h in a 9 cm diameter petri dish. Usually, 20 female parasitoids were used in each round of parasitization of hosts. After being parasitized, caterpillars were transferred to cups with artificial diet and reared individually. Pupal cocoons were collected, and emerged adults were kept in a container (27 cm x 15 cm x 11 cm) provided with a 20% honey solution. Insect colonies were reared in a growth incubator (25± 2°C, 16L:8D).

Plant

Tomato seeds (*Solanum lycopersicum* cv. Microtom) were sown in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) in a greenhouse (16L:8D) at Pennsylvania State University. Microtom is a small tomato variety,

which is ideal for plant fitness tests due to its overall small size and faster generation time than many cultivars (Lima et al. 2004; Martí et al. 2006) and is a self-pollinated tomato variety (Medina et al. 2013; Ueta et al. 2017). Two weeks after germination, seedlings were transferred to pots (10 cm x 10 cm x 9 cm) and 3 gm of fertilizer (Osmocote, 15-9-12) was applied. Plants were watered daily. Plants with three fully expanded leaves (5-6 weeks old) were used in the following experiments.

Plant defense responses

To evaluate how parasitized caterpillars influence plant defense responses, plant defense-related gene expression and enzyme activities were tested. There were three treatments: control plants with no treatment (C); plants treated with parasitized (P) caterpillar saliva, and plants treated with non-parasitized (NP) caterpillar saliva. The use of saliva-treated plants was used as a proxy for herbivory to standardize both the timing and amount of herbivory. This treatment produces a uniform herbivore phenotype and previously showed similar differential plant defense responses as observed by caterpillar feeding (Tan et al., 2018).

On the last day of second instar *H. zea* caterpillars (with head capsule slippage) were exposed to *M. croceipes* females. Caterpillars were removed immediately once parasitized by a female parasitoid and reared individually. Six days after being parasitized, labial glands were collected, homogenized and applied on the tomato plants (Tan et al. 2018). Labial glands from non-parasitized caterpillars were collected at the same developmental stage. Parasitized and non-

parasitized caterpillars were placed on ice for 20 min before dissection, labial glands were collected under a dissecting microscope and homogenized with phosphate buffer (0.1 M, pH 7.0). Supernatant was collected after centrifugation (4°C, 7,500 x g, 10 min). Protein in parasitized and non-parasitized insect saliva samples were quantified by Bradford assay using BSA (bovine serum albumin, Fraction V, Omnipur) as the protein standard (Bradford 1976). Briefly, a serrated wounding tool was used to wound the third terminal leaflet (counting from the bottom) and immediately 15 µl (1 µg/µl protein) of insect saliva from parasitized or non-parasitized caterpillars was applied with a pipette.

Twenty-four hours after saliva application, plant tissues (1 g) were collected from the treated leaflet. RNA extraction, cDNA synthesis and qRT-PCR analysis were processed as described (Tan et al., 2018). Reference genes (actin and ubiquitin) were used and the relative expression of target genes was compared with intact control (C) by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Primers used in this assay are listed (Table 3-1).

Table 3-1. Primer pairs used for tomato gene expression

Gene name	Description	Species	Forward	Reverse	Accession No.
CysPI	Cysteine proteinase inhibitor	Tomato	GGTGAAGGAATGGG AGGACTTCAA	GGAGGTTTGGGAA TGGAACATTGG	AF198390
PPOB	Polyphenol oxidase B	Tomato	TTCGCGAGTGGGAAT ACCTCGTTT	AGTCAGGGACTGT TTGGACACGAA	Z12834
UBI	Ubiquitin	Tomato	GCCAAGATCCAGGA CAAGGA	GCTGCTTTCCGGCG AAA	X58253
ACT	Actin-7	Tomato	AGGTGTTATGGTCGG AATGG	TCATCCCAATTGCT GACTATACC	AB199316

Forty-eight hours after saliva application, plant tissues (50 mg) were collected from the third terminal leaflet for peroxidase (POD), polyphenol oxidase (PPO) and trypsin inhibitor (TI) assays. PPO and POD assays were performed as described by Felton et al. (1989). Briefly, samples were powdered with a Genogrinder and a phosphate butter (1.25 ml, 0.1 M, pH 7) with 5% PVP (Alfa Aesar 41631) was added to each sample. Samples were set on ice for 10 min. Supernatant was collected after centrifugation (4°C, 11,000 rpm, 10 min). Five microliters of sample was added to 200 µl of caffeic acid (3 mM, Sigma C0625) for PPO activity; 5 µl of supernatant was mixed with 10 µl of hydrogen peroxide (3%, CareOne) and 190 µl of guaiacol (3mM, Sigma G5502) for the POD assay. The change in absorbance at 450 nm was recorded in a plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA) for both the PPO and POD assays. The protein concentration in each sample was quantified using the Bradford assay with BSA (bovine serum albumin, Fraction V, Omnipur) as the standard (Bradford 1976).

For trypsin inhibitor activity assays, samples were powdered with a Genogrinder and 1.25 ml of assay buffer (0.046M Tris and 0.0115M $CaCl_2$; pH 8.1; 5% PVP) was added. Supernatant (4°C, 11,000 rpm, 10 min) was collected for the TI activity measurement. Ten microliters of sample was mixed with 10 µl of Trypsin (20 µg/ml, Sigma T1426) and assay buffer (80 µl). Ten minutes later, 100 µl of TAME (p-toluene-sulfonyl-l-arginine methyl ester, 0.002 M, Sigma T4626) was added and absorbance values were read at 247 nm (Chung et al., 2013). The percent inhibition of each sample was calculated by comparing to the activity of trypsin and assay buffer alone. The protein concentration in each sample was

quantified using the Bradford assay (Bradford 1976).

Plant fitness

To determine if parasitized or non-parasitized caterpillar treatments differently influence plant fitness, three treatments were used: control plants with no treatment (C); plants treated with parasitized (P) caterpillar saliva, and plants treated with non-parasitized (NP) caterpillar saliva.

Labial glands were collected and homogenized from parasitized and non-parasitized caterpillars as described above. Briefly, a serrated wounding tool was used to wound the third terminal leaflet of tomato plants (counting from the bottom, 37 day-old) and immediately 15 µl (1 µg/µl protein) of insect saliva from parasitized or non-parasitized caterpillars was applied with a pipette. Two days later, the same process was repeated on two leaflets of the 4th leaf. Four days after the first application, insect saliva was applied to two leaflets of the 5th leaf. In total, insect saliva was applied three times to five leaflets. Plants were watered as needed and pots were rotated randomly every week.

The first flowering date, flower number, fruit weight, seed weight and seed number were recorded to represent plant fitness. The first flowering date is the number of days between the first saliva application and appearance of the first flower. The experiment was ended (133 day-old) when more than half of the leaves turned brown and no green fruit ripened within 1 week.

Second generation performance

To determine if herbivore treatment of maternal plants influenced offspring

performance, seed germination rate and plant defense responses were measured.

To determine seed germination speed and rates, seeds were collected from the plant fitness experiments described above. There were three treatments: seeds from maternal plants with no treatment (C), seeds from maternal plants treated with parasitized caterpillar saliva (P), and seeds from maternal plants treated with non-parasitized caterpillar saliva. Thirty seeds from each maternal plant were sown in potting soil in the greenhouse and a total 10 maternal plants of each treatment were used. Pots were placed on trays and water was added to the tray to maintain soil moisture. Seeds were observed daily for germination speed. Germination rate of seed were calculated at 11 days after sawing as follows: (germinated seed #/ total seed # (30 seeds)) x 100.

In the offspring defense response assay, there were three maternal plant treatments (C, P, and NP) and two herbivore treatments (N: no herbivore treatment, and H: herbivore treatment). Seeds were planted as described above. Plants with 3 fully expanded leaves (5-6 weeks old) were used for the experiment. Labial glands were collected and homogenized from non-parasitized caterpillars as described above. For the herbivore treatment (H), 15 µl (1 µg/µl protein) of insect saliva were applied after mechanical wounding on the third terminal leaflet (counting from the bottom) of tomato plants, while the other half plants were without herbivore (N) treatment.

Twenty-four and 48 hours after saliva application, plant tissues (50 mg) were collected from the third terminal leaflets for polyphenol oxidase (PPO), peroxidase (POD) and trypsin inhibitor (TI) assays as described above. Each plant was only

used once.

Statistical analyses

Data were transformed as needed to obtain a normal distribution and to address residuals with heterogeneity of variance; SAS 9.4 (SAS Institute Inc) was used for data analyses. Plant fitness was analyzed by One-way ANOVA (Proc GLM), and plant defense enzyme activities and seed germination rate were analyzed by Two-way ANOVA (Proc GLM), followed by means comparisons using the Tukey's Least Significant Difference (LSD) test (significance level, $P<0.05$). Seed germination days were analyzed by Kruskal-Wallis, followed by pairwise multiple comparison (DSCF) test (significance level, $P<0.05$).

Results

To evaluate how parasitized caterpillars influence plant defense responses, we applied saliva from parasitized (P) and non-parasitized (NP) caterpillars to wounded leaflets of plants and then measured plant defense-related gene expression and proteins activities. Saliva from P-caterpillars triggered significantly lower defense gene (*PPOB* and *CysPI*) expression and protein (PPO, POD and TI) activities on tomato plants than saliva from NP- caterpillars treatment (Fig. 3-1).

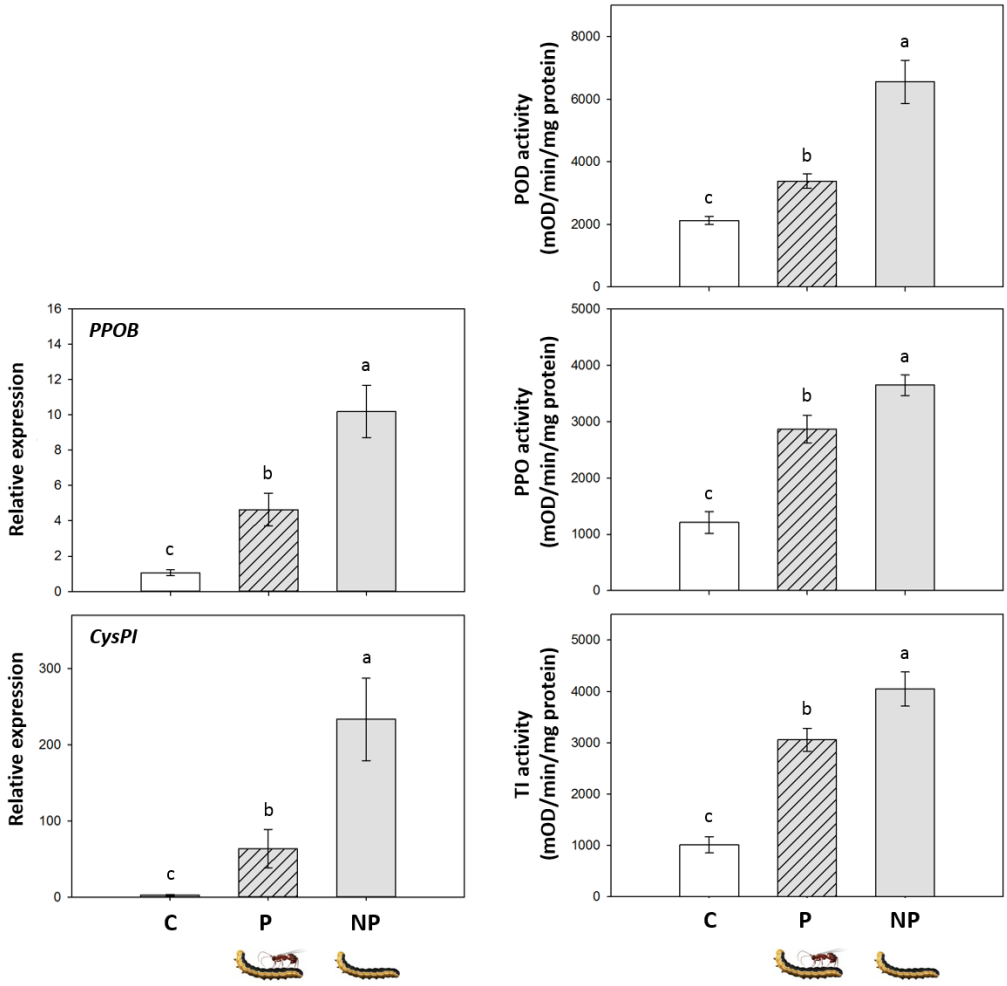

Fig. 3-1. Effects of H. zea caterpillar parasitism on induction of tomato defensive responses. Values are untransformed mean ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, α = 0.05; *PPOB*, n = 10, $F_{(2, 27)} = 25.98$, $P < 0.0001$; *CysPI*, n = 10, $F_{(2, 27)} = 44.79$, $P < 0.0001$; POD, n = 14–15, $F_{(2, 41)} = 46.75$, $P < 0.0001$; PPO, n = 14–15, $F_{(2, 41)} = 33.78$, $P < 0.0001$; TI, n = 14, $F_{(2, 39)} = 47.28$, $P < 0.0001$. C, intact control plant; P, plant treated with parasitized caterpillar saliva (15 µl (1 µg/µl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 µl (1 µg/µl protein)).

