THE INSECT IMMUNE SYSTEM AS A TARGET FOR PROTECTING BENEFICIAL INSECTS AND CONTROLLING PESTS

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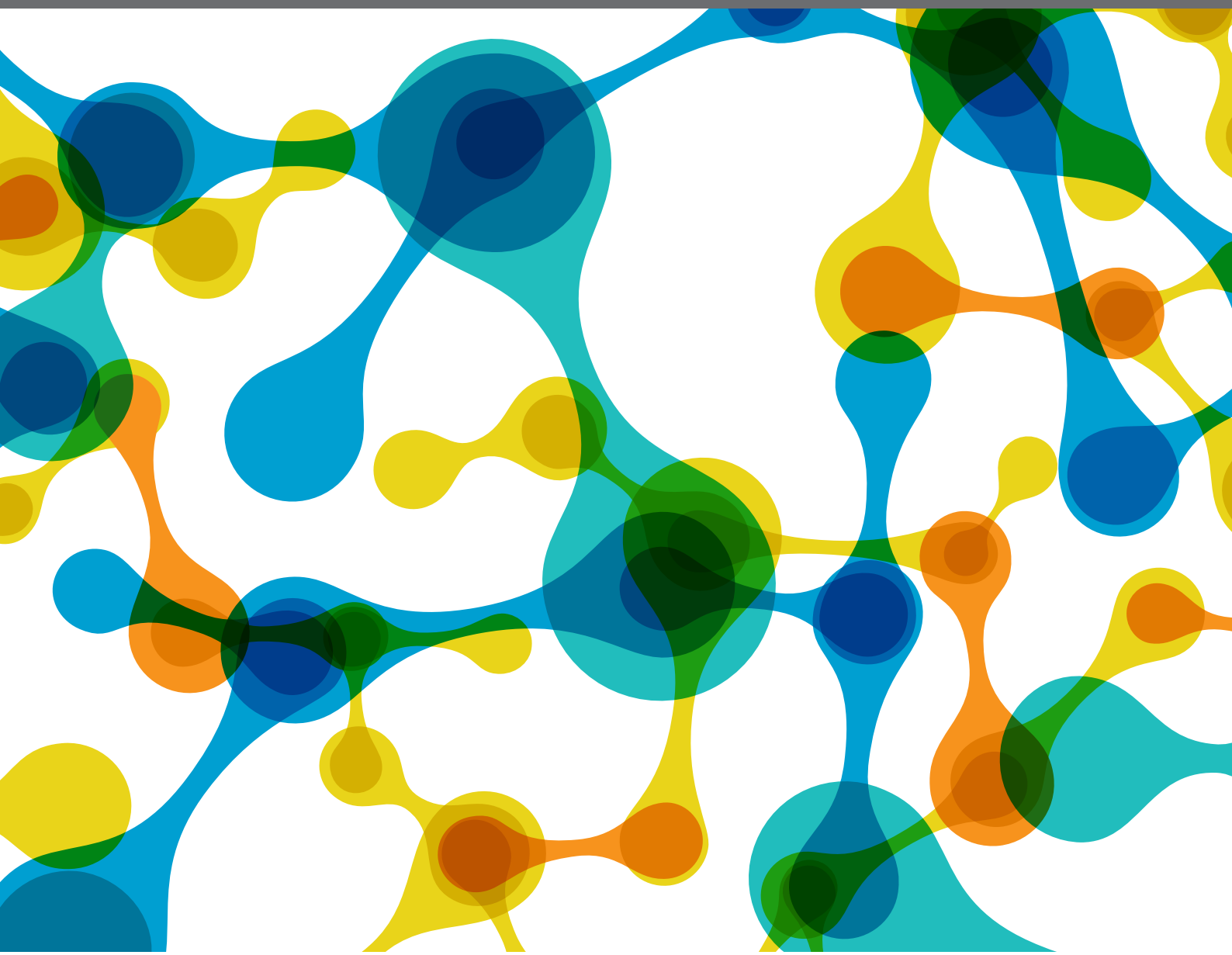

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THE INSECT IMMUNE SYSTEM AS A TARGET FOR PROTECTING BENEFICIAL INSECTS AND CONTROLLING PESTS

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Mosquito Hemocytes Associate With Circulatory Structures That Support Intracardiac Retrograde Hemolymph Flow

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A powerful immune system protects mosquitoes from pathogens and influences their ability to transmit disease. The mosquito's immune and circulatory systems are functionally integrated, whereby intense immune processes occur in areas of high hemolymph flow. The primary circulatory organ of mosquitoes is the dorsal vessel, which consists of a thoracic aorta and an abdominal heart. In adults of the African malaria mosquito, *Anopheles gambiae*, the heart periodically alternates contraction direction, resulting in intracardiac hemolymph flowing toward the head (anterograde) and toward the posterior of the abdomen (retrograde). During anterograde contractions, hemolymph enters the dorsal vessel through ostia located in abdominal segments 2–7, and exits through an excurrent opening located in the head. During retrograde contractions, hemolymph enters the dorsal vessel through ostia located at the thoraco-abdominal junction, and exits through posterior excurrent openings located in the eighth abdominal segment. The ostia in abdominal segments 2 to 7—which function in anterograde intracardiac flow—are sites of intense immune activity, as a subset of hemocytes, called periostial hemocytes, respond to infection by aggregating, phagocytosing, and killing pathogens. Here, we assessed whether hemocytes are present and active at two sites important for retrograde intracardiac hemolymph flow: the thoraco-abdominal ostia and the posterior excurrent openings of the heart. We detected sessile hemocytes around both of these structures, and these hemocytes readily engage in phagocytosis. However, they are few in number and a bacterial infection does not induce the aggregation of additional hemocytes at these locations. Finally, we describe the process of hemocyte attachment and detachment to regions of the dorsal vessel involved in intracardiac retrograde flow.

Keywords: immunity, circulation, phagocytosis, heart, dorsal vessel, *Anopheles gambiae*

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INTRODUCTION

The dorsal vessel of an insect is the main pulsatile organ that drives hemolymph circulation throughout the hemocoel (Jones, 1977; Chapman et al., 2013; Klowden, 2013; Wirkner et al., 2013; Hillyer, 2015). It is a contractile tube that traverses the length of the body and is comprised of the aorta in the head and thorax, and the heart in the abdomen. Hemolymph enters the

dorsal vessel through valves called ostia and is propelled by the wave-like contractions of heart muscle. Depending on the insect or life stage, the dorsal vessel propels hemolymph toward the head (anterograde) or periodically alternates between propelling hemolymph toward the head and toward the posterior of the body (retrograde).

The heart of the adult malaria mosquito *Anopheles gambiae* alternates between contracting in the anterograde and retrograde directions (**Figures 1A,B**; Glenn et al., 2010). When the heart contracts anterograde, hemolymph enters the lumen of the dorsal vessel through 6 pairs of incurrent ostia located in the anterior portion of abdominal segments 2–7, and exits the vessel through an excurrent opening located in the head (Glenn et al., 2010). When the heart contracts retrograde, hemolymph in the venous channels of the thorax and in the hemocoel of the first abdominal segment enters the dorsal vessel through a pair of thoraco-abdominal ostia, and exits the vessel through a pair of excurrent openings located in the 8th abdominal segment (Glenn et al., 2010; Sigle and Hillyer, 2018b). The thoraco-abdominal ostia are in a region of the heart called the conical chamber, which is adjacent to the location where the heart, aorta, and venous channels converge. Though the heart is the primary circulatory pump, the aorta persistently contracts in the anterograde direction (Sigle and Hillyer, 2018b). However, hemolymph does not flow through the aorta during periods of retrograde heart contractions.

The circulatory and immune systems of insects are functionally integrated, and this integration has been described in detail in *A. gambiae* (Hillyer, 2015; League and Hillyer, 2016). In adult mosquitoes, a population of sessile hemocytes—called periostial hemocytes—are always present in the regions surrounding the abdominal ostia (the periostial regions), where they phagocytose pathogens in regions of high hemolymph flow (King and Hillyer, 2012; Sigle and Hillyer, 2016). Upon infection, additional hemocytes actively migrate to the periostial regions, where they aggregate and continue the phagocytosis and killing of pathogens. The aorta lacks ostia, and hence, even though a few hemocytes are distributed across its surface, infection does not induce the aggregation of hemocytes in that portion of the dorsal vessel (Sigle and Hillyer, 2018b).

Whereas the presence and aggregation of hemocytes at the periostial regions of abdominal segments 2–7 has been clearly established, it is unclear whether hemocytes are present at the thoraco-abdominal ostia. This distinction is important because the abdominal ostia function in anterograde heart flow whereas the thoraco-abdominal ostia function in retrograde heart flow (Glenn et al., 2010; Sigle and Hillyer, 2018b). Furthermore, whereas few or no hemocytes are present in the anterior excurrent opening of the aorta—a structure that functions during anterograde heart flow—it remains unknown whether hemocytes are present at the posterior excurrent openings of the heart—a structure that functions during retrograde heart flow (Glenn et al., 2010; Sigle and Hillyer, 2018b). In this study we examined hemocyte presence and function at sites of the dorsal vessel that are important during periods of intracardiac retrograde hemolymph flow. We uncovered immunologically active hemocytes around the thoraco-abdominal ostia and the posterior excurrent openings of the heart. However, these hemocytes are few in number, and infection does not induce their aggregation at these locations. Finally, by means of intravital video imaging we revealed that hemocyte associations with circulatory structures involved in intracardiac retrograde flow are dynamic in that hemocytes attach to and detach from these structures.

