

Symbiotic polydnavirus of a parasite manipulates caterpillar and plant immunity

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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 a polydnavirus 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.

herbivore | plant defense | parasitoid | host immunity | phytobiome

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” (1). 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 (2). 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 (3, 4). 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) (3, 5). However, microbes present in the herbivore’s gut can alter the composition of oral secretions and thus may trigger or suppress plant defensive responses (6, 7).

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 (8). PDV genomes are stably integrated in the genomes of parasitoid wasps (8). 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 (9). PDVs use virulence factors to manipulate the immune systems of their caterpillar hosts to enable the survival of parasitoid eggs and larvae (8, 10, 11). PDVs are associated with parasitic wasps belonging to the Braconidae and Ichneumonidae families, respectively (8).

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. (12) 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 (12). 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 (13). The differential induction in this case was attributed to increased feeding in the parasitized caterpillars (13). In contrast to parasitoids that carry polydnavirus symbionts, this particular parasitic wasp maintains and even enhances the host immune system (14). 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

Significance

The role of herbivore-associated microbes in mediating plant-herbivore interactions has gained recent attention. We show that a parasitoid associated with its caterpillar host not only suppresses the immune system of the caterpillar but also suppresses the induced defenses of the caterpillar’s host plant. Parasitoids inject eggs into their hosts but also inject polydnaviruses that suppress the caterpillar’s immunity. Immunosuppression enables eggs to hatch and develop as larvae within caterpillars. Additionally, the polydnavirus reduces salivary glucose oxidase, the primary elicitor found in the caterpillar’s oral secretions. Caterpillars injected with polydnavirus induce lower plant defenses than untreated caterpillars. Our results reveal a dimension to the complexity of plant-herbivore interactions indicating that polydnaviruses mediate the phenotypes of the parasitoid, herbivore, and plant.

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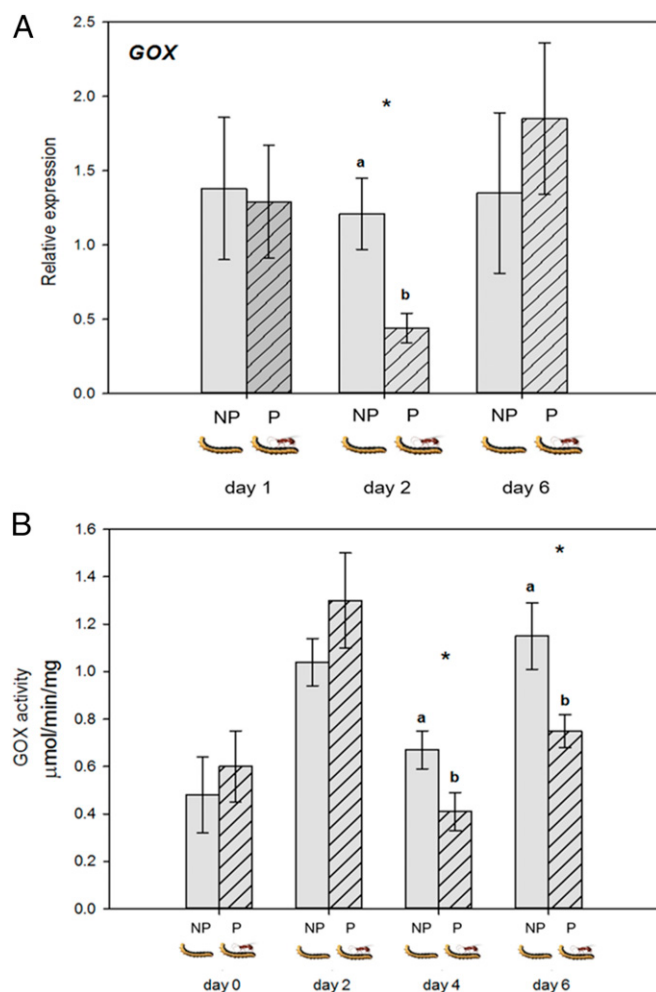


Fig. 2. GOX transcript levels and enzyme activity at different time points after parasitism. Values are untransformed mean $\pm$ SEM. Different letters indicate significant differences between treatments. Treatments include P- and NP-caterpillars. Relative expression of GOX (A) was measured 1, 2, and 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, $n = 15$, $F(1, 28) = 5.57$, $P = 0.027$.

performance was shown to be reduced when caterpillar hosts are reared on induced tomato foliage compared with unwounded foliage (29). 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 (*SI Appendix, Table S1*). 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 (30, 31). In tomato, the glycoalkaloid α -tomatine has been shown to be toxic to an endoparasitoid larva when ingested by its *H. zea* host (32) and to cause disruption of pupation (33), 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 (*SI Appendix, Fig. S1*). GAME4 plays a key regulatory role in glycoalkaloid biosynthesis in tomato (34).