To determine if parasitized caterpillars differentially influence plant fitness, we applied saliva from parasitized (P) and non-parasitized (NP) caterpillars to a wounded plant leaflets and recorded the first flowering day, flower number, fruit weight, seed number and seed weight to represent plant fitness. Plants without

treatment (C) showed the best fitness, followed by the P-caterpillar treatment, and then NP-caterpillar treatment. There was no significant difference between C and P treatments in flower number, fruit weight, seed number and seed weight. However, C plants flowered 5 days earlier than plants treated with herbivore saliva (P, NP) (Fig.3-2). Plants treated with P-caterpillar saliva showed significantly higher flower numbers (16.3% more) and fruit weights (13.5% higher) compared to the NP-caterpillar treatment.

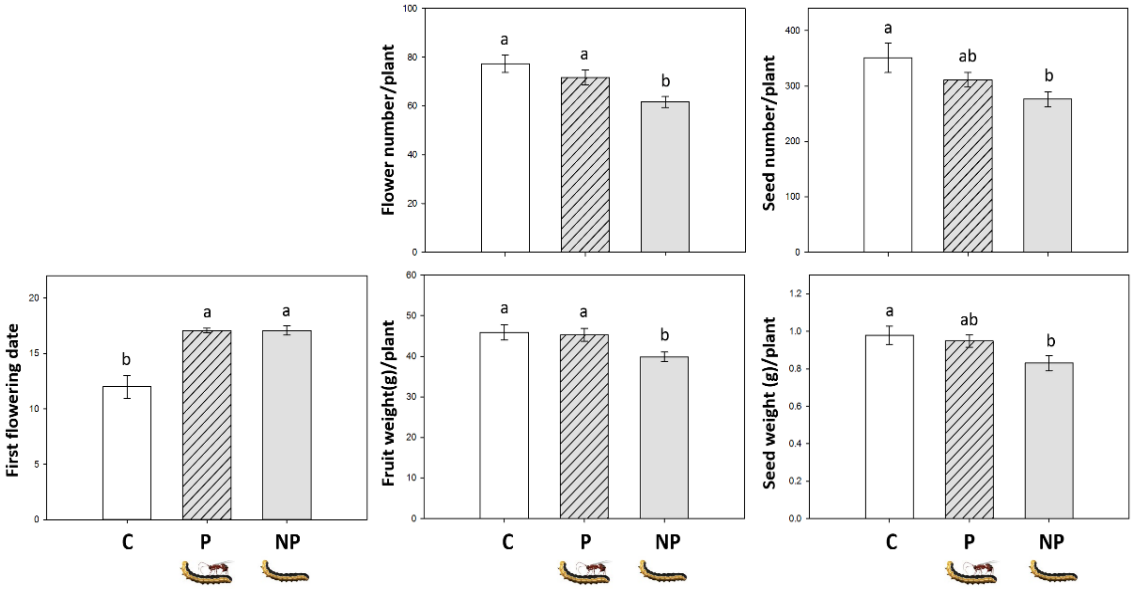

Fig. 3-2. Tomato fitness in different treatments. First flowering date, flower number, fruit weight, seed number and seed weight were recorded to represent plant fitness. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, α = 0.05; first flowering date, n = 13-15, $F_{(2, 39)} = 19.38$, $P < 0.0001$; flower number, n = 13-15, $F_{(2, 39)} = 7.50$, $P = 0.0018$; fruit weight, n = 13, $F_{(2, 36)} = 4.54$, $P = 0.0175$; seed number, n = 13, $F_{(2, 36)} = 3.88$, $P = 0.0297$; seed weight, n = 13, $F_{(2, 36)} = 3.62$, $P = 0.0371$. C, intact control plant; NP, plant treated with non-parasitized caterpillar saliva; P, plant treated with parasitized caterpillar saliva.

To determine if herbivore treatment of the maternal generation can influence offspring seed germination, we recorded seed germination speed and germination rate from different maternal plant treatments. The germination speed and

germination rates were significantly higher for seeds from maternal plants that were exposed to parasitized herbivore treatments (P) compared to control (C) plants (Fig. 3-3, 3-4). There are no difference of seed germination rate and speed between seeds from P maternal plants and NP seeds.

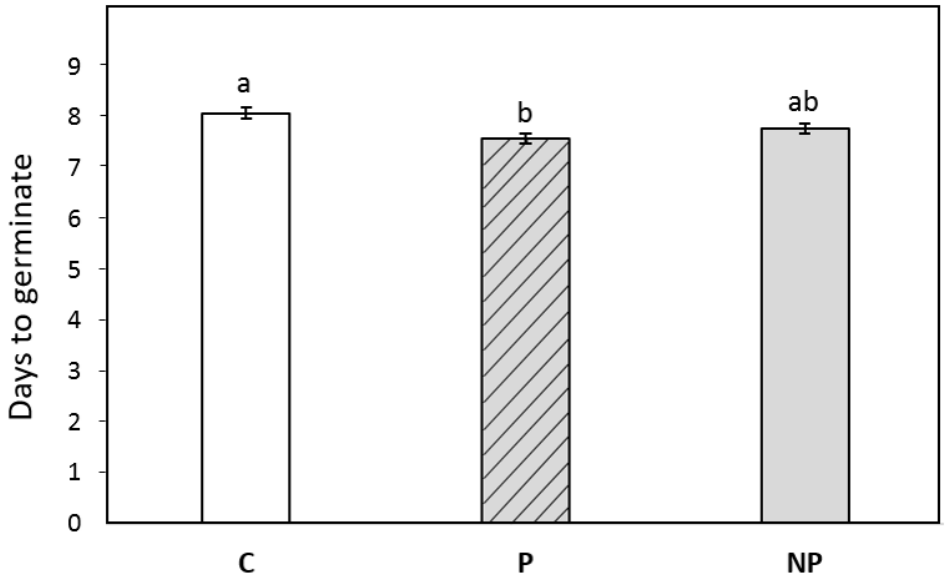

Fig. 3-3. Maternal effects on seed germination speed. Values are untransformed means ± SEM. Different letters indicate significant difference between treatments: Kruskal-Wallis followed by DSCF test, $\alpha = 0.05$; n = 198-249, $F_{(2, 667)} = 5.73$, $P = 0.0034$. C, seed from maternal plant without any treatment; P, seed from maternal plant treated with parasitized caterpillar saliva; NP, seed from maternal plant treated with non-parasitized caterpillar saliva.

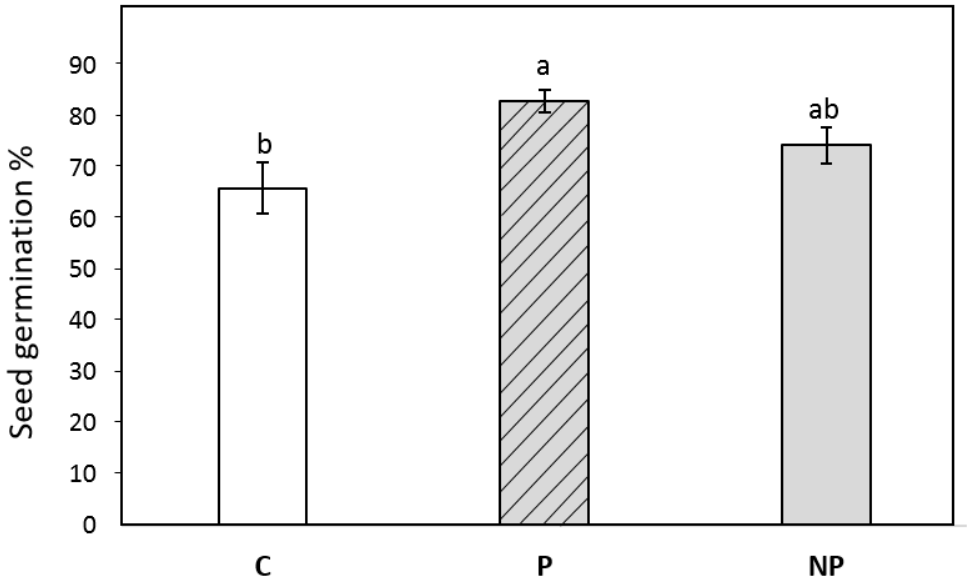

Fig. 3-4. Maternal effects on seed germination rate. Germination rates were measured at 11 days post planting. Values are untransformed means ± SEM. Different letters indicate significant difference between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; n = 10, $F_{(2, 27)} = 4.92$, $P = 0.015$. C, seed from maternal plant without any treatment; P, seed from maternal plant treated with parasitized caterpillar saliva; NP, seed from maternal plant treated with non-parasitized caterpillar saliva.

To determine if herbivore treatment in the maternal plant can influence offspring defense response, we treated the offspring plants with herbivore saliva and examined plant defense-related protein activities at 24 and 48 h post treatment. Polyphenol oxidase (PPO), peroxidase (POD) and trypsin inhibitor (TI) activities were significantly induced after herbivore (H) treatment compare to no-herbivore control (N) plants (Fig. 3-4). Among these three defense proteins, PPO activity was increased significantly higher in offspring from maternal plants that were treated with P-caterpillar (P) and untreated control (C) compared to NP plants 24 h after saliva application (Fig. 3-4A, Table 3-2). Overall, there was no maternal effect

detected for POD and TI activities at 24 h and 48 h after herbivore saliva treatment.