MATERIALS AND METHODS

Mosquito Rearing and Maintenance

Anopheles gambiae Giles sensu stricto (G3 strain) were reared and maintained at 27°C and 75% relative humidity under a 12 h: 12 h light: dark photoperiod as previously described (Estévez-Lao et al., 2013). Larvae were fed a mixture of koi food and yeast, and adults were fed 10% sucrose solution *ad libitum*. Experiments were initiated on adult female mosquitoes at 5 days post-eclosion, an age when infection is known to induce the aggregation of hemocytes at the periostial regions of the heart (King and Hillyer, 2012; Sigle and Hillyer, 2016, 2018a).

Mosquito Injections and Bacterial Infection

For all injections, mosquitoes were anesthetized on ice and then injected into the hemocoel a volume of 0.15–0.20 μl using a glass needle that had been inserted through the thoracic anepisternal cleft as previously described (Coggins et al., 2012). For infections, tetracycline resistant/GFP-expressing *Escherichia coli* (DH5 alpha) were grown in Luria-Bertani media (LB) and injected. The infection dose was determined immediately after each experiment by plating serial dilutions of the bacterial cultures (Coggins et al., 2012), and averaged 72,560 *E. coli* per mosquito. To control for the effect of injection, a subset of mosquitoes was injected sterile LB (injury group).

Mosquito Dissections

Mosquitoes were anesthetized on ice, injected with 16% formaldehyde to fix hemocytes and other tissues (Electron Microscopy Sciences, Hatfield, PA, USA), and the head, legs and wings were removed by cutting with a fine blade. To isolate the dorsal thoraco-abdominal junction and abdomen, a mosquito was placed in PBS containing 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA, USA), the abdomen and thorax were bisected along the coronal plane, and the thorax was bisected at the anterior-posterior midline of the transverse plane. The internal organs were removed to expose the dorsal vessel, and the specimen was placed in Aqua Poly/Mount (Polysciences, Warrington, PA, USA) on a microscope slide. To resect the heart, the heart was pulled away from the dorsal cuticle after disrupting all alary muscles with an insect pin.

Fluorescence Labeling

To label muscle, mosquitoes were anesthetized, injected with 16% formaldehyde, and incubated for 5 min. Mosquitoes were dissected to expose the relevant structures and then incubated in a solution of 0.6 μM phalloidin-Alexa Fluor 488 (to label muscle green; Invitrogen, Carlsbad, CA, USA), 0.75 mM Hoechst 33342 (to label nuclei blue; Invitrogen) and 0.1% Triton X-100 (Fisher

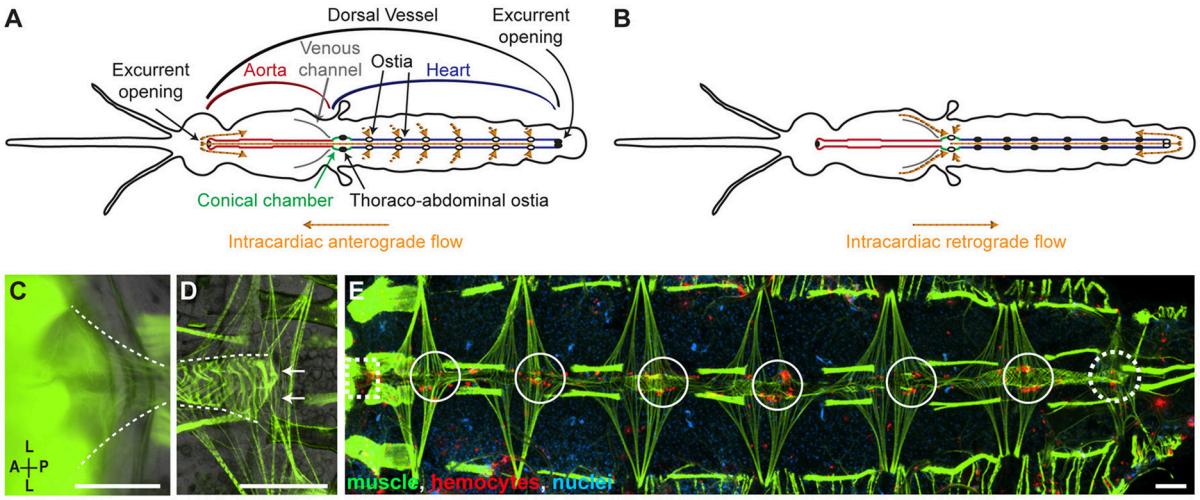

FIGURE 1 | The hemocytes of naïve mosquitoes are present at sites of incurrent and excurrent intracardiac retrograde hemolymph flow. **(A,B)** Dorsal view of the structure of the circulatory system of an adult mosquito showing the flow of hemolymph during anterograde **(A)** and retrograde **(B)** heart contractions. Diagrams are adapted from Sigle and Hillyer, 2018b. **(C,D)** Dissected specimens showing the muscles (phalloidin; green) of the conical chamber at the thoraco-abdominal junction **(C**; dotted lines) and the posterior excurrent openings (arrows) of the heart (**D**; dotted lines). **(E)** Dissected naïve mosquito where muscle (green) and hemocytes (CM-DiI; red) have been fluorescently labeled, showing the presence of hemocytes around the thoraco-abdominal ostia (dotted square), the periostial regions of abdominal segments 2–7 (solid circles), and the posterior excurrent openings of the heart (dotted circle). Scale bars: 100 μm.

Scientific) in PBS for 30 min. Afterwards, specimens were washed 3 times in PBS.

The hemocytes of live mosquitoes were fluorescently labeled red with CM-DiI, which is a dye that when injected into the hemocoel becomes incorporated in hemocytes and no other cells (King and Hillyer, 2012). Briefly, mosquitoes were anesthetized, injected a solution of 75 mM Vybrant CM-DiI Cell-Labeling Solution (Invitrogen) and 0.75 mM Hoechst 33342 in PBS, and incubated at 27°C for 20 min. Mosquitoes were then either imaged intravitally through the dorsal cuticle, further processed to label muscle, or dissected to either visualize structures or count hemocytes. In specimens where muscle and hemocytes were both labeled, following CM-DiI labeling, muscle was stained as described above or by intrathoracic injection of phalloidin-Alexa Fluor 488 as previously described (Glenn et al., 2010), with the exception that Triton X-100 was not included in the solution (Sigle and Hillyer, 2018b).

Hemocytes were also labeled by means of their phagocytosis of *E. coli* bacterial bioparticles conjugated to pHrodo (Sigle and Hillyer, 2016). For these experiments, *E. coli*-pHrodo-Red bioparticles (Invitrogen) were reconstituted in PBS at 1 mg/ml, injected into live mosquitoes, and mosquitoes were maintained at 27°C for 24 h. Mosquitoes were then anesthetized, injected with 16% formaldehyde, and either imaged through the dorsal cuticle or were dissected and washed 3 times in PBS. These specimens were either mounted for imaging or further processed for muscle staining.

Acquisition of Still Images

Images were acquired on a Nikon 90i compound microscope (Nikon Corp, Tokyo, Japan) equipped with a linear encoded Z-motor, a Nikon Intensilight C-HGFI fluorescence illumination unit, a Nikon DS-Qi1Mc CCD camera, and Nikon Advanced Research NIS-Elements software. Three-dimensional Z-stack images were acquired and rendered into focused two-dimensional images using the Extended Depth of Focus tool in NIS-Elements.

Counting of Hemocytes

CM-DiI-labeled hemocytes were observed and counted at 24 h post-treatment in naïve, injured (LB injected) and *E. coli* infected mosquitoes. At the thoraco-abdominal junction, hemocytes were visualized through the dorsal cuticle of intact mosquitoes. This approach was taken because disruption of the thoracic flight muscles during dissection causes the non-specific incorporation of CM-DiI into the myofibers, and this interferes with hemocyte visualization in the thorax and the first abdominal segment. This technique was validated by comparing hemocyte counts in intact mosquitoes and the same mosquitoes after dissection, which revealed that more hemocytes could be identified in the first abdominal segment of intact mosquitoes. In the 8th abdominal segment, hemocyte counts were similar regardless of whether specimens were visualized before or after dissection, so hemocytes were counted in intact specimens.