In nature, a large percentage of *H. zea* caterpillars may be parasitized (35), indicating that PDVs may be important and ubiquitous mediators of the interactions of *H. zea* with its host plants. Remarkably, *McBV* 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 (8). 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 herbivore-induced plant volatiles (36). 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.

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 (37) 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 (38). At the last day of the second instar stage (head capsule slippage stage), *H. zea* larvae were offered to one female parasitoid

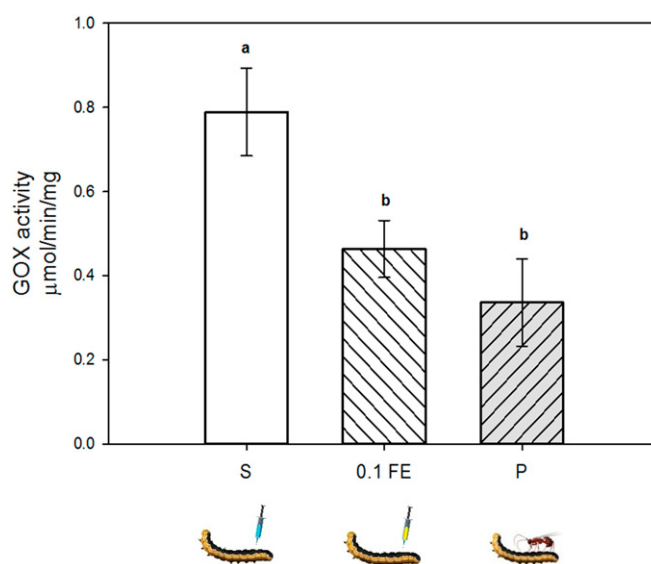


Fig. 3. Effects of parasitism and polydnavirus on salivary GOX activity of caterpillars. Treatments include saline (S), 0.1 FE and P-treated caterpillars. Values are untransformed mean $\pm$ SEM. Different letters indicate significant differences between treatments: ANOVA followed by LSD test, $\alpha = 0.05$: $n = 13-16$, $F(2, 41) = 6.33$, $P = 0.004$. P, parasitized caterpillars; S, Pringle's saline-injected caterpillars; 0.1 FE, caterpillars injected with 0.1 FE purified McBV.