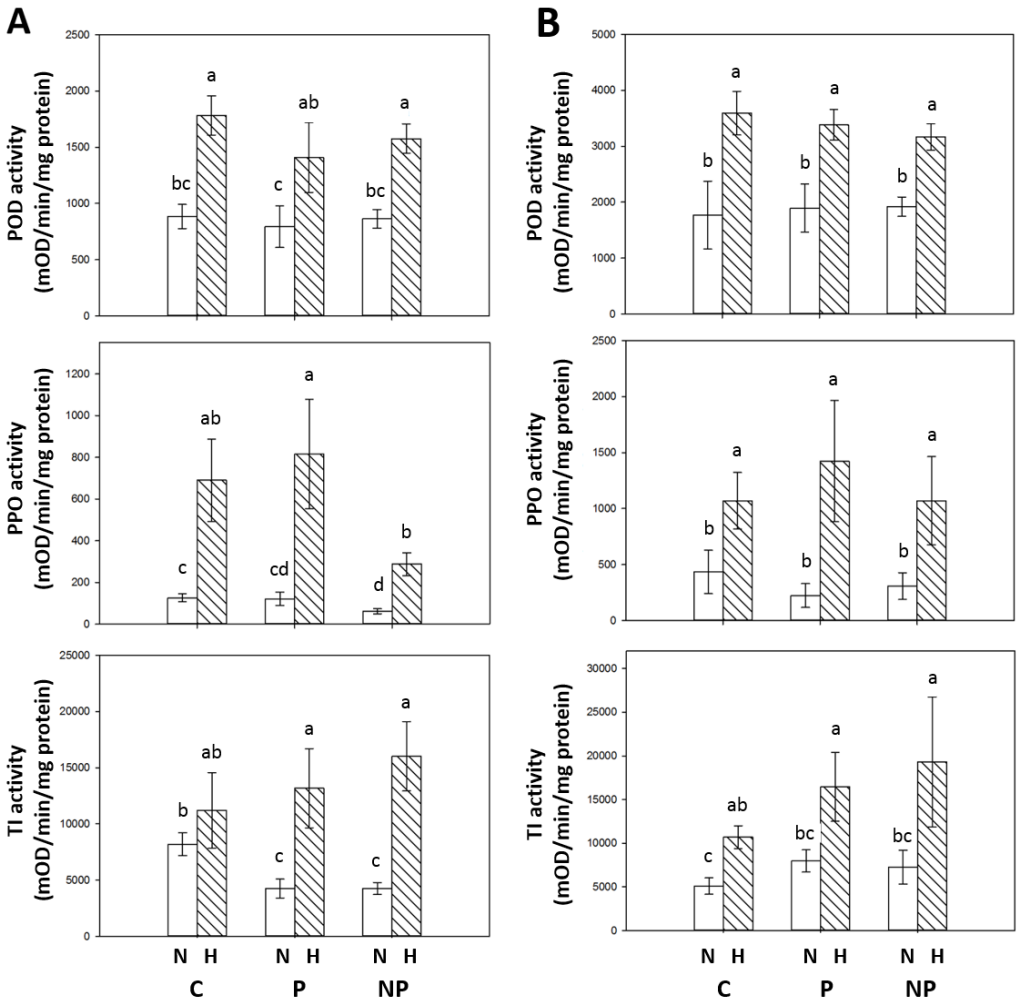

Fig. 3-5. Second generation induced plant defense responses. Plant defense protein, PPD, PPO and TI, activities were measured at 24 h (A) and 48 h (B) post herbivore saliva application to wounded leaflets. Values are untransformed means ± SEM. Different letters indicate significant difference between treatments: ANOVA followed by LSD test, $\alpha = 0.05$; 24 h POD, n = 5-7, $F(5, 30) = 4.55$, $p = 0.0033$; PPO, n = 6, $F(5, 30) = 14.11$, $p < 0.0001$; TI, n = 6, $F(5, 30) = 9.81$, $p < 0.0001$; 48 h POD, n = 4-7, $F(5, 31) = 5.14$, $p = 0.0015$; PPO, n = 4-7, $F(5, 31) = 4.77$, $p = 0.0024$; TI, n = 4-9, $F(5, 30) = 4.16$, $p = 0.0055$. C, seeds from maternal plants with no treatment; P, seeds from maternal plants treated with parasitized caterpillar saliva; NP, seeds from maternal plants treated with non-parasitized caterpillar saliva; N, plants without herbivore treatment; H, plants treated with herbivore saliva. POD, peroxidase; PPO, polyphenol oxidase; TI, trypsin inhibitor.

Table 3-2. Two-way ANOVA analysis of seedling induced defense responses

Time	Variable	df	*p*	F	N
24 h	PPO		***p*<0.0001**	F=14.11	36
	Maternal plant	2	***p*=0.0093**	F=5.50	
	Herbivore treatment	1	***p*<0.0001**	F=59.35	
	Maternal x Herbivore	2	*p*=0.9077	F=0.10	
	POD		***p*=0.0033**	F=4.55	36
	Maternal plant	2	*p*=0.4987	F=0.71	
	Herbivore treatment	1	***p*<0.000**1	F=21.09	
	Maternal x Herbivore	2	*p*=0.7669	F=0.27	
	TI		***p*<0.0001**	F=9.81	36
	Maternal plant	2	*p*=0.1977	F=1.71	
	Herbivore treatment	1	***p*<0.0001**	F=34.67	
	Maternal x Herbivore	2	***p*=0.0095**	F=5.46	
48 h	PPO		***p*=0.0038**	F=4.38	38
	Maternal plant	2	*p*=0.8593	F=0.15	
	Herbivore treatment	1	***p*=0.0001**	F=19.59	
	Maternal x Herbivore	2	*p*=0.5439	F=0.62	
	POD		***p*=0.0015**	F=5.14	37
	Maternal plant	2	*p*=0.9899	F=0.01	
	Herbivore treatment	1	***p*<0.0001**	F=24.01	
	Maternal x Herbivore	2	*p*=0.7263	F=0.32	
	TI		***p*=0.0055**	F=4.61	36
	Maternal plant	2	*p*=0.1340	F=2.81	
	Herbivore treatment	1	***p*=0.0003**	F=16.79	
	Maternal x Herbivore	2	*p*=0.9477	F=0.05	

PPO, polyphenol oxidase; POD, peroxidase; TI, trypsin inhibitor. Values are untransformed means ± SEM (Two-way ANOVA, GLM).

Discussion

Parasitism of caterpillars commonly occurs in nature, depending on location and host plant species. For example, larval parasitism rates of the large white butterfly (*Pieris brassicae*) can exceed 35% on cabbage (Razmi et al. 2011). More than 70% of fall armyworm (*Spodoptera frugiperda*) larvae were parasitized on corn (Ashley et al. 1983; Ashley 1986), and corn earworm (*Helicoverpa zea*) parasitism rates can reach 50% to 82% (Young and Price 1975; King and Coleman 1989; Tipping et al. 2005) in the field. High rates of parasitism may protect plants from damage and ultimately increase plant fitness due to the reduction of herbivore populations. This benefit of natural enemies on plant fitness has been documented in several systems. For instance, corn plants had significantly lower leaf damage (30%), and higher fitness (1.3~1.5-fold) (seed number, ear number and seed biomass) when fed on by *Cotesia marginiventris* parasitized *Spodoptera littoralis* larvae compared with non-parasitized caterpillars (Hoballah and Turlings 2001). Charlock mustard (*Sinapis arvensis*) had more seed pod (>4 fold) and seed number when fed on by (*Hyposoter ebeninus* or *Cotesia glomerata*) parasitized *Pieris brassicae* larvae compared with non-parasitized caterpillars, due to the lower percentage of seed pod damage (Gols et al., 2015).

To our knowledge, indirect effects of parasitism on plant fitness have not been documented before. Because parasitized caterpillars may induce lower plant defenses during feeding than their non-parasitized counterparts (as observed in tomato, Tan et al., 2018 and cabbage, Cusamano et al. 2018), I hypothesized that the reduction in defense-related costs may contribute to greater plant fitness.

Parasitization of *H. zea* by the solitary parasitoid *M. croceipes* reduced levels of salivary glucose oxidase (GOX), an important salivary elicitor that mediates plant defense expression in tomato (Tan et al., 2018). Consequently, feeding by parasitized caterpillars elicited lower levels of expression of tomato plant defense-related genes and activities of defense proteins (PPO and TI), which may reduce plant defense energetic costs compared to responses mediated by non-parasitized caterpillars. The synthesis of defense-related traits can be costly. For example, the glucosinolate synthesis process drains energy (15%) away from leaf growth and development in *Arabidopsis thaliana* (Bekaert et al. 2012). *Arabidopsis* had lower fruit production when plants showed enhanced levels of physical (trichome density) and chemical (glucosinolate concentration) defenses (Mauricio 1998). Likewise, in *Brassica rapa*, plants with higher glucosinolate concentrations had lower flower and seed numbers (Stowe and Marquis 2011). In Solanaceae, transgenic tobacco plants had large reductions in seed capsule numbers (25-53%) when plants produced higher levels of trypsin proteinase inhibitor (TPI) activity (Zavala et al. 2004). When tomato plants were treated with exogenous JA (jasmonic acid), the high JA (10 mM) treated plants showed significant reductions in the numbers of fruit (34.4%) and seed (32.9%) compared with plants treated with lower (1 mM) JA concentrations (Redman et al. 2001).

My results reveal a negative correlation between plant induced defense responses and plant fitness. Tomato plants produced more flowers and yielded heavier fruit when treated with parasitized caterpillar saliva compare with non-parasitized ones. These results illustrate the possible trade-off between plant defense responses and plant fitness (Herms and Mattson 1992; Zangerl and Bazzaz 1992; Huot et al. 2014). The trade-off between defense and fitness can be found in

many systems, including pathogen-mediated plant defense (Heil et al. 2000) and natural enemy-caterpillar defense (Higginson et al. 2011). The higher fitness seen in tomato plants treated with parasitized caterpillar saliva compared with the non-parasitized treatment was not caused by either the higher pest parasitism rate or lower levels of herbivore damage; instead, it was principally due to the downregulation of induced plant defense responses. This is a previously undocumented benefit to parasitism: that plants fed on by parasitized caterpillars can benefit from reduced defense costs and increased plant fitness. Additionally, the possibility of plant compensatory growth was not measured in this study.

Flowering timing is crucial to the reproduction and offspring success of plants; altering the timing of flowering may cause negative effects on plant fitness by changing interactions with mutualist (pollinators) and antagonist (seed predators) herbivores (Elzinga et al. 2007). Herbivore-treated plants showed a 5-day delay in the first flowering date compared with control plants. Previous studies also found that damage by vertebrate and invertebrate herbivores postponed plant flowering dates (McClay 1994; Strauss et al. 1996; Juenger and Bergelson 1998, 2000; Tooker and Hanks 2006; Kettenring et al. 2009). The delay of flowering may be due to the damage to vegetative or reproductive tissues. However, in this study, insect saliva was applied on plant leaves and there was no removal of plant tissue. Thus, in this study plants diverted energy to induced defenses resulting in a delay of initial flowering time. Early flowering may promote better fitness due to a longer reproductive period (Kelly and Levin 2000). Although the first flowering date was delayed in P-treated plants, plants produced similar flower numbers and fruit weight in the end compared with the control plants. Thus, the P-treated plants had the plasticity required to compensate for the delayed flowering time by

accelerating flower and fruit production.

A meta-analysis of 55 studies suggested that early seed germination can benefit seedling growth and fertility (Verdú and Traveset 2005). In this study, caterpillar saliva treatments resulted in faster and greater seed germination rates compared with the control seeds. Moreover, seeds from P-maternal plants germinated faster and had higher germination rate than NP seeds. Thus, it seems that the moderately induced responses may not influence tomato fitness, for example, the lower concentration of jasmonic acid (<1.5 mM) application did not negatively affect fruit number, seed numbers and fruit weight on tomato compared with untreated plants (Redman et al. 2001; Thaler 1999). Likewise, a lower level (15-30%) of defoliation did not influence fruit weight in wild and cultivated tomato plants (Welter and Steggall 1993). Thus, moderately induced responses caused by parasitized caterpillars may benefit plants through accelerated growth in the second generation.

The transgenerational effect in plant induced defense responses was observed in PPO activity at 24 h after herbivore treatment. P and C-maternal plant seedlings showed higher PPO activity compared with NP treatment and there was a significant difference between P and NP-maternal plant seedlings. Tansgenerational priming may help offspring deal with stress by triggering plant defense responses in a shorter time period and/or by producing a greater response (van Hulten et al. 2006; Rasmann et al. 2012; Holeski et al. 2012). However, there was no transgenerational priming effect in plant defense-related protein activities found in herbivore-maternal plant seedlings (P and NP) when compared with the control treatment. It is possible that the NP-maternal plant seedlings produced

lower PPO activity than the other two treatments because of differences in energy cost allocations.