Following CM-DiI labeling and incubation at 27°C, mosquitoes were anesthetized on ice, injected with 16% formaldehyde, and the head, legs, and wings were removed by cutting with a fine blade. Specimens were placed dorsal-side-up for visualization under epi-fluorescence illumination at 200–400X magnification and the number of hemocytes at the thoraco-abdominal ostia and the posterior excurrent openings were counted. For a cell to be considered a hemocyte it had to be

9–18 μm in diameter (King and Hillyer, 2012; Hillyer and Strand, 2014; Sigle and Hillyer, 2016), and it had to be labeled with both CM-DiI and Hoechst 33342. A minimum of 25 mosquitoes across 7 independent trials were analyzed for each treatment group, and for each mosquito, data were collected for both the thoraco-abdominal ostia and the posterior excurrent openings. Data were analyzed with the non-parametric Kruskal-Wallis test and Spearman correlation analysis using Prism 6 Software (GraphPad, La Jolla, CA, USA).

Intravital Video Imaging

Live mosquitoes were visualized in real-time through the dorsal abdominal cuticle. Hemocytes were labeled with CM-DiI, and mosquitoes were restrained dorsal-side-up using a non-invasive method previously described (Boppana and Hillyer, 2014). Mosquitoes were imaged on the Nikon 90i compound microscope, and real-time videos were acquired under low-level epi-fluorescence illumination (using an ND 2 filter) at a magnification of 100X.

RESULTS

Hemocytes Attach to the Regions Surrounding the Thoraco-Abdominal Ostia and the Posterior Excurrent Openings

When the heart contracts in the retrograde direction, hemolymph enters the lumen of the dorsal vessel through a single pair of ostia located at the thoraco-abdominal junction, and exits the heart via the posterior excurrent openings (**Figures 1A–D**). To determine whether hemocytes associate with structures involved in intracardiac retrograde hemolymph flow, we examined whether—similar to what occurs in the periostial regions of abdominal segments 2 through 7—hemocytes are present at the thoraco-abdominal junction. In addition, we examined whether hemocytes are present at the excurrent openings of the 8th abdominal segment. In naïve mosquitoes, hemocytes labeled with CM-DiI were often observed in the areas surrounding the thoraco-abdominal ostia as well as near the posterior excurrent openings of the heart (**Figure 1E**). However, not all mosquitoes had hemocytes at these locations. At the thoraco-abdominal ostia and the excurrent openings, hemocytes were present in 79 and 46% of mosquitoes, respectively, and presence at the thoraco-abdominal ostia was not always a predictor of presence at the posterior excurrent openings (and vice versa; Spearman's correlation $p = 0.850$). Together, these findings show that hemocytes often surround circulatory structures involved in intracardiac retrograde hemolymph flow.

Infection Does Not Increase the Number of Hemocytes on the Thoraco-Abdominal Ostia and the Posterior Excurrent Openings

Because infection induces the aggregation of hemocytes at the periostial regions (King and Hillyer, 2012; Sigle and Hillyer, 2016), we hypothesized that infection increases the number of hemocytes at the thoraco-abdominal ostia, and perhaps the posterior excurrent openings. To test this hypothesis, we visualized and counted the hemocytes present at these locations in naïve, injured, and *E. coli* infected mosquitoes at 24 h post-treatment. Similar to naïve mosquitoes, some infected mosquitoes had hemocytes at these structures whereas others did not (**Figures 2A–E**). Specifically, mosquitoes had a median of 2.5 and 2.0 hemocytes at the thoraco-abdominal ostia of naïve and injured mosquitoes, respectively (**Figures 3**). At 24 h following *E. coli* infection, the median number of hemocytes was also 2.0, indicating that infection does not cause hemocyte aggregation at this location (Kruskal-Wallis $p = 0.458$). A similar trend was observed at the posterior excurrent openings. Naïve, injured and *E. coli*-infected mosquitoes had a median number of 0, 0.5, and 0 hemocytes at the posterior excurrent openings, respectively (**Figures 3**; Kruskal-Wallis $p = 0.965$), and never more than 5. There was also no correlation between the number of hemocytes at the thoraco-abdominal ostia and the number of hemocytes at the posterior excurrent openings (Spearman $r = -0.189$, 0.180, and 0.338 for naïve, injured and infected mosquitoes, respectively; Spearman's correlation $p = 0.336$, 0.378, and 0.098 for naïve, injured and infected mosquitoes, respectively). Even though few hemocytes were present at the structures involved in intracardiac retrograde hemolymph flow, examination of intact, dissected or resected specimens consistently confirmed the presence of numerous periostial hemocytes in abdominal segments 2–7, and that their number increases following infection (**Figures 1E**, **2A**, **4**).

Hemocytes at the Thoraco-Abdominal Ostia and the Posterior Excurrent Openings Are Phagocytic

To determine whether the hemocytes present at the thoraco-abdominal junction and the posterior excurrent openings are immunologically active, we tested their phagocytic activity. After infection, hemocytes at the thoraco-abdominal ostia and the posterior excurrent openings co-localized with *E. coli*-GFP, suggesting that pathogens are actively phagocytosed and killed by hemocytes at these locations (**Figures 5A,B**). To confirm phagocytosis, mosquitoes were injected pHrodo-conjugated *E. coli* bacterial bioparticles. These dead bacteria are conjugated to a pH-sensitive dye that only fluoresces in acidic environments, such as the phagolysosome. As expected, many hemocytes—including those present at the thoraco-abdominal ostia, the posterior excurrent openings, and the periostial regions—readily phagocytosed *E. coli*-pHrodo (**Figures 5C–G**).

Hemocyte Interactions With Structures Involved in Intracardiac Retrograde Flow Are Dynamic

During retrograde heart contractions, hemolymph flowing through the venous channels of the thorax or the dorsal hemocoel of the first abdominal segment enters the conical chamber of the heart via the thoraco-abdominal ostia **Figure 1B**; Sigle and Hillyer, 2018b). Intravital imaging of the thoraco-abdominal ostia revealed that most hemocytes enter the conical chamber by first flowing through the venous channels of the thorax, and a smaller proportion of hemocytes enters the heart by first flowing in the hemocoel of the first abdominal

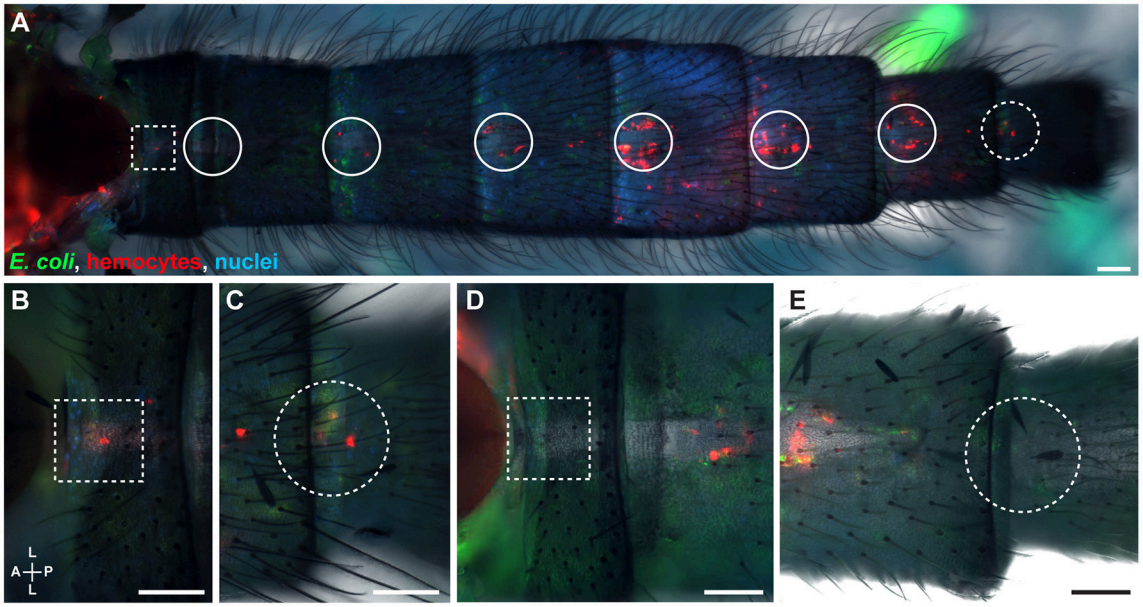

FIGURE 2 | The hemocytes of infected mosquitoes are present at sites of incurrent and excurrent intracardiac retrograde hemolymph flow. **(A–C)** Intact infected mosquito showing hemocytes (red) and *E. coli*-GFP (green) around the thoraco-abdominal ostia **(A**, magnified in **B**; dotted squares) and the posterior excurrent openings of the heart (**A**, magnified in **C**; dotted circles). **(D,E)** Images of another infected mosquito showing that hemocytes do not always surround the thoraco-abdominal ostia **(D**; dotted square) or the posterior excurrent openings **(E**; dotted circle). Nuclei were labeled blue with Hoechst 33342. Scale bars: 100 μm.