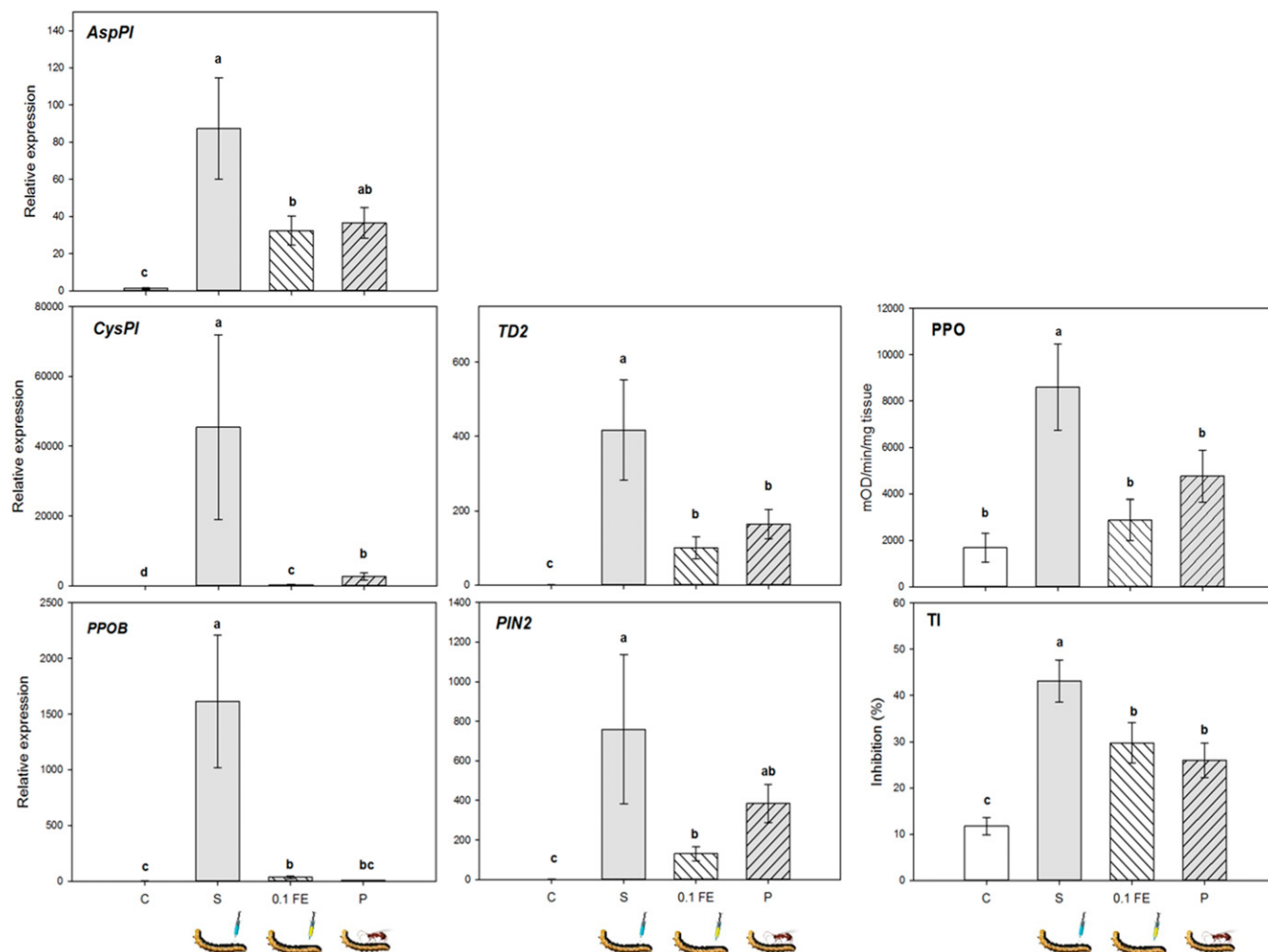


Fig. 4. Gene expression levels and activities of defense proteins in plants fed on by saline-injected (S), 0.1 FE McBV-injected, parasitized (P)-treated caterpillars, and intact control plants (C). Relative expression of all genes was measured 24 h and protein activities 48 h after insect feeding. Values are untransformed mean $\pm$ 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.

(>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 ($25 \pm 2^\circ\text{C}$, 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 (38). Following pupal eclosion, each female was maintained with several males in a container [9.5 (diameter) cm $\times$ 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 $\times$ 10 $\times$ 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 (from 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) (7). Gene primers of defense genes used for quantitative real-time PCR (qRT-PCR) analysis were designed by Primer Quest Software (Applied Biosystems) and listed (SI Appendix, Table S2). 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 (39).

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 ground with the Genogrinder. Immediately, 1.25 mL of 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 4450 was recorded in the Spectramax 190 plate reader (Molecular Devices) (22).

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 (7). 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 (40), 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 (41).

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* (37). 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 (McBV). McBV was purified from the calyx region of the ovaries of *M. croceipes* (SI Appendix, Fig. S6) following an established protocol (42) 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 was 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. 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 McBV segment are shown in SI Appendix, Table S2. All caterpillars (*n* = 6) that were injected with McBV showed viral gene expression in the labial glands (SI Appendix, Fig. S7). Six days after parasitism (third day of the fifth instar stage and second day of the fifth instar stage of S- or McBV-treated *H. zea* caterpillars) were caged on plants for measuring plant-defense gene expression and PPO and TI activities as described above. 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 McBV is the factor reducing *H. zea* salivary gland GOX gene expression, a GOX relative expression experiment was conducted. McBV were collected and purified as described above. There were three groups treated: S-injected, 0.1 FE McBV-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.

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. McBV 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, $P < 0.05$).

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