These results support my hypothesis that plants can distinguish damage between non-parasitized and parasitized caterpillars and alter their defense response accordingly. Moreover, plants benefit from parasitized caterpillar herbivory by inducing lower defense responses, which can minimize plant defense costs and favor plant fitness. This is a previously unidentified benefit of parasitoids on plant productivity/fitness. This study provides a novel perspective of mutualism between plants and parasitoids.

Acknowledgements

We thank Dr. Henry Fadamiro (Auburn University) for providing *M. croceipes* pupae; Ju-Che Lo for assistance the experiment and maintain insect colonies. This research was supported by National Science Foundation Grant IOS-1645548.

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Chapter 4

Parasitoid influence plant perception of insect herbivores in two different systems

Abstract

Microplitis croceipes is a solitary parasitoid that specializes on noctuid larvae of *Helicoverpa zea* and *Heliothis virescens.* Both the parasitoid and its hosts are naturally distributed across a large part of North American. When parasitoids deposit their eggs into hosts, venom and polydnaviruses (PDVs) are also injected into the caterpillars, which can suppress host immune responses, thus allowing parasitoid larvae to develop. Besides suppressing the host's immune system, PDVs can regulate host oral cues, such as glucose oxidase (GOX). The purpose of this study is to determine if parasitized caterpillars differently induce plant defenses compared to non-parasitized caterpillars using two different host/plant systems. First, I looked at the interaction among tomato plants, *Heliothis virescens* caterpillar and *M. crocipes* parasitoid. Secondly, we looked at the interaction among tobacco plants, *H. zea* and *M. croceipes*. *H. virescens* caterpillars parasitized by *M. crocipes* had significantly lower salivary GOX activity than non-parasitized caterpillars, resulting in lower levels of tomato defense responses, which benefited parasitoid performance by increasing growth rate of parasitized caterpillars. In tobacco plants, parasitized *H. zea* caterpillars had lower GOX activity but induced higher plant defense responses. The higher tobacco defense responses negatively affected parasitoid performance by reducing the growth rate of parasitized caterpillars, causing longer developmental periods, reducing cocoon mass, and lowering the survival rate of parasitoids. These studies demonstrate a

species-specific effect in different plant-insect systems. Based on these results, plants' perception of insect herbivores can be affected by parasitoids and lead to positive or negative consequences to higher trophic levels depending upon the particular host-plant system.

Introduction

The oviposition by parasitoids such as braconid and ichneumonid wasps massively transforms their host's physiology when they inject a cocktail of eggs, venom and polydnavirus (PDV) into caterpillar hosts (Asgari 2006; Burke and Strand 2012). The mutualistic PDV express virulence genes in host caterpillars to ensure parasitoid larval development by suppressing the host's immune defense responses (Tanaka and Vinson 1991; Fathpour and Dahlman 1995; Hegazi et al. 2005; Asgari 2006; Asgari and Rivers 2011; Burke and Strand 2012). Besides the immunological effects, PDVs can influence caterpillar hosts more broadly where *Microplitis demolitor* associated PDV genes are expressed in the haemocytes, fat body, gut, nervous system and salivary glands of its host larvae *Pseudoplusia includens* within 24 h post-infection (Bitra et al. 2011). In the case of *Heliothis virescens* labial gland proteins are significantly reduced after parasitism by *Microplitis croceipes* (Rana et al. 2002) and similarly in *Pieris brassicae* caterpillars parasitized by *Cotesia glomerata* (Cusumano et al. 2018). The labial gland proteins play a principle role in providing cues that trigger/suppress plant defense responses (Acevedo et al. 2015; Rivera-Vega et al. 2017; 2018). Thus, parasitoids may strongly influence the perception of insect herbivores by plants via manipulating these caterpillar salivary cues.

Several recent papers show that parasitoids can indirectly affect plant defense responses through changing host caterpillar's oral secretions. Poelman et al. (2011) found that the color of oral regurgitant from *Pieris rapae* and *P. brasscae* caterpillars changed after parasitization (*Cotesia glomerata* and *Hyposoter ebeninus*). The regurgitant of parasitized caterpillars induced higher transcriptomic levels of plant defense-related genes in cabbage, thus reducing diamondback moth ovipositional preference. More recently it was reported that cabbage plants expressed unique transcriptional levels and produce different volatile compounds while being fed on by parasitized caterpillars (*Pieris spp.*) (Zhu et al. 2015).

PDVs play an important and complex role in plant-insect interactions by not only suppressing the host caterpillar's immune system, but also by indirectly downregulating plant defenses for their own benefit (Tan et al. 2018). *Microplitis croceipes* parasitized corn earworm larvae (*Helicoverpa zea*) had lower elicitor activity in their saliva (i.e., glucose oxidase, GOX) compared with non-parasitized caterpillars, and significantly downregulated tomato defense-related gene expression and defense protein activities during feeding. The ultimate cause of downregulation of plant defense responses was due to the obligate mutualist PDV of the parasitoid. PDVs suppress GOX gene expression and activity in parasitized caterpillar labial glands thereby downregulating plant defense responses. The lower induced plant defenses benefitted the parasitoid by promoting parasitized caterpillar growth rate, producing heavier cocoon masses and overall higher parasitoid survival rates (Tan et al. 2018). The downregulation of insect saliva elicitors was also found in *Pieris brassicae* parasitized by *Cotesia glomerata*; the PDV and venom of the parasitoid suppressed elicitor gene (glucose dehydrogenase,

GDH) expression and enzyme (β-glucosidase) expression in the caterpillars' salivary glands (Cusumano et al. 2018; Zhu et al. 2018). Additionally, PDV and venom of the parasitoid increased exposure of the parasitoid to its natural enemies, hyperparasitoid *Lysibia nana*, by changing plant volatile composition and increasing plant attraction to the hyperparasitoid (Zhu et al. 2018).

These studies demonstrate that plants can distinguish parasitized and non-parasitized caterpillars and respond accordingly. However, more studies are needed to verify if this phenomenon is commonly present in different plant and insect systems. *Microplitis croceipes* (Hymenoptera, Braconidae) is a solitary endoparasitoid specializing on several noctuid species, including *Helicoverpa zea*, *Heliothis virescens* and *Heliothis subflexa*, which are naturally distributed in North America (Lewis and Snow 1971; Hopper and King 1984; Smith et al. 1976). *Helicoverpa zea* and *H. virescens* are generalist herbivores that feed on many plant families, including Solanaceae, Fabaceae, Malvaceae, Plantaginaceae, Geraniaceae and Asteraceae (Neunzig 1963; Fitt 1989; Stadelvacher et al. 1984).

To determine if parasitoids affect plant defenses in different plant and insect systems, two sets of experiments were conducted. First, I looked at the interaction in tomato -with the caterpillar *H. virescens* and the parasitoid -*M. croceipes* and secondly in tobacco using the-*H. zea* as host of- *M. croceipes*. Activity of GOX, an elicitor produced in the saliva of many insects, plant defense responses and parasitoid performance were investigated in this study to evaluate the effect of the parasitoid on plant defenses and the consequences for insect fitness.

Materials and methods

Insects

Corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) eggs were purchased from Benzon Research (Carlisle, PA). Larvae of both species were fed on artificial diet (Peiffer & Felton 2005) and reared individually until pupal formation. Thirty to 40 pupae were kept in a container [19 cm (diameter) x 28 cm (height)] and a 10% sugar solution was provided as food for adults. Eggs were collected daily for the experiments. The two colonies were kept in our lab for multiple generations.

Microplitis croceipes pupae were kindly provided by Dr. Henry Fadamiro (Auburn University, Auburn, AL) and a colony was established and maintained in our lab. Briefly, 10 *H. zea* caterpillars (second and/or third instars) were exposed to one female parasitoid for 1 h in a petri dish (9 cm diameter). Usually, 20-40 female parasitoids were used for every group of larvae parasitism. After parasitization, caterpillars were fed on artificial diet and reared individually. Parasitoid pupal cocoons were collected, and emerged adults were kept in a container (27 cm x 15 cm x 11 cm) with a 20% honey solution. All insect colonies were reared in a growth incubator (25± 2°C, 16L:8D).

Plants

Tomato (*Solanum lycopersicum* cv. Betterboy) and tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were sown in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) in a greenhouse (16L:8D) at Pennsylvania State University. After germination, tomato (2 week-old) and tobacco (3 week-old) were

transferred to pots (10 cm x 10 cm x 9 cm), and 3 gm of fertilizer (Osmocote, 15-9-12) was applied. Plants were watered daily. Tomato plants with five fully expanded leaves (4-5 week-old) and tobacco plants with six fully expanded leaves (7-8 week-old) were used in the following experiments.

Caterpillar salivary glucose oxidase enzyme activities

To determine how parasitism affects insect GOX enzyme activity, labial glands were collected and examined from parasitized and non-parasitized caterpillars. The last day of the second instar stage (with head capsule slippage), *H. zea* and *H. virescens* larvae were parasitized by *M. croceipes* females. Caterpillars were removed immediately following a single oviposition by the female parasitoid and reared individually.

Salivary glands were collected from parasitized (P) caterpillars 6 d after parasitization (Tan et al. 2018). Non-parasitized (NP) caterpillar salivary glands were collected at the same developmental stage as P-caterpillars. GOX enzyme activity was analyzed as described previously (Eichenseer et al., 1999). Briefly, labial glands were homogenized with 30 µL of phosphate buffer (0.1 M, pH 7) using a grinder (Pellet Pestle motor). Supernatant was collected after centrifugation (4°C, 11,000 rpm, 10 min). Five µL of each sample was mixed with 200 µL of substrate (1.3 mg dianisidine-HCL (Sigma D-3252), 2.5 mL of phosphate buffer (0.1 M, pH 7), 0.5 ml of D-glucose (100 mg/mL, Aldrich 253073), and 20 µL of horseradish peroxidase (1 mg/mL, Sigma P2088)) and the change in absorbance value was measured at 460 nm by microplate reader (Molecular Devices, SpectraMax 190). Protein concentration in each sample was quantified by

Bradford assay using BSA (bovine serum albumin, Fraction V, Omnipur) as the protein standard (Bradford, 1976).

Plant defense responses

To determine how parasitized caterpillars influence plant-defense responses, I used three treatments in the experiment: Parasitized-caterpillar feeding (P), non-parasitized caterpillar feeding (NP), and intact control plants (C). One P- or NP-caterpillar was placed in a clip cage on the third (counting from the bottom) terminal leaflet of each tomato plant. This method controlled for caterpillar leaf consumption (3.15 cm^2), location and duration during feeding. In the control treatment, an empty cage was placed on the plant. Leaf cages were removed when the *H. virescens* caterpillar consumed the entire tomato leaf tissue inside the cage within 10 h. The same methods were used for the *H. zea*-tobacco system. Briefly, one P- or NP-caterpillar was caged on the 4^{th} leaf of each tobacco plant and the clip cage was removed when the caterpillar had consumed the entire leaf area inside the cage within 12 h.

Twenty-four hours after placing the caterpillars on the plants, 100 mg of the treated leaflet was collected. RNA extraction, cDNA synthesis and qRT-PCR analysis were performed as described (Tan et al., 2018). Reference genes (actin and ubiquitin) were used and the relative expression of target genes was compared with intact control (C) by using the $2-\Delta\Delta ct$ method (Livak and Schmittgen 2001). Primers used in this assay are listed in Table 4-1.