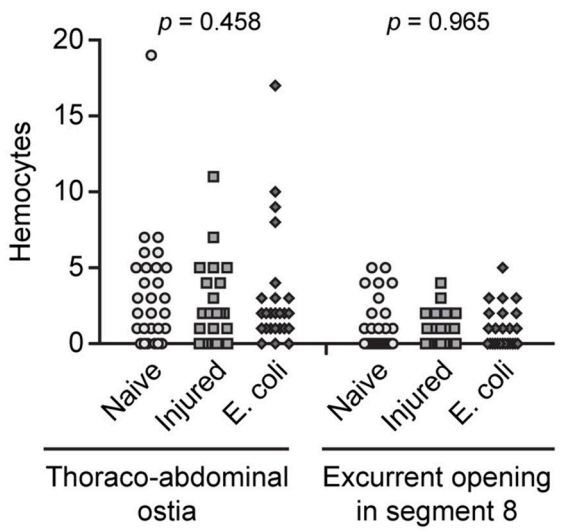

FIGURE 3 | Hemocytes are few in number at sites of incurrent and excurrent intracardiac retrograde hemolymph flow and do not increase in response to infection. Number of hemocytes at the thoraco-abdominal ostia and the posterior excurrent openings of the heart of naïve, injured and *E. coli* infected adult mosquitoes at 24 h following treatment. Each point represents the number of hemocytes in an individual mosquito, and *p*-values result from a Kruskal-Wallis test.

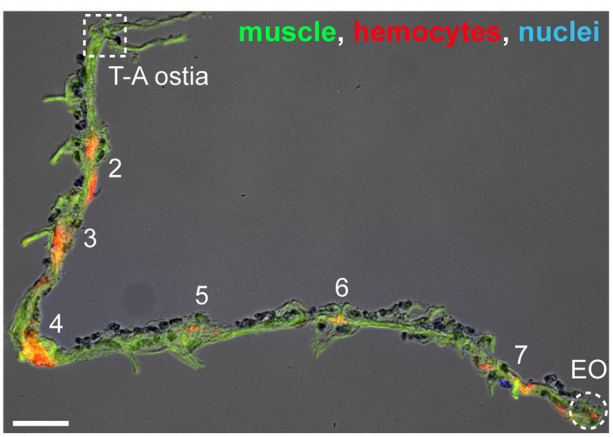

FIGURE 4 | Hemocytes on a resected heart. Image of a resected heart where muscle (phalloidin; green), hemocytes (CM-DiI; red), and nuclei (Hoechst 33342; blue) have been labeled. In this specimen there are no hemocytes at the thoraco-abdominal ostia (T-A ostia), but there are hemocytes at the periostial regions (abdominal segments are numbered) and at the posterior excurrent openings (EO). Scale bar: 100 μm.

segment (**Supplementary Video 1**). Hemocytes move through the venous channels at high speeds, which may hinder their ability to attach to the thoraco-abdominal ostia. Imaging of the 8th abdominal segment revealed that there is significant movement of hemocytes at the posterior excurrent openings of the heart. Hemocytes exiting the heart swiftly flow through the excurrent openings, and sessile hemocytes on the posterior surface of the heart are periodically released back into circulation (**Supplementary Video 2**). Thus, these data show the dynamic movement of hemocytes at locations of intracardiac retrograde

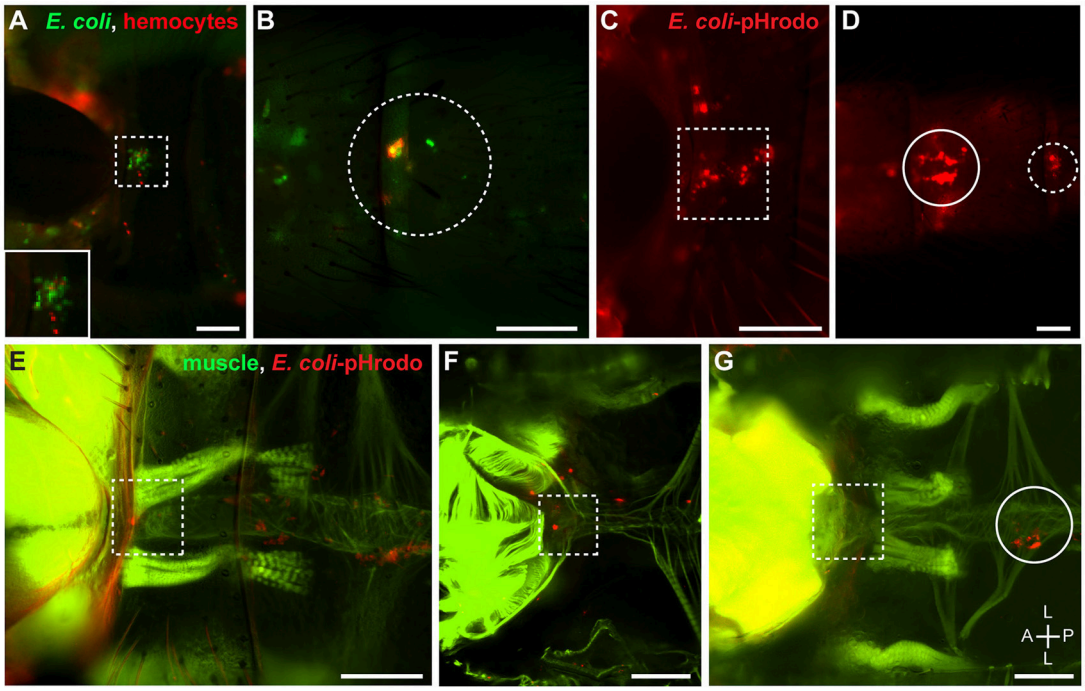

FIGURE 5 | Hemocytes at the thoraco-abdominal ostia and the posterior excurrent openings are phagocytic. **(A,B)** Intact adult mosquito showing that *E. coli*-GFP (green) co-localizes with hemocytes (CM-DiI; red) that surround the thoraco-abdominal ostia **(A**; dotted square; inset) and the posterior excurrent openings of the heart **(B**; dotted circle). **(C,D)** Intact adult mosquito showing that hemocytes that surround the thoraco-abdominal ostia **(C)** and the posterior excurrent openings of the heart **(D)** phagocytose *E. coli*-pHrodo (red). **(E,F)** Adult mosquitoes showing phagocytic hemocytes (*E. coli*-pHrodo) on the muscle (phalloidin; green) surrounding the thoraco-abdominal ostia in intact **(E)** and dissected **(F)** specimens. **(G)** Dissected adult mosquito showing that phagocytic hemocytes are not always present at the thoraco-abdominal ostia. In panels **(D,G)**, phagocytic hemocytes can be seen in the periostial regions of the 7th and 2nd abdominal segments, respectively (solid circles). Scale bars: 100 μm.

hemolymph flow, and their association and dissociation with circulatory structures.

DISCUSSION

In this study we show that hemocytes surround structures that support intracardiac retrograde hemolymph flow. Though not always present, a small number of hemocytes are often located at the thoraco-abdominal ostia and the posterior excurrent openings of the heart. Infection does not induce the aggregation of hemocytes at these locations; however, the hemocytes present at the thoraco-abdominal ostia and the posterior excurrent openings are phagocytic and immunologically active.

Prior studies showed that mosquito hemocytes aggregate near circulatory structures involved in intracardiac anterograde flow (King and Hillyer, 2012; Sigle and Hillyer, 2016). Specifically, a population of hemocytes, called periostial hemocytes, surround the ostia of abdominal segments 2–7, where they phagocytose pathogens in regions of high hemolymph flow. Because these locations are only functional when the heart contracts anterograde (Glenn et al., 2010), we hypothesized that hemocytes are also present in circulatory structures involved in intracardiac retrograde hemolymph flow, such as the thoraco-abdominal ostia. We found that hemocytes are present at the thoraco-abdominal ostia of most but not all mosquitoes, but that they are few in number. The average naïve mosquito has two hemocytes at this location, which is fewer than the fifty or so hemocytes present at the periostial regions (King and Hillyer, 2012; Sigle and Hillyer, 2016). Furthermore, infection does not recruit additional hemocytes to the thoraco-abdominal ostia, which is different from the more than doubling of hemocytes that occurs at the periostial regions following infection. Thus, although all hemocytes, regardless of location, are immunologically active, the thoraco-abdominal ostia are not locations of intense immune activity, and infection-induced hemocyte aggregation is restricted to the abdominal periostial regions. Given that the heart of 5-day-old adult *A. gambiae* spends a significant amount of time (∼1/3) contracting retrograde (Glenn et al., 2010; Estévez-Lao et al., 2013), and that the proportion of retrograde contractions increases with age (Doran et al., 2017), the lack of concerted immune activity at the thoraco-abdominal ostia was unexpected. We speculate that the absence of infection-induced hemocyte aggregation at the thoraco-abdominal ostia is due to the shear force of flow at this region, relative to the periostial regions of the abdomen. Another possible explanation is that hemocyte aggregation at this location would restrict flow at the only entry-point for hemolymph during retrograde heart contractions. Hemocytes

assume an asymmetric distribution across the 6 periostial regions of the abdomen, with most hemocytes aggregating in segments 4–6. This asymmetric distribution is less pronounced in infected mosquitoes, suggesting that periostial hemocyte aggregation results in the partial obstruction of flow at the ostia of the mid-abdominal segments, which results in the partial redirection of hemolymph to other ostial pairs (Sigle and Hillyer, 2016). Perhaps obstructing hemolymph flow at the thoraco-abdominal ostia is costlier than obstructing flow at any of the 6 pairs of abdominal ostia.