Table 4-1. Primer pairs used for tomato gene expression

Gene name	Description	Species	Forward	Reverse	Accession No.
CysPI	Cysteine proteinase inhibitor	Tomato	GGTGAAGGAATGGG AGGACTTCAA	GGAGGTTTGGGAA TGGAACATTGG	AF198390
PPOB	Polyphenol oxidase B	Tomato	TTCGCGAGTGGGAAT ACCTCGTTT	AGTCAGGGACTGT TTGGACACGAA	Z12834
UBI	Ubiquitin	Tomato	GCCAAGATCCAGGA CAAGGA	GCTGCTTTCCGGCG AAA	X58253
ACT	Actin-7	Tomato	AGGTGTTATGGTCGG AATGG	TCATCCCAATTGCT GACTATACC	AB199316

Forty-eight hours after caterpillar feeding, 50 mg of plant tissues were collected from the treated leaf for polyphenol oxidase (PPO), peroxidase (POD) and trypsin inhibitor (TI) enzyme assays. PPO and POD assays were performed as described by Felton et al. (1989). Briefly, samples were powdered with a Genogrinder and a phosphate butter (1.25 mL, 0.1 M, pH 7) with 5% PVP (Alfa Aesar 41631) added to each sample. Samples were set on ice for 10 min. Supernatant was collected after centrifugation (4°C, 11,000 rpm, 10 min). For PPO activity, 5 µL of sample was added to 200 µL of caffeic acid (3mM, Sigma C0625); and 5 µL of supernatant was mixed with 10 µL of hydrogen peroxide (3%, CareOne) and 190 µL of guaiacol (3mM, Sigma G5502) for POD assays. The change in absorbance at 450 nm was recorded in a plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA) for both PPO and POD assays. Protein concentration in each sample was quantified by Bradford assay using BSA as the protein standard (Bradford, 1976).

For trypsin inhibitor activity assays, samples were powdered with a Genogrinder and 1.25 mL of assay buffer (0.046M Tris and 0.0115M $CaCl_2$; pH

8.1; 5% PVP) was added. Supernatant (4 °C, 11,000 rpm, 10 min) was collected for TI activity measurement. Ten microliters of each sample was mixed with 10 μL of Trypsin (20 μg/mL, Sigma T1426) and assay buffer (80 μL). Ten minutes later, TAME (p-toluene-sulfonyl-l-arginine methyl ester, 100 μL, 0.002 M, Sigma T4626) was added and absorbance values were read at 247 nm (Chung et al., 2013). Percent inhibition of each sample was calculated by comparing to the activity of trypsin and assay buffer alone. Protein concentration in each sample was quantified by the Bradford assay (Bradford, 1976) using BSA as the standard.

Insect saliva and plant defense responses

To verify if saliva is responsible for the different levels of plant defense responses that we observed from P- and NP-caterpillar feeding, plant defense responses were evaluated after applying insect saliva to wounded leaves. Caterpillars were parasitized as described above (plant defense responses). Salivary glands were collected from P-caterpillars 6 d after parasitization (Tan et al. 2018). NP-caterpillar salivary glands were collected at the same developmental stage as P-caterpillars. Glands were homogenized with phosphate buffer (0.1 M, pH 7), and then supernatant was collected after centrifugation (4 °C, 7,500 × g, 10 min). Protein concentration in the supernatant was quantified by Bradford assay (Bradford, 1976) using BAS as the standard. Briefly, a serrated wounding tool was used to wound the third terminal leaflet of tomato plants and forth leaf of tobacco plants (counting from the bottom) and 15 mL (1 μg/μL protein) of saliva homogenate was applied immediately from P- or NP-caterpillars. The third group was intact control plants without treatment. For gene expression experiments, samples of the wounded leaf were collected 24 h after treatment. PPO, POD and TI

activities were analyzed 48 h after treatment as described above. For all subsequent experiments, treatment of leaves with saliva from caterpillars was done on wounded leaves or leaflets.

Caterpillar host performance

To evaluate the effect of plant-defense responses on P-caterpillar performance, larval relative growth rate experiments were performed. There were three groups in the experiment: caterpillars feeding on plants treated with P-caterpillar saliva or NP-caterpillars saliva, and intact control plants (C). Plants were treated with labial salivary gland homogenate as described above. Forty-eight hours after treatment, the treated leaf was collected for bioassay. Third instar *H. zea* and *H. virescens* of similar body size were selected and parasitized by *M. croceipes*. Caterpillars were weighed and then fed on the treated tomato/tobacco leaves in plastic cups lined with 2% agar to keep leaves moist. Forty-eight hours later, caterpillars were reweighed and relative growth rate was calculated as follows: (final weight − initial weight)/(average weight × no. of days).

Parasitoid performance

To determine if different levels of induced plant defense responses caused by parasitized and non-parasitized caterpillars influence parasitoid development, we conducted parasitoid performance experiments. Tobacco plants were treated with labial salivary gland homogenate from *H. zea* caterpillars as described above. Forty-eight hours after treatment, the treated leaf was collected and placed in a plastic cup lined with 2% agar to keep leaves moist. Third instar *H. zea* of similar body size were parasitized by *M. croceipes* and fed in one of three treatments: P-

caterpillar–treated plants, NP-caterpillar–treated plants or intact control plants (*H. zea*: total n = 35; five replicates of seven individuals per treatment). Leaves were changed every other day to keep food fresh until parasitoid larvae pupated. Larval duration, cocoon weight, pupal duration, total development time, larval mortality, cocoon formation failure rate, adult emergence rate, and survival rate were recorded. Cocoon weight was measured 2 d after cocoon formation, and adult emergence rate was calculated 30 d after cocoon formation. For the percentage of larval mortality, percentage of cocoon formation failure rate, percentage of adult emergence, and percentage of total survival, data were calculated from five replicates with n = 7 individuals per treatment.

Statistical analyses

Data were transformed as needed to obtain a normal distribution and to address residuals with heterogeneity of variance; SAS 9.4 (SAS Institute Inc) was used for data analyses. Insect labial gland GOX enzyme activities were compared between treatments using Student's t test. Plant-defense responses (gene expression and TI and PPO activities), caterpillar performance bioassays (RGR) and parasitoid performance (larval duration, cocoon weight, pupal duration, total development period, percentage of larval mortality, cocoon formation, adult emergence, and total survival) were analyzed using one-way ANOVA (Proc GLM), followed by means comparisons using Tukey's Least Significant Difference (LSD) test (significance level, $P < 0.05$).

Results

Tomato-*Heliothis virescens*-*Microplitis croceipes* system

To determine if *M. croceipes* can influence *H. virescens* salivary enzyme activity with subsequent effects on induced plant defenses from parasitized caterpillar feeding, insect labial gland GOX activity and plant defense responses were measured. Parasitized *H. virescens* caterpillars had significantly lower GOX activity in their labial glands compared with non-parasitized caterpillars (Fig 4-1). Tomato plants showed dramatically lower defense protein activities (POD and PPO) when fed on by P-caterpillars compared with NP-caterpillars (Fig 4-2).

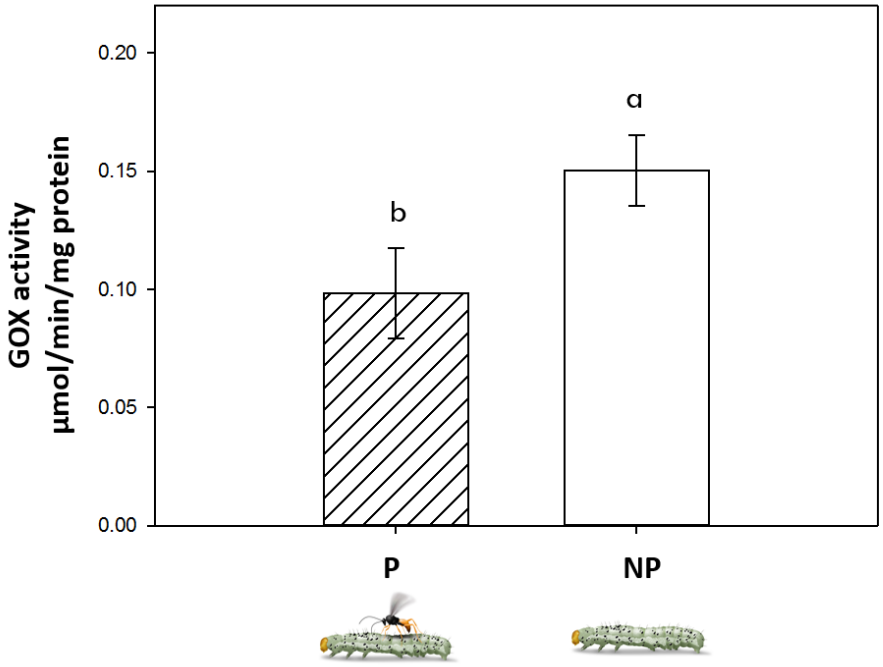

Fig. 4-1. Heliothis virescens GOX enzyme activity at six days after being parasitized. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments. Treatments include parasitized (P) and non-parasitized (NP) caterpillars. ANOVA, α = 0.05; followed by Student's t test; n = 13, $F_{(1, 24)} = 4.58$, $P = 0.044$.

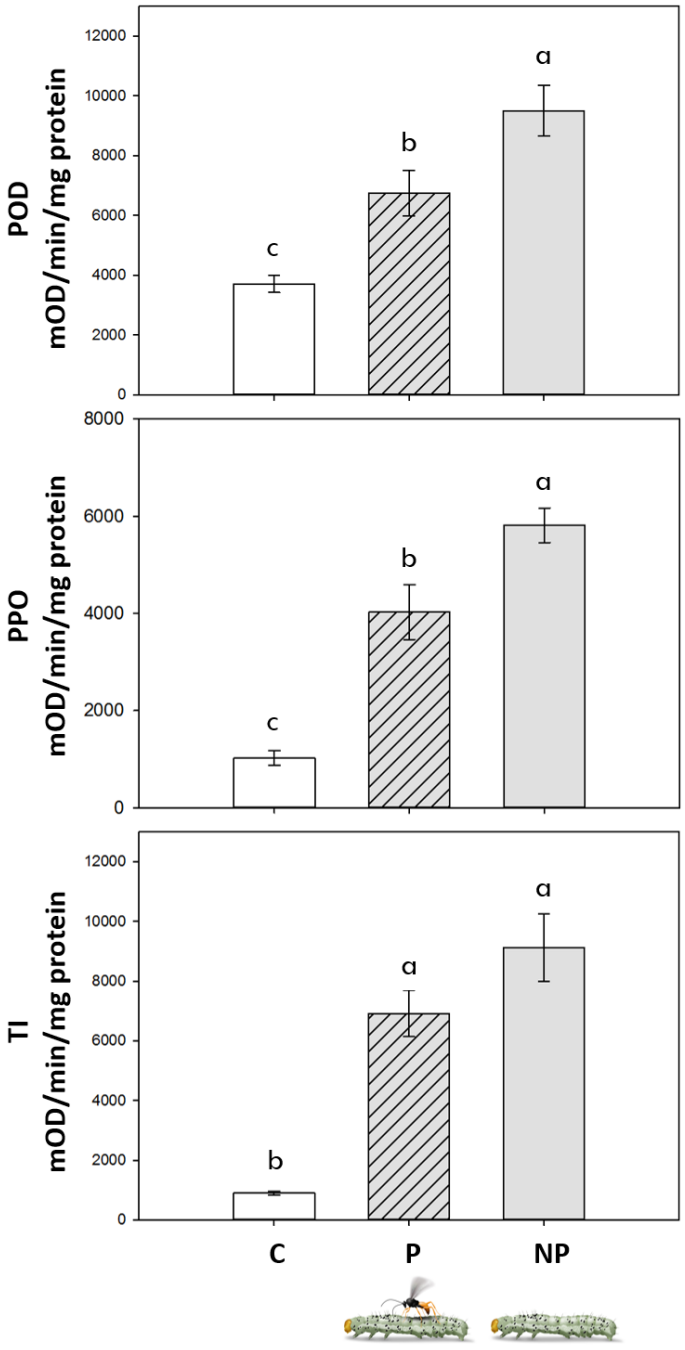

Fig. 4-2. Effect of H. virescens caterpillar parasitism on induction of tomato defensive proteins. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, α = 0.05; POD, n = 6–9, $F_{(2, 21)} = 23.59$, $P < 0.0001$; PPO, n = 7–9, $F_{(2, 21)} = 61.50$, $P < 0.0001$; TI, n = 6–9, $F_{(2, 23)} = 139.92$, $P < 0.0001$. C, intact control plant; NP, plant fed on by non-parasitized caterpillar; P, plant fed on by parasitized caterpillar; POD, peroxidase PPO, polyphenol oxidase; TI, trypsin inhibitor.

To determine if insect saliva was responsible for difference in induced plant defenses, tomato defense-related gene expression and defense protein activities were measured after insect saliva application. Tomato plants showed dramatically lower transcript (*PPOB* and *CysPI*) levels and enzymatic activities (POD, PPO and TI) in plant defenses when saliva was applied from P-caterpillars saliva compared with NP-caterpillars (Fig 4-3). These results indicate that saliva is the factor responsible for the lower induction of tomato defense responses after parasitisation.

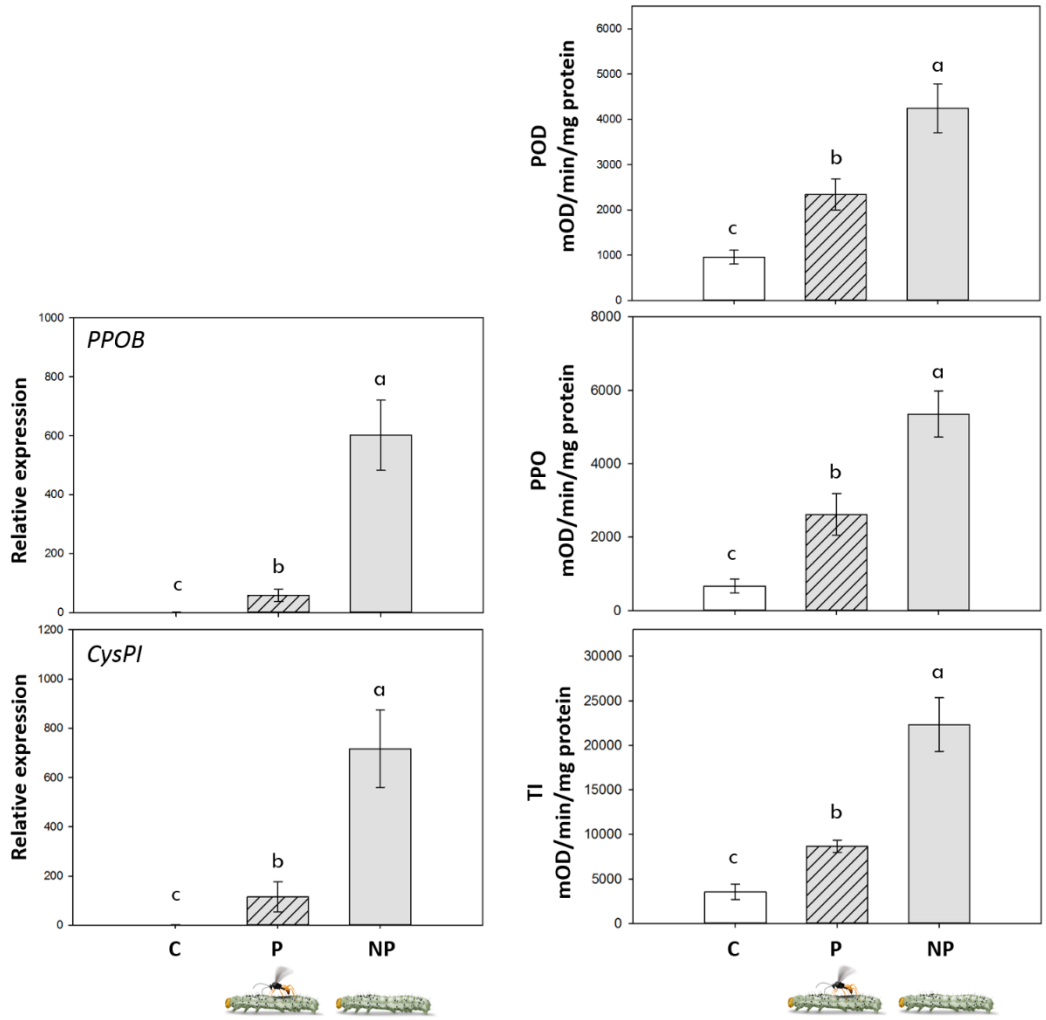

Fig. 4-3. Plant defense gene expression levels and protein activities in tomato plants treated with saliva of non-parasitized caterpillars (NP), parasitized caterpillars (P) and unwounded control (C). Gene expression levels and protein

activities were measured 24 h and 48 h after saliva application respectively. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, α=0.05: *PPOB*, $F_{(2, 7)} = 76.38$, $P < 0.0001$; *CysPI*, $F_{(2, 24)} = 40.10$, $P < 0.0001$; POD, $F_{(2, 41)} = 27.54$, $P < 0.0001$; PPO, $F_{(2, 41)} = 28.19$, $P < 0.0001$; TI, $F_{(2, 41)} = 43.19$, $P < 0.0001$). C, intact control plant; P, plant treated with parasitized caterpillar saliva (15 µl (1 µg/µl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 µl (1 µg/µl protein)).

To determine if difference in induced plant defenses influence parasitoid performance, parasitized caterpillar relative growth rate (RGR) and parasitoid development were measured. Parasitized *H. virescens* caterpillars had lower RGR when fed on NP-caterpillar treated tomato plants (higher plant defenses) than the P-caterpillar treatment (Fig. 4-4). These results indicate that parasitized *H. virescens* caterpillars had lower GOX activity in their labial glands after being parasitized and induced lower defense responses in tomato plants. The parasitoid *benefitted* from the lower induced plant defenses when treated with parasitized *H. virescens* caterpillars by promoting parasitized caterpillar growth.

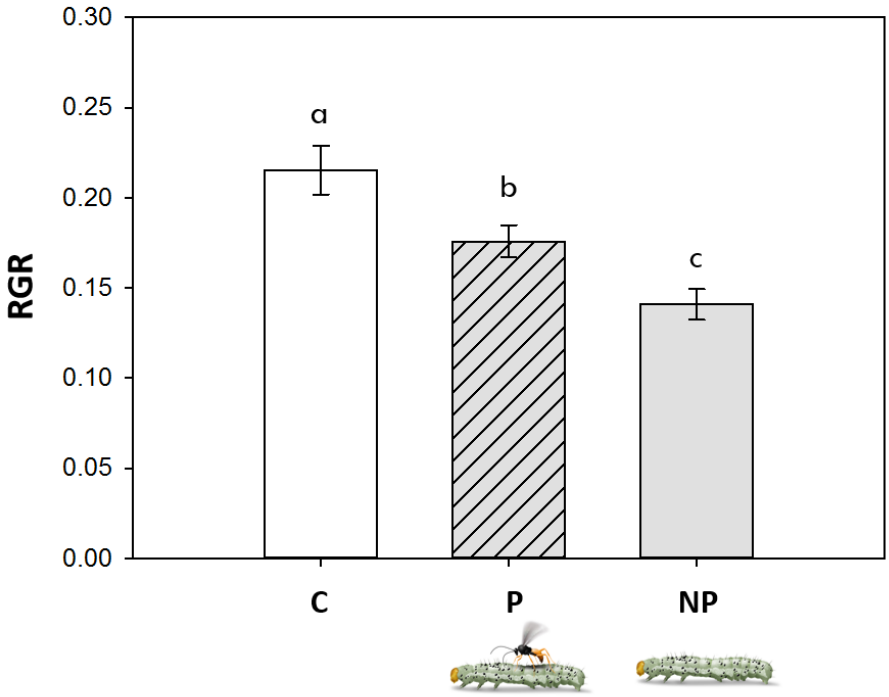

Fig. 4-4. Relative growth rate of parasitized H. virescens caterpillars feeding on tomato plants previously treated with saliva from parasitized caterpillars (P), saliva from non-parasitized caterpillars (NP) or untreated control plants (C) for 48 h. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, α = 0.05: n =49-53, $F_{(2, 148)}$ =11.68, P <0.0001. C, intact control plant; NP, plant treated with non-parasitized caterpillar saliva; P, plant treated with parasitized caterpillar saliva.

Tobacco-*Helicoverpa zea-Microplitis croceipes* system

To determine if *M. croceipes* parasitized *H. zea* caterpillars can affect plant defense responses in different plant system, tobacco plants were used in this study. In tobacco, GOX suppresses plant induced defense responses (Musser et al. 2002; Zong and Wang 2004). Therefore, we expected to see opposite results when compared to tomato where defenses are induced by GOX (Tian et al. 2012). In this system, parasitized *H. zea* caterpillars had significantly lower GOX activity in the labial glands (Fig. 2-6) and then triggered higher plant defense protein activities (PPO and TI) in tobacco plants compared with the NP-caterpillar feeding treatment (Fig. 4-5).

To determine if insect saliva was responsible for the observed difference in induced plant defenses, tobacco defense protein activities were measured after application of insect saliva. Saliva from P-caterpillars induced higher plant defense protein activities (PPO and TI) in tobacco plants than plants treated with saliva from NP-caterpillars (Fig. 4-6). These results indicate that saliva is the factor responsible for the higher induction of tobacco defense responses after caterpillars were parasitized.

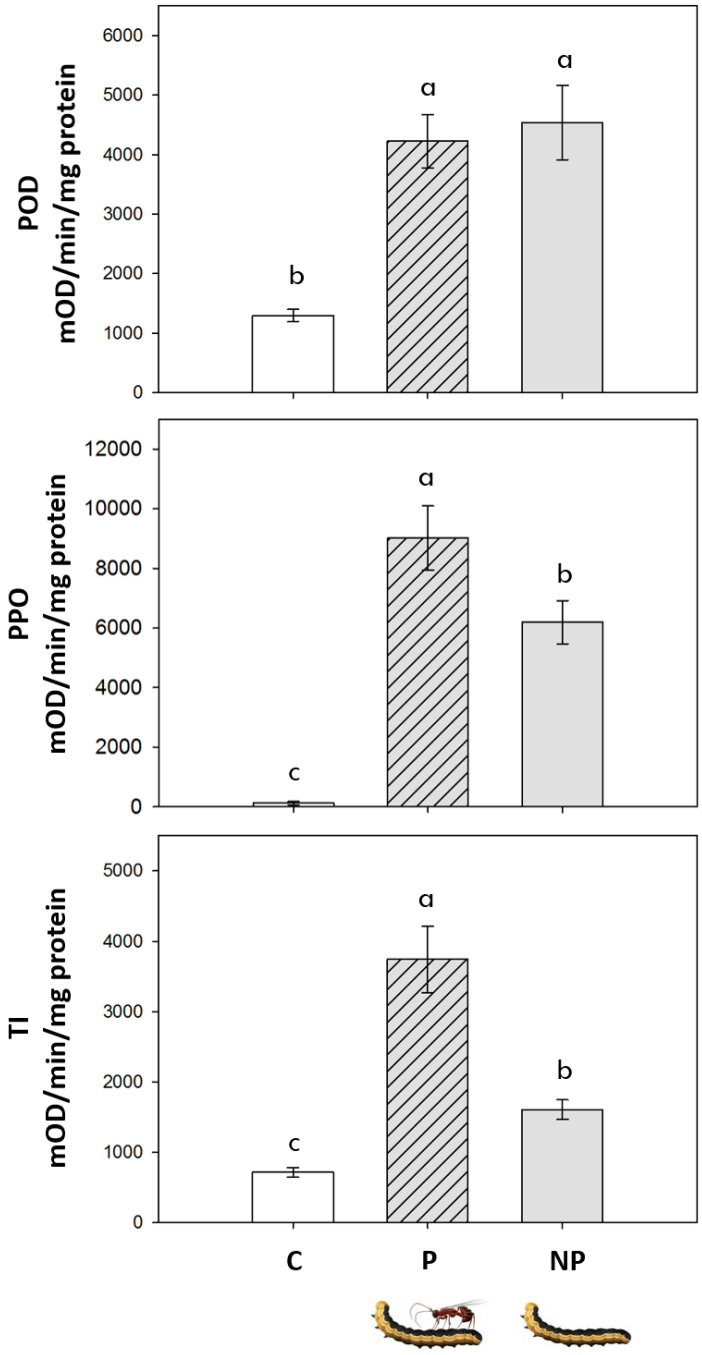

Fig. 4-5. Effect of caterpillar parasitism on induction of plant defensive proteins. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, α = 0.05; POD, n = 11–15, $F_{(2, 37)} = 43.43$, $P < 0.0001$; PPO, n = 11–14, $F_{(2, 35)} = 89.75$, $P < 0.0001$; TI, n = 7–15, $F_{(2, 30)} = 54.61$, $P < 0.0001$. C, intact control plant; NP, plant fed on by non-parasitized caterpillar; P, plant fed on by parasitized caterpillar; POD, peroxidase; PPO, polyphenol oxidase; TI, trypsin inhibitor.