On the surface, it appears that our finding that few hemocytes are present at the thoraco-abdominal ostia is in contrast to findings made in *Drosophila melanogaster*, where hemocytes and pathogens are aggregated—in high numbers—at the conical chamber (Elrod-Erickson et al., 2000; Horn et al., 2014; Ghosh et al., 2015). However, there are two clear distinctions between mosquitoes and fruit flies. The first distinction is that the conical chamber of mosquitoes has only one ostial pair—the thoraco-abdominal ostia—whereas the conical chamber of fruit flies has two ostial pairs—the thoraco-abdominal ostia and the first abdominal ostial pair (Wasserthal, 2007; Glenn et al., 2010; Sigle and Hillyer, 2018b). That posteriormost ostial pair of the conical chamber of fruit flies is functionally similar to the ostial pair of the second abdominal segment of mosquitoes—which is a location where periostial hemocytes aggregate. The second distinction is that a region surrounding the conical chamber of fruit flies serves as a hematopoietic hub (Ghosh et al., 2015). Such a hub has not been described in mosquitoes. Instead, mosquito hemocytes have been found to divide while in circulation, although it is possible that they replicate at a yet to be described sessile location (Christensen et al., 1989; Castillo et al., 2011; King and Hillyer, 2013; Bryant and Michel, 2014).

We also found that hemocytes are present at the posterior openings of the heart, but again, they are few in number and they do not aggregate at this location in response to infection. This is in contrast to what occurs in mosquito larvae (League and Hillyer, 2016), and some lepidopteran larvae (Locke, 1997), where numerous hemocytes exist attached to the tracheal tufts that surround the posterior of the heart. At least in mosquitoes, the reason for this difference pertains to changes in circulatory physiology that occur during development (League et al., 2015). That is, the adult heart contracts bidirectionally and the posterior openings have excurrent function whereas the larval heart only contracts anterograde and the posterior openings have incurrent function. In that sense, the posterior of the larval heart is functionally analogous to the abdominal ostia of adults. Thus, our findings at the posterior of the adult heart were not surprising, especially because hemocytes do not aggregate at the anterior end of the mosquito aorta (Sigle and Hillyer, 2018b), and experiments in fruit flies have not detected the aggregation of hemocytes or pathogens on the posterior of the heart (Horn et al., 2014; Ghosh et al., 2015).

Intravital video imaging of hemocytes revealed that their movement near the posterior excurrent openings is dynamic. Hemocytes can bind and detach from this location, indicating that hemocytes can readily change from circulating to sessile states and vice versa. We have previously detected similar attachment and detachment of hemocytes in the periostial regions of adult mosquitoes (Sigle and Hillyer, 2016). Furthermore, in addition to immunity, hemocytes are critical in development and wound healing (Krautz et al., 2014; Wood and Martin, 2017). In these instances, cells actively migrate to their sites of action (Wood et al., 2006; Babcock et al., 2008), and this migration is not visually dissimilar to the migration of mosquito hemocytes. Although the molecular basis of hemocyte migration has received significant attention in the *Drosophila* system (Evans and Wood, 2014), such information is unknown for mosquitoes. Significant strides have been made to uncover the transcriptome and proteome of mosquito hemocytes (Bartholomay et al., 2004; Baton et al., 2009; Pinto et al., 2009; Smith et al., 2016; Thomas et al., 2016; He et al., 2017; Severo et al., 2018), and we recently revealed that Nimrod family genes are involved in periostial hemocyte aggregation, but the relative roles of these genes—specifically *eater* and *draper*—in hemocyte migration requires further study (Sigle and Hillyer, 2018a). Further studies should seek to elucidate the relative contributions of the two major hemocyte populations—the phagocytic granulocytes and the melanizing oenocytoids (Hillyer and Strand, 2014)—on the immune responses that take place on the surface of the heart.

Mosquitoes transmit disease-causing pathogens. Many of these pathogens, such as *Plasmodium* sp., circulate with the hemolymph prior to invading their target organ: the salivary glands (Hillyer et al., 2007; Douglas et al., 2015). Hemocytes and hemocyte-derived factors attack these parasites while in the hemocoel, including at the periostial regions (Clayton et al., 2014; Hillyer and Strand, 2014; Severo and Levashina, 2014; Bartholomay and Michel, 2018). In this study we assessed hemocyte activity at two structures important for intracardiac retrograde hemolymph flow and found that infection does not induce the aggregation of hemocytes at the thoraco-abdominal ostia or the posterior excurrent openings of the heart. Together with data assessing immunity at the aorta and the periostial regions of the heart (King and Hillyer, 2012; Sigle and Hillyer, 2016, 2018b), these data show that the primary sites of immune activity on the dorsal vessel of adult mosquitoes are the incurrent structures involved in intracardiac anterograde hemolymph flow.

AUTHOR CONTRIBUTIONS

LS and JH conceived and designed the study. LS conducted the experiments. LS and JH analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Bacillus thuringiensis Suppresses the Humoral Immune System to Overcome Defense Mechanism of *Plutella xylostella*

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Background: *Plutella xylostella* has become a notorious pest of cruciferous crops all over the world. Delta-endotoxins of *Bacillus thuringiensis* are widely used insecticidal proteins for controlling *P. xylostella*. However, the interaction mechanism of *B. thuringiensis* with the immune system of *P. xylostella*, at the genomic level, is still unclear. This study explored the immune response of *P. xylostella* to *B. thuringiensis*, at different time intervals, 6 h, 12 h, 18 h, 24 h, and 36 h, by using RNA-Sequencing (RNA-Seq) and RT-qPCR.

Results: In total, 167 immunity-related genes were identified and placed into different families, including pattern recognition receptors (PRRs), signal modulators, immune pathways (Toll, IMD, and JAK/STAT), and immune effectors. It is worth mentioning that the analyses of the differentially expressed immunity-related genes revealed that most of the differentially expressed genes (DEGs) (87, 56, 76, 67, and 73 genes) were downregulated in *P. xylostella* following *B. thuringiensis* oral infection at 6 h, 12 h, 18 h, 24 h, and 36 h. Interestingly, our RNA-Seq analysis also revealed reduced expression of antimicrobial peptides, that play a vital role in the humoral immune system of *P. xylostella*.

Conclusion: This study demonstrates that *B. thuringiensis* plays a novel role in controlling *P. xylostella*, by suppressing the immune system.

Keywords: *Plutella xylostella*, *Bacillus thuringiensis*, insect immunity, transcriptome, digital gene expression profiling, antimicrobial peptides

INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.), (Lepidoptera: Plutellidae), is the main pest of cruciferous crops and is the most widely distributed of all lepidopteran pests (Talekar and Shelton, 1993). The annual management cost of DBM has reached approximately US$ 4–5 billion worldwide (Tabashnik et al., 1990; Zalucki et al., 2012). Despite the availability of modern integrated pest management approaches (Furlong et al., 2008; Grzywacz et al., 2010), most of the *Brassica* crops are treated prophylactically with insecticides (Grzywacz et al., 2010). However, the extensive use of broad-spectrum insecticides against DBM promotes the selection of insecticide

resistance (Li et al., 2012; Mohan and Gujar, 2003), destroys natural enemies (Furlong et al., 2004), and pollutes the environment (Shakeel et al., 2017a). To reduce the harmful effects of insecticides, alternative control strategies have been suggested (Liu et al., 2001; Sutherland et al., 2002; Rubilar et al., 2007; Hussain et al., 2009; Diez et al., 2012), including biopesticides like *Bacillus thuringiensis*.

B. thuringiensis, a spore-forming Gram-positive bacterium, present in soil, leaf litter, and the microflora on the surface of leaves, is widespread in nature (Aptosoglou et al., 1997). *B. thuringiensis* produces many kinds of insecticidal crystal proteins, including proteins which are toxic to lepidopterans, and are encoded by crystal (*cry*) and cytolytic (*cyt*) genes (Crickmore et al., 1998). Cry proteins have not only been used in formulated sprays but also expressed in transgenic plants to protect them from insect attacks (Shelton et al., 2002). Cry toxins, unlike most chemical insecticides, have a distinct mode of action that involves toxin solubilization, proteolytic activation in the midgut of the insect, and binding to larval midgut proteins. In the pore formation model, toxin binding results in the formation of pores in membranes and the lysis of cells in the midgut ultimately, resulting in the death of the insect (Pigott and Ellar, 2007). Until now, insects have developed several resistance mechanisms to *B. thuringiensis* toxins, including the modification of the receptor site, alteration of binding ability and proteolysis of protoxin and/or toxin, and an elevated immune status (Pardo-Lopez et al., 2012; Zhu et al., 2016).

Like many insects, *P. xylostella* opposes microbial invaders by mounting well-adjusted immune responses. Insects, unlike their mammalian counterparts, solely rely on innate immunity, which is divided into cellular and humoral immune responses (Hoffmann, 2003). The cellular innate immune response is mediated by strong phagocytic activities of plasmatocytes while melanin synthesis, clotting, and the production of antimicrobial peptides mediated by fat body are collectively known as humoral innate immunity (Hoffmann, 2003). Although our knowledge of insect-pathogen interaction (fungi as a pathogen) has increased in recent years (Shakeel et al., 2017c; Xu et al., 2017), there are only a few reports on the interaction between *B. thuringiensis* and insects (Grizanova et al., 2014; Contreras et al., 2015); for example, when *Galleria mellonella* was infected by the oral administration of *B. thuringiensis*, an elevated immune response was observed, indicating an increased immune resistance to *B. thuringiensis* in *G. mellonella* (Grizanova et al., 2014).

Keeping the importance of insect-pathogen interaction in mind to understand the innate immune response of *P. xylostella* to *B. thuringiensis*, we investigated whether *B. thuringiensis* has the ability to suppress the immune system of *P. xylostella*. To address this question, fourth instar larvae of *P. xylostella* were fed *B. thuringiensis* at five different time points (6 h, 12 h, 18 h, 24 h, and 36 h) with a control using high-throughput Illumina sequencing and real-time quantitative PCR (RT-qPCR) techniques at the genomic level. Our findings reveal that *B. thuringiensis* has the ability to overcome the immune defense mechanism mounted by *P. xylostella* by suppressing the humoral immune system.

MATERIALS AND METHODS

Insect Rearing and *B. thuringiensis* Preparation

A susceptible population of *P. xylostella* was obtained from the Engineering Research Center of Biological Control, Ministry of Education, South China Agricultural University, China and kept in an insecticide-free environment for 10 generations. Adults were fed 10% honey solution, and larvae were reared on Chinese broccoli. All populations were maintained at 25 ± 1°C under a photoperiod of 16: 8 h (light: dark) and 65% relative humidity. The highly pathogenic *B. thuringiensis* HD-73 strain was kindly provided by Mr. Zhang Jie of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Bacteria from a glycerol stock (stored at −80°C) were plated on Luria-Bertani (LB) agar and grown overnight. Later, the bacterial suspension was inoculated into fresh LB medium with a proportion of 1:100, incubated for 12 h at 30°C, and finally the bacteria were centrifuged and subsequently resuspended in phosphate buffered saline (PBS). The spores were counted using a Thoma counting chamber (0.02 mm depth) and immediately used for feeding.

Feeding *P. xylostella* Larvae With *B. thuringiensis* and RNA Sample Preparation

The lethal concentration 50 (LC_{50}) dose of bacteria was determined in a pilot experiment, in which fourth instar larvae of *P. xylostella* were selected to be fed on nine increasing doses of *B. thuringiensis* treated leaf discs (by leaf dip bioassay method). The dose that came closest to killing 50% of the larvae (1.0×10^8/ml) within 36 h was then selected.

The selected LC_{50} was used for the experiments. All the larvae used for the feeding experiment were starved for 2 h prior to feeding and then exposed to *B. thuringiensis* treated leaf discs at 6 h, 12 h, 18 h, 24 h, and 36 h. Larvae treated with PBS were used as a control. Twenty larvae were used in each treatment, and the whole body of the surviving larvae that ate (as determined by observing the food bites) treated leaves were used to extract RNA. Experiments were carried out in triplicate.

The Trizol Total RNA Isolation Kit (Takara, Japan) was used to extract RNA from the whole body of *P. xylostella,* following the manufacturer's protocol. To determine the concentration and integrity of RNA, Nanodrop (Bio-Rad, United States) and Agilent 2100 Bioanalyzer (Agilent, United States) were used.

cDNA Library Preparation and Illumina Sequencing

A total of six libraries (6 h, 12 h, 18 h, 24 h, 36 h, and control) were created by the Illumina Gene Expression Sample Prep Kit (Illumina, San Diego, CA, United States). 10 μg of total RNA, extracted from each treatment and control, was incubated with oligo (dT) magnetic beads for the isolation of the polyadenylated RNA fraction. To synthesize first- and second-strand cDNAs, random hexamers and RNase H and DNA polymerase I were used. The double stranded cDNA was purified with magnetic

beads following ligation of fragments with sequencing adaptors enriched by PCR amplification. Finally, to qualify and quantify the sample libraries, Agilent 2100 Bioanalyzer and ABI Step One Plus Real-Time PCR System were used following the sequencing on the Illumina HiSeq^{TM}2000 system (Illumina, United States). Illumina sequencing was performed at the Beijing Genomics Institute (BGI-Shenzhen, China).

Genome Mapping and Analysis of Differentially Expressed Genes

The filtration process was carried out to remove raw reads with adapters and unknown bases >10%. After filtration, Bowtie and HISAT (Kim et al., 2015) were used to map the remaining clean reads with the reference gene and reference genome. Finally, all data were normalized as fragments per kilobase of transcript per million fragments mapped (FPKM). Differential expression analysis was carried out by a strict approach, and the threshold *p*-value was determined by using the false discovery rate (FDR) methodology for analyzing multiple tests (Kim and van de Wiel, 2008). A standard threshold (FDR <0.001 and log2 ratio ≥1) was set to identify significantly differentially expressed genes (DEGs) in the libraries. The number of differentially expressed immunity-related genes and the ratio of pairwise comparison of all the treatments with the control are represented in **Figure 1**.

Identification and Hierarchical Clustering of Differentially Expressed Immunity-Related Genes

To identify *P. xylostella* immunity-related genes, the BLASTX algorithm search was compared against the Nr database using a cutoff *E*-value of 0.1. The potential candidates of *P. xylostella* immunity-related genes were confirmed by aligning the available immunity-related gene sequences to other model insect species. The reference insect species included *Danaus plexippus*, *Ostrinia nubilalis*, *Bombyx mori*, *Spodoptera frugiperda*, and *Manduca sexta*. Hierarchical clustering of differentially expressed immunity-related genes was performed using the pheatmap package in an R environment.

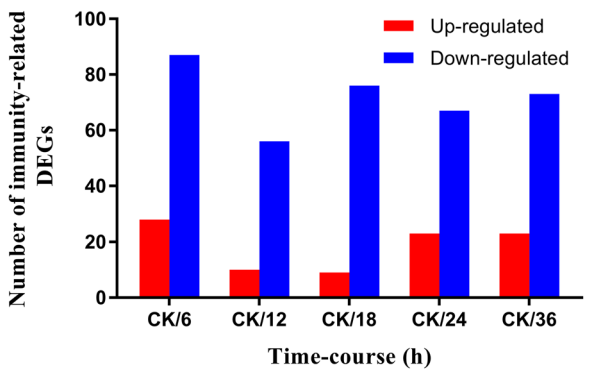

FIGURE 1 | Screening of immunity-related DEGs of *Plutella xylostella* in response to *Bacillus thuringiensis* at 6 h, 12 h, 18 h, 24 h, and 36 h postinfection. CK denotes control. *Y*-axis indicates the number of differentially expressed immunity-related genes, and *X*-axis shows the ratio of pairwise comparison of all the treatments with the control.

Functional Analysis of Differentially Expressed Immunity-Related Genes

Plutella xylostella genome (GCA_000330985.1) was set as the background to identify significantly enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways within the DEGs dataset using a hypergeometric test and a corrected *P*-value of ≤0.05 as the threshold.

Validation of DEG Libraries by RT-qPCR

Real-time quantitative PCR is the method of choice for analyzing the expression of genes and to confirm the results of RNA-Sequencing (RNA-Seq) (Shakeel et al., 2017b). The mRNA expression patterns of control vs. treatment groups were validated by RT-qPCR, by randomly choosing 15 immunity-related DEGs. The total RNA was isolated from the fourth instar larvae, following the same method as described earlier. First-strand cDNA (1 μg) was prepared using M-MLV reverse transcriptase (Promega, United States), following the instruction manual. RT-qPCR was carried out on a Bio-Rad iQ2 optical system (Bio-Rad) using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, United States), following the instruction manual. Purity of the PCR products was confirmed by generating a dissociation curve from 65°C to 95°C (Shakeel et al., 2015) with the following PCR conditions: 95°C for 30 s, 40 cycles at 95°C for 5 s, and 55°C for 10 s. Ribosomal protein S13 (RPS13) was used as an internal control for normalization (Fu et al., 2013), and the relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Each treatment included three replicates, and each reaction was run in triplicate. Primers used for RT-qPCR were designed by Primer Premier 5 (**Table 1**).

RESULTS

Summary of Illumina Sequencing and Mapping to Reference Genome

A total of 11,930,289, 12,243,539, 12,207,944, 11,905,098, 12,375,994, and 11,709,506 clean reads were generated, after filtering out adapter sequences and low-quality reads (tags with the unknown nucleotide "N"), from the six libraries (6 h, 12 h, 18 h, 24 h, 36 h, and the control). Among the data of the six clean read libraries, 75.36% to 78.22% of the clean reads were successfully mapped to the reference genome (**Table 2**).

Dynamics of *B. thuringiensis*-Responsive Immunity-Related DEGs

A differential gene expression analysis was carried out to identify the variations in gene expression patterns between the control (PBS-treatment) and the treated groups (*B. thuringiensis*-infected) at different time points (6 h, 12 h, 18 h, 24 h, and 36 h). Our results showed that, compared to the control, there were 115 [28 (24.35%) upregulated and 87 (75.65%)

TABLE 1 | Primers used in RT-qPCR verification for immunity-related genes.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
Trypsin12	CCAGCCAGCGTCCATCAATGC	ACTCGGCGTTGTTCACTGTGTATAG	146
Serpin1	TTCAGGCAAGGACTCAAGTAATAGACG	TTCTTCTACGCCATTCTTCATCAGGAC	133
PP01	CAACTATGGCTTCTCTGTGGCTCTG	GAGAACACTTGCGAGTCCAGGAAC	111
Defensin	ACAGGAGACAGTGGTTGAGGAGTC	TTGTATCTTCAGTGGCGTCTTCGTAC	98
cd-SP1	GCCAGGAGCTTCGAGAACTACAC	TTGGAGGTGCGATGCTGATGTG	84
Cecropin5	TCTGCTGCGCCTAGGTGGAAG	CGCTGGACCTGCCTTGATGATG	87
PPO5	GATGATGGTGGTGAAGATGGTGACTAC	TCCGATCAGAGCCAGACGAAGAC	106
PGRP1	GTTCATCTCAGTAACGCAACACATCAC	AGACAGCAGGCGACCAGGAG	150
SP23	GGCTCGCTACCAGAACATCAGAATG	CCACGATGAGACTCCAATGACCAC	145
Integrin7	AGTGCGACGGACTCAAGGTAGG	GAACTGCTGCCGCCACTCAC	98
βGBP8	CATCTCAGTCAGCACGGCATCAG	CGGCTCATCAGGCATCATAATCTCC	130
Lysozyme	AGTTGATAACTGACGACATCACGAAGG	CCATCCATACCAGGCGTTGAAGC	86
C-Jun2	AGCCTACCTTCTATGACGAGCAGTAC	AAGTCCAGGTCCAGCGTGAGG	82
Catalase2	TCAGAACATCACCAACAACCAGGAAG	CTTAGTGTAGAAGACGGTCGCTTGAG	184
PGRP6	CTTACGGCTACAACAGGAAGTCTATCG	TCTCACTCCACACTTCAGCAATGC	116
RPS13	TCAGGCTTATTCTCGTCG	GCTGTGCTGGATTCGTAC	100

TABLE 2 | DGE sequencing statistics.

Sample	Clean reads	Total mapped clean reads (%)
6 h	11,930,289	75.36
12 h	12,243,539	77.79
18 h	12,207,944	78.12
24 h	11,905,098	77.68
36 h	12,375,994	77.11
Control	11,709,506	78.22

downregulated], 66 [10 (15.15%) upregulated and 56 (84.85%) downregulated], 85 [9 (10.59%) upregulated and 76 (89.41%) downregulated], 90 [23 (25.56%) upregulated and 67 (74.44%) downregulated], and 96 [23 (23.96%) upregulated and 73 (76.04%) downregulated] immunity-related genes that were significantly altered in *P. xylostella* after 6 h, 12 h, 18 h, 24 h, and 36 h, respectively (**Figure 1**). A Venn diagram analysis showed that only 25 genes of these immunity-related DEGs were commonly expressed among all the treatments, whereas 17, 7, 3, 6, and 6 immunity-related genes were specifically expressed at 6 h, 12 h, 18 h, 24 h, and 36 h, respectively (**Figure 2**).

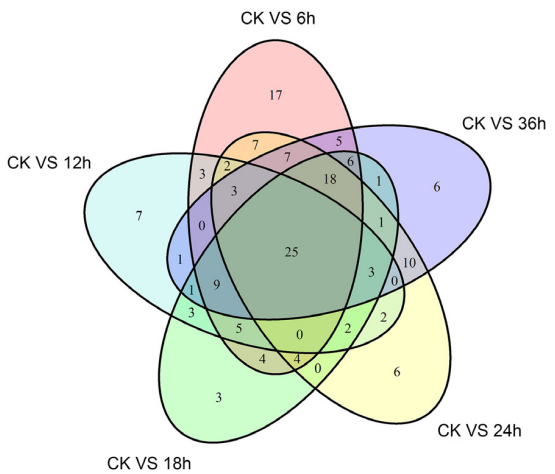

FIGURE 2 | A Venn diagram of immunity-related differentially expressed genes (DEGs) in *P. xylostella* at 6 h, 12 h, 18 h, 24 h, and 36 h postinfection. The numbers in each circle show immunity-related differentially expressed genes in each comparison treatment, and the overlapping regions display genes that are commonly expressed among the comparison treatments.

Identification and Functional Analysis of *B. thuringiensis*-Responsive Immunity-Related Genes

A comprehensive analysis was carried out to identify *B. thuringiensis*-responsive immunity-related genes in *P. xylostella* by BLAST searches against the non-redundant sequence database and by combining GO and KEGG annotation results. To get more reliable results, genes annotated as hypothetical or unknown proteins were filtered out. Finally, the identified immunity-related genes (167) were classified into different groups, including signal recognition, signal modulation, signal transduction, effectors, and other immune molecules (**Figure 3** and **Supplementary Table S1**).

To further analyze the functions of all significantly differentially expressed immunity-related genes in their corresponding pathways, GO enrichment and KEGG pathway analyses were performed. Our results showed that catalytic activity (32.37%) was the most enriched term following response to stimulus (19.12%), biological regulation (10.74%), regulation of biological process (9.71%), and metabolic process (8.50%) (**Figure 4**), whereas the digestive system (57.42%), signal molecules and interaction (36.42%), viral infectious diseases (31.94%), parasitic infectious diseases (20.23%), and signal transduction (18.16%) were identified as highly enriched categories by the KEGG pathway enrichment analysis (**Figure 5**).

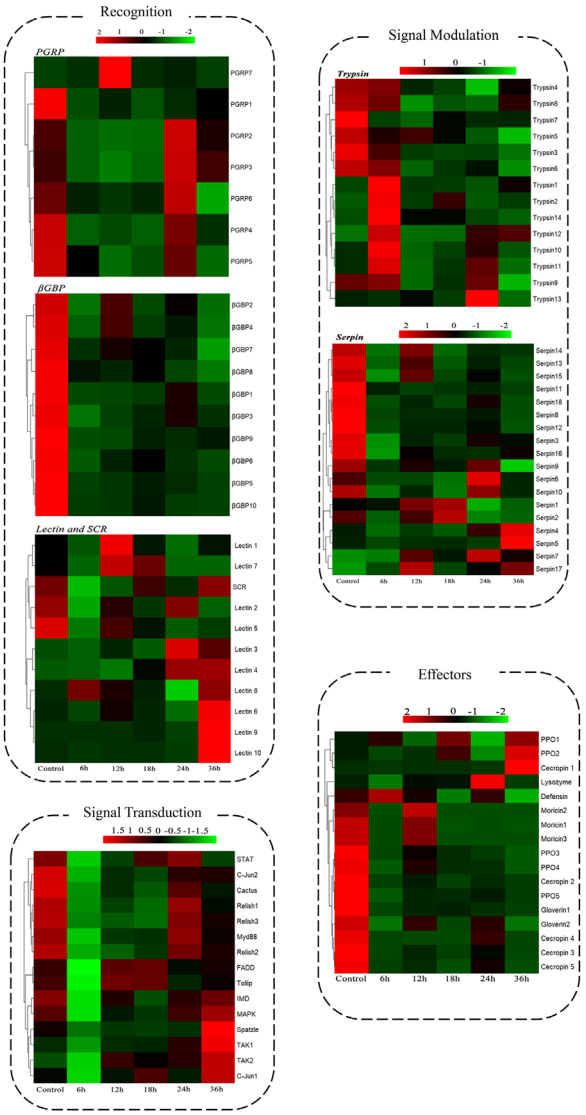

FIGURE 3 | Expression patterns of the immunity-related genes of *P. xylostella* expressed in response to *B. thuringiensis* challenge. Cluster analysis expression profiles of immunity-related genes data are organized into four groups: recognition, signal modulation, signal transduction, and effectors. Six datasets were included: 6 h, 12 h, 18 h, 24 h, and 36 h postinfection with *B. thuringiensis* and one control (6 h after PBS treatment). Gene families and functional pathways (Toll, IMD) are categorized within the group. Gene names are shown on the right side, Gene ID and details are shown in the **Supplementary Table S1**.

Genes Involved in Microbial Recognition

In the signal recognition group of this study, most of the peptidoglycan recognition proteins (PGRPs), β-glucan-binding proteins (β-GBPs), and scavenger receptors were downregulated in response to *B. thuringiensis*; for example, PGRP4 (px-105386207), β-GBP2 (px-105389999), and β-GBP10 (px-105380182) showed a downregulated expression of 9.90-fold, 9.17-fold, and 10.01-fold, 6 h postinfection. However, a few members of the lectin family exhibited upregulated expression at 36 h with lectin6 (px-105383612), lectin9 (px-105392416), and lectin10 (px-105398492) upregulated by 3.97-fold, 12.69-fold, and 18.99-fold, respectively, postinfection.

Genes Involved in Signal Modulation

In this study, 67 serine protease genes showed a significant difference in expression in response to *B. thuringiensis* in the signal modulation group, including 45 serine proteases, 2 clip-domain serine proteinases, 14 trypsin-like serine proteinases, and 6 chymotrypsin-like serine proteinases. Among these serine proteases, a mixed response of upregulation and downregulation in expression after *B. thuringiensis* infection was observed at all time courses.

In this study, 18 serine protease inhibitors (serpins) were identified, and most of them were downregulated after *B. thuringiensis* infection at different time courses with serpin18 (px_105383822) significantly downregulated by 4-fold when compared with the control; however, a few serpins like serpin7 (px_105383829) and serpin5 (px_105387001) were upregulated by 2.4-fold and 2.26-fold, respectively.

Genes Involved in Immune Signaling Pathways

In this study, components of Toll pathway such as Spätzle, Myd88, cactus, and toll-interacting protein showed a downregulated expression in response to *B. thuringiensis* with Spätzle showing an 8-fold downregulation at the early stage of infection 6 h postinfection, compared to the control. A similar downregulated expression pattern was observed in the components of IMD (FADD, TAK1, TAK2, and relish), JNK (C-jun1 and C-jun2), and JAK-STAT (STAT) pathways.

Genes Involved in Immune Effector Families

Intriguingly, among immune effectors, a significantly downregulated expression of antimicrobial peptides, such as cecropins, moricins, and gloverins, was observed after *B. thuringiensis* infection, at different time courses (**Figure 3** and **Supplementary Table S1**). A gradual variation in the expression of gloverins was observed at different time courses with gloverin1 (px-105389810) downregulated by 4.9-fold at 6 h, 3.08-fold at 12 h, 2.8-fold at 18 h, 3.3-fold at 24 h, and 4.1-fold at 36 h. Similarly, cecropin1 and moricin2 were significantly downregulated by 10-fold and 7-fold, respectively, at 6 h postinfection of *B. thuringiensis*.

RT-qPCR Validation of Immunity-Related DEGs

To verify the changes in FPKM values between different samples, expression levels of 15 DEGs were selected and verified by RT-qPCR. Consistent with the DGE sequencing data, RT-qPCR results of the 15 randomly selected genes showed a similar pattern of expression in the six libraries (**Figure 6**), which further confirmed that our sequencing data were reliable.

DISCUSSION

The use of *B. thuringiensis* based bioinsecticides is considered a promising biological alternative to control insect pests. A wide range of insecticidal proteins, with wide pathogenicity, produced

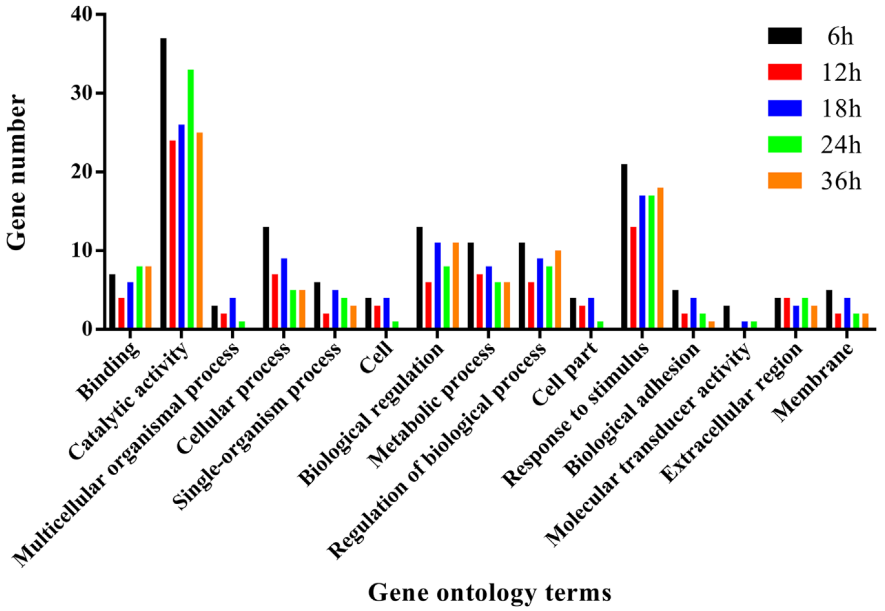

FIGURE 4 | Summary of gene ontology annotations. Functional classification of immunity-related DEGs in *P. xylostella* at 6 h, 12 h, 18 h, 24 h, and 36 h postinfection using gene ontology terms.

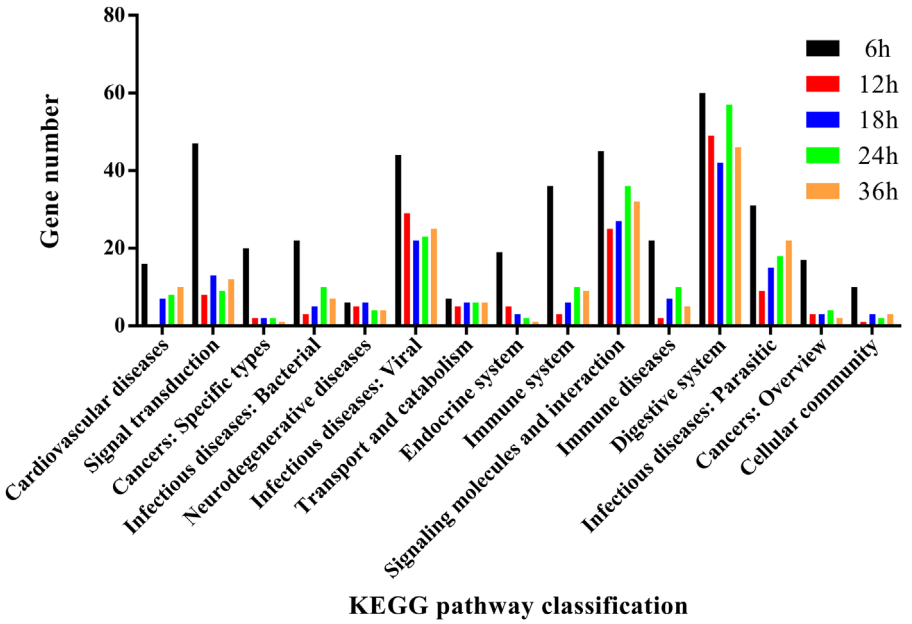

FIGURE 5 | KEGG pathway annotation classification of immunity-related genes in *P. xylostella* fed with *B. thuringiensis* at 6 h, 12 h, 18 h, 24 h, and 36 h. The abscissa is the KEGG classification, and the ordinate left is the gene number.

by this bacterium are active against numerous pest species (van Frankenhuyzen, 2013). Presently, among the toxins produced by this bacterium, Cry toxins are the most popular biocontrol agents (Lacey et al., 2001). The pathogenesis mechanism of *B. thuringiensis* against insects is a complex process that involves several factors as well as the activation of immune responses of insects to combat infection, which is also considered as a factor contributing to tolerance against *B. thuringiensis* (Contreras et al., 2015). To better understand whether the larval immune response helps the insect to reduce the damage produced by *B. thuringiensis* (HD-73) or to overcome the immune defense mechanism mounted by the insect, we aimed to conduct a genome-wide transcriptional profiling of *P. xylostella* challenged with *B. thuringiensis* at different time courses (6 h, 12 h, 18 h,

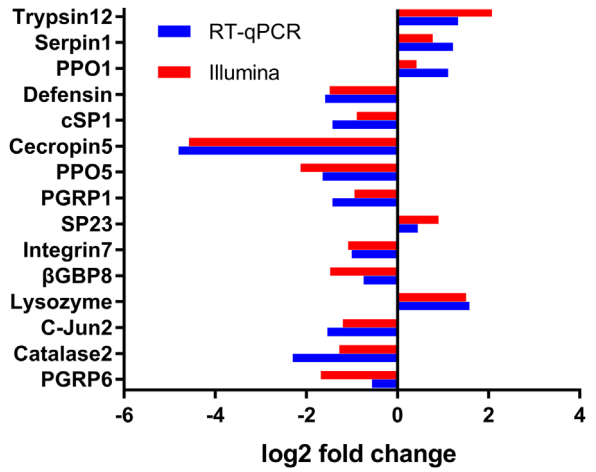

FIGURE 6 | Validation of the differential expression ratio (log2) achieved by RT-qPCR and RNA-Seq for immunity-related genes. PGRP6, Peptidoglycan Recognition Protein (Px_105391041); Catalase2, Catalase (Px_105390515); c-Jun2, c-Jun(Px_105388607); Lysozyme, Lysozyme (Px_105382813); β-