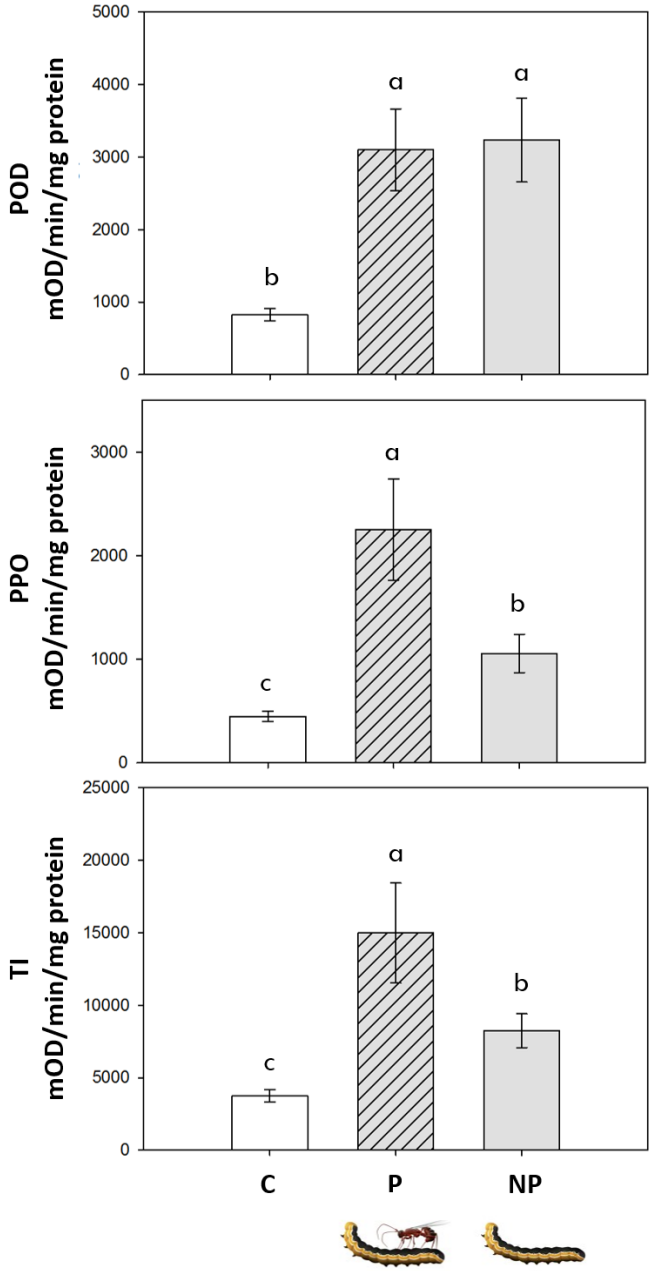

Fig. 4-6. Plant defense protein activities in tobacco plants treated with saliva of non-parasitized caterpillars (NP), parasitized caterpillars (P) and unwounded control (C). Defense protein activities were measured 48 h after saliva application. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, α=0.05: POD, n = 10–13, $F_{(2, 35)} = 15.60$, $P < 0.0001$; PPO, n = 10–13, $F_{(2, 35)} = 25.60$, $P < 0.0001$; TI, n = 14, $F_{(2, 41)} = 14.05$, $P < 0.0001$. C, intact control plant; P, plant treated with parasitized caterpillar saliva (15 µl (1 µg/µl protein)); NP, plant treated with non-parasitized caterpillar saliva (15 µl (1 µg/µl protein)). POD, peroxidase; PPO, polyphenol oxidase; TI, trypsin inhibitor.

To determine if difference in induced plant defenses influence parasitoid performance, the relative growth rate (RGR) of parasitized caterpillars and parasitoid performance were measured. Parasitized *H. zea* caterpillars had lower RGR when fed on P-caterpillar treated tobacco plants (higher plant defense responses) (Fig. 4-7). Moreover, parasitoids performed worse when their host caterpillars fed on P-caterpillar treated plants compared to NP-caterpillar treatments. They had longer development times, lower cocoon weights, and lower total survival compared with those whose host caterpillars fed on NP-caterpillar treated tobacco plants (Table 4-2).

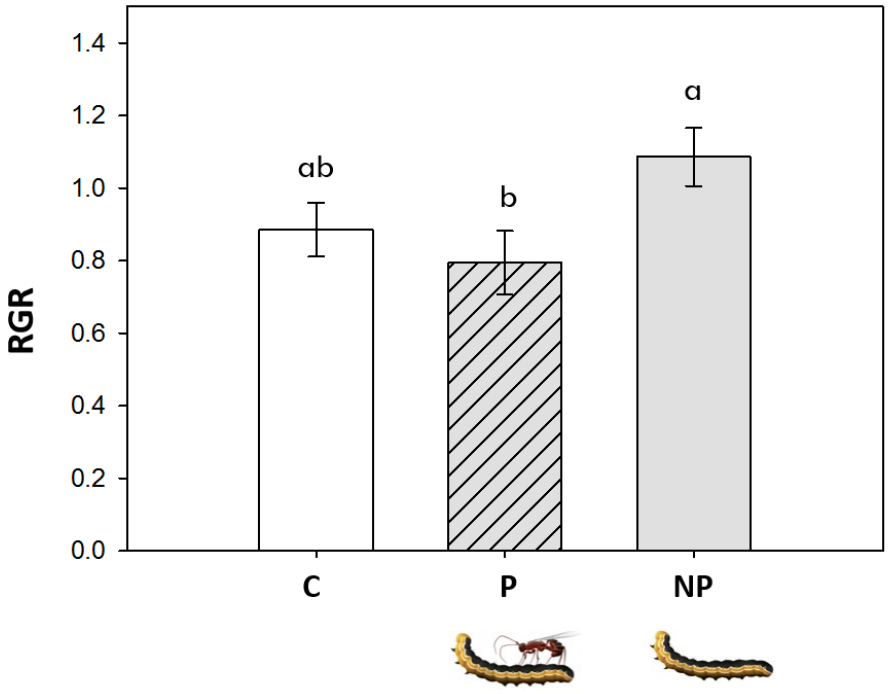

Fig. 4-7. Relative growth rate of parasitized H. zea caterpillars feeding on tobacco plants previously treated with saliva from parasitized caterpillars (P), saliva from non-parasitized caterpillars (NP) or untreated control plants (C) for 48 h. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (ANOVA followed by LSD test, α = 0.05: n = 29-33, $F_{(2, 93)} = 3.40$, $P = 0.0377$. C, intact control plant; NP, plant treated with non-parasitized caterpillar saliva; P, plant treated with parasitized caterpillar saliva.

Table 4-2. Parasitoid performance with *Helicoverpa zea* host feeding on different tobacco plant treatments

	Larval duration (day)	% Larval mortality	% Cocoon formation failure	Cocoon weight (mg)	Pupal duration (day)	Total development (day)	% Adult emergence	% Total survival
C	15.11 ± 0.28 ^{ab}	20.00 ± 5.71 ^{b}	38.33 ± 5.00 ^{a}	12.98 ± 0.33 ^{ab}	10.80 ± 0.20	25.50 ± 0.58 ^{ab}	60.00 ± 8.90	28.57 ± 4.52 ^{b}
P	15.96 ± 0.47 ^{a}	37.14 ± 3.50 ^{a}	17.00 ± 7.68 ^{b}	12.32 ± 0.51 ^{b}	11.42 ± 0.36	27.83 ± 0.96 ^{a}	66.67 ± 4.56	34.29 ± 3.50 ^{b}
NP	14.59 ± 0.23 ^{b}	17.14 ± 5.35 ^{b}	20.86 ± 3.30 ^{b}	14.00 ± 0.34 ^{a}	11.13 ± 0.24	25.19 ± 0.44 ^{b}	72.00 ± 8.46	45.71 ± 2.86 ^{a}
	$p = 0.035$	$p = 0.030$	$p = 0.044$	$p = 0.011$	$p = 0.357$	$p = 0.045$	$p = 0.525$	$p = 0.019$
	F = 3.50	F = 4.78	F = 4.09	F = 4.85	F = 1.06	F = 3.41	F = 0.68	F = 5.60

C: plant without any treatment; P: saliva from parasitized *Helicoverpa zea* treated plant; NP: saliva from non-parasitized *Helicoverpa zea* treated plant. Values are untransformed means ± SEM. Different letters indicate significant differences between treatments (one-way ANOVA).

These results indicated that parasitized *H. zea* caterpillars had lower GOX activity in their labial glands, thereby inducing higher defense responses in tobacco plants resulting in lower parasitoid performance.

Discussion

Glucose oxidase is a multifunctional enzyme that occurs widely in larvae of lepidopteran species and it tends to have higher expression in generalist/polyphagous species (Eichenseer et al. 2010). Salivary GOX has been shown to suppress defenses in several host plant species (Musser et al. 2002; Bede et al. 2006; Diezel et al. 2009). Salivary GOX also occurs in folivore hymenopteran sawflies (Eichenseer et al., 2010) and in other Hymenoptera such as the honeybee, *Apis mellifera*, where it plays a crucial role in social immunity by sterilizing larval food and contributing to the antiseptic properties of honey (López-Uribe et al. 2017). Thus, GOX plays a dual role in mediating insect and plant immunity.

However, the effects of salivary GOX on plant immunity/defense are species specific: in tobacco GOX in several caterpillar species, including *H. zea, Helicoverpa armigera* and *H. assulta* (Musser et al. 2002; Zong and Wang 2004) suppresses jasmonate-regulated plant defenses (defense protein activities and nicotine concentration). The suppression of JA-defenses is likely due to hormonal cross-talk with the salicylic acid signaling. Application of *H. zea* GOX triggered significantly higher levels of the SA-mediated PR-1a protein in *N. tabacum* (Musser et al. 2005), while salivary GOX levels of *Spodoptera exigua* glucose oxidase activity (but not *β*-glucosidase activity) were sufficient to trigger a SA burst and attenuate the JA and ethylene levels in *Nicotiana atteunata* (Diezel et al. 2009). In contrast, GOX *triggers* JA-regulated defenses such as proteinase inhibitors in tomato (Tian et al. 2012), but does not elicit an SA burst (unpublished data; Tian et al. 2012). In other plant species the effects of GOX have been shown to be dose dependent: basal levels of salivary GOX in the European corn borer *Ostrinia nubilalis* (Louis et al. 2013) or *H. zea* (Wang et al. 2018) were not sufficient to trigger defenses in maize. However, because GOX is involved in insect immunity, it is expected that microbes influence its expression (Wang et al. 2017). Bacteria present in the digestive tract of *H. zea* caused caterpillars to secrete more than twice as much GOX during feeding on maize leaves, which was a sufficient dose to trigger defenses in maize (Wang et al. 2018).

Polydnaviruses associated with parasitoids play a critical role in suppressing the immune systems of their caterpillar hosts (Burke and Strand, 2012). I previously showed that the polydnavirus associated with *M. croceipes* (*Mc*BV) caused a suppression of immune related gene *gox* expression in *H. zea* (Tan et al.

2018). The downregulation of GOX by *Mc*BV has cascading effects across trophic levels. The lower expression of GOX results in an attenuation of plant defenses during caterpillar feeding, which in turn improves the survival and fitness of the parasitoid (and *Mc*BV). Here I report a similar phenomenon in another host of *M. croceipes*, *H. virescens*, where parasitized caterpillars had lower GOX activity and triggered lower levels of plant defenses. Because GOX has different effects on different host plants, we predicted that the effects of the parasitoid could be quite different in other *H. zea* host plants. Indeed, when tobacco is the host plant for *H. zea*, the downregulation of salivary GOX by parasitoids can have detrimental effects on the developing parasitoid by reducing their overall survival due to the enhanced induction of plant defenses.

There is an emerging body of evidence that insect herbivore-associated microbes not only mediate insect immunity, but could directly (Chung et al. 2013) or indirectly mediate plant immunity by altering the expression of salivary cues such as GOX (Wang et al. 2017; 2018; Tan et al. 2018). Whereas the bottom up effects of plant defense traits on parasitoids and microbes are generally well appreciated (Peterson et al. 2016; Shikano et al. 2017), the top down effects of parasitoids and herbivore-associated microbes on plant defense traits has only recently become recognized (Poelman et al. 2011; Tan et al. 2018; Cusamano et al. 2018; Zhu et al. 2018). Due to the large number of lepidopteran species (>180,000) and their associated parasitic braconid (upwards to 50,000) and ichneuomonid wasp species (60,000 to 100,000) which carry PDVs, we predict that the top down regulation of plant defense traits by parasitoids and PDVs may be a widely occurring phenomenon impacting multiple trophic levels.

Acknowledgements

We thank Dr. Henry Fadamiro (Auburn University) for providing *M. croceipes* pupae; Ju-Che Lo for assistance the experiment and maintain insect colonies. This research was supported by National Science Foundation Grant IOS-1645548.

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Chapter 5

Conclusions

This Chapter summarizes significant research results that answers questions proposed in the first chapter and suggests of future research directions.

Living organisms are surrounded and/or interact (i.e., beneficial, harmful, or neutral) with microorganisms in the natural environment (Hacquard et al. 2015; Eisthen and Theis 2016). These microbes can mediate plant-herbivore interactions through many different ways including plant-beneficial microbes, entomopathogens, phytopathogens and herbivore associated microbes (Shikano et al. 2017). This dissertation research highlights the potential top-down effects of parasitoids and their symbiotic microbes (PDVs) on plant-herbivore interactions (Fig. 5-1), including potential mechanism behind these effects and ultimate consequences for plant and insect fitness.

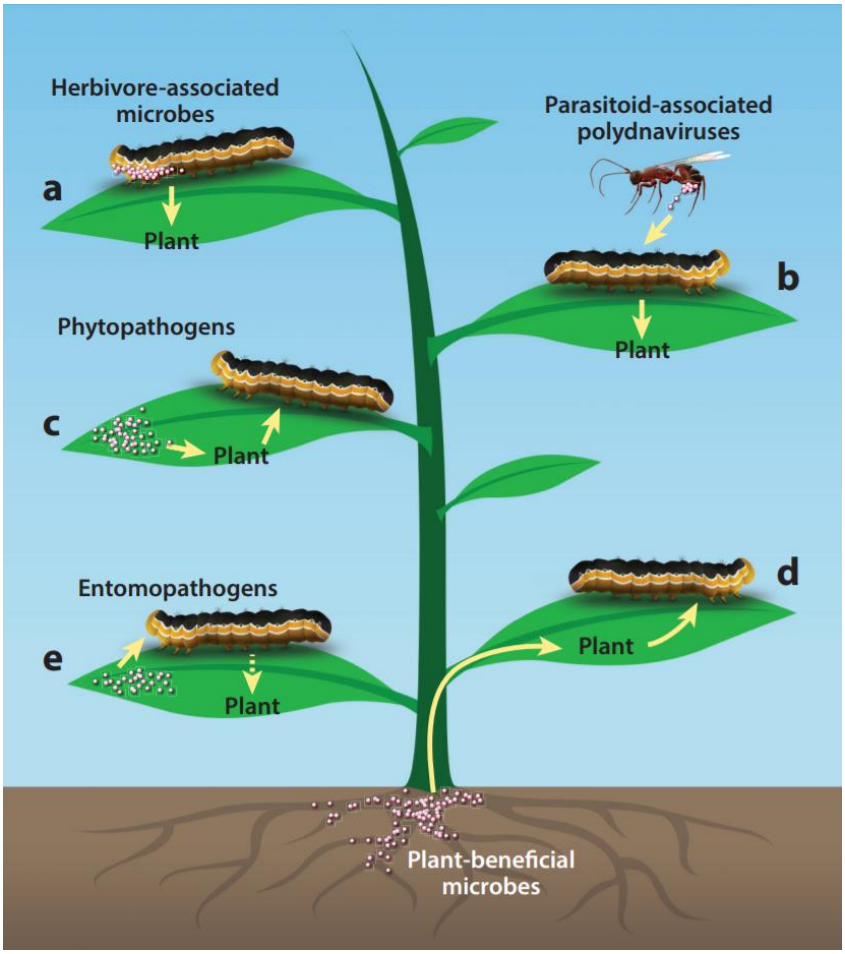

Fig. 5-1. Microbes mediated plant-herbivore interactions. (a) Insect-associated microbes can suppress plant defenses and detoxify defensive phytochemicals that enhance insect fitness. (b) Symbiotic polydnaviruses of some parasitoids that are released into the host during parasitization can modify the expression of herbivore-associated molecular patterns (HAMPs) and effectors, thereby suppressing plant defenses to promote host growth and parasitoid fitness. (c) Phytopathogens and (d) plant-beneficial microbes can influence insect behavior and fitness through changes in plant biomass, nutritional quality, defensive properties, and attraction of natural enemies. (e) Entomopathogens may potentially influence plant defenses directly as endophytes or indirectly by modifying the composition of insect oral secretions. Illustration courtesy of Nick Sloff, Pennsylvania State University, University Park, PA, USA. (in Shikano et al. 2017).

Higher trophic level causes top-down effects on plant and herbivore interactions

One mechanism of how parasitoids influence plant defenses is through interference with host caterpillar oral cues. *Microplitis croceipes* parasitized corn earworm larvae (*Helicoverpa zea*) had lower elicitor activity in their saliva (i.e., glucose oxidase) compared with non-parasitized caterpillars, which significantly downregulated tomato defense-related gene expression and defense protein activities during feeding.

The ultimate cause of downregulation of plant defense responses is due to the obligate mutualist PDVs of the parasitoid. PDVs are transferred to host caterpillars when parasitoids deposit eggs (Strand and Burke 2014, 2015). The expression of PDV viral genes suppressed host immune systems and provide a more suitable environment to nurse the developing parasitoid larvae (Strand and Burke 2014; Beckage 1998; Kroemer and Webb 2004). Injection of purified PDVs into caterpillars suppresses glucose oxidases gene expression and activity in caterpillar salivary glands thereby downregulating plant defense responses.

From the bottom-up perspective, changes in plant defenses can impact herbivore feeders and even organisms in higher trophic levels (Harvey et al. 2005). A long-term growth performance of parasitoids was performed to evaluate the effects of lower induced plant defenses on herbivore growth. My results showed that parasitoids *benefitted* from the lower induced plant defenses by promoting parasitized caterpillar growth performance, producing heavier cocoon mass and overall higher parasitoid survival rate. These results demonstrate that PDVs play

an important and complex role by not only suppressing the host caterpillar immune system, but also indirectly downregulating plant defenses for their own benefit.

The lower defense responses induced by parasitized caterpillar improve plant fitness

From a plant's perspective, induced defenses can be costly (Mauricio 1998, Redman et al. 2001, Zavala et al. 2004, Stowe and Marquis 2011, Bekaert et al. 2012). My study tested the hypothesis that the lower induced responses elicited by parasitized caterpillars may benefit the plants by reducing defense energy costs compared to responses to non-parasitized caterpillars. A long-term plant reproduction experiment showed that tomato plants had higher fitness (flower number and fruit weight) when they were treated with saliva from parasitized caterpillars rather than non-parasitized caterpillars. The higher plant fitness in parasitized caterpillar treated plants was due to the lower induced defense costs (transcriptomic and enzymatic levels) rather than reduced food consumption by the herbivore. In addition, the seed germination speed and rate were higher when its maternal plants were previously exposed to parasitized herbivore treatments compared to control plants. Therefore, the lower induced defense responses caused by parasitized caterpillars *benefitted* plant reproduction and the germination of progeny. These results also reveal a new perspective of mutualism between parasitoids and plants.

Parasitoids effects on plant defenses may be a widely occurring phenomenon with species-specific effects

Two different systems, *Microplitis croceipes*-*Helicoverpa zea*-tobacco and *M. c*-*Heliothis virescens*-tomato, were evaluated to determine if parasitoids commonly affect plant defenses in different herbivore and plant systems. Based on the results, plants perception of insect herbivores can be affected by the parasitoid and lead to positive/negative consequences at higher trophic levels (ex. parasitoid performance) depending upon the particular host plant system. These studies demonstrate species-specific effects in different plant-insect systems.

Conclusions

A series of biochemical, physiological and molecular experiments provide solid evidence to support proposed hypotheses that parasitized (P-) caterpillars induce lower plant defense responses than non-parasitized (NP-) caterpillars. In other words, plants can distinguish between feeding by parasitized and non-parasitized caterpillars and thus alter their defense responses accordingly. Second, this suppression of plant defenses can benefit both the plant and the parasitoid by minimizing plant defense costs and providing better host quality for the parasitoid. Furthermore, the manipulation of plant defenses by parasitoids may be a widely occurring phenomenon impacting multiple trophic levels.

Research impact

The findings of my dissertation research have revealed a very novel aspect of microbe-mediated interactions between plants and insects. The symbiotic PDV not only alters the phenotype of its primary host (i.e., parasitoid) and secondary host (i.e., caterpillar), but also the host plant of the caterpillar. This is the most extreme example of the extended phenotype known: a virus phenotype that extends across three trophic levels. This work has important implications for the evolutionary ecology of plant-herbivore-parasitoid interactions and points out a new perspective of mutualism between plants and parasitoids.

Further directions

The results presented in this dissertation provide background information for further studies. For example, proteomic and metabolomics approaches are needed to more comprehensively identify the difference in salivary composition between parasitized and non-parasitized caterpillars, which will provide a more global analysis of how parasitoids/PDVs affect insect oral cues. The complexity of caterpillar host ranges (specialist and generalist), parasitoid reproductive types (solitary and gregarious) and plant species may lead to unique results. Furthermore, the effects of modulated plant defenses may cause impacts to the fourth trophic level, i.e., hyperparasitoids. More studies are needed to uncover these fascinating connections whereby higher trophic levels directly and indirectly mediate plant defensive responses and plant fitness.

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Selected Publications

Tan C-W, Peiffer M, Hoover K, Rosa, C, Acevedo FE, Felton GW 2018. Symbiotic polydnavirus of a parasite manipulates caterpillar and plant immunity. PNAS 155:5199-5204.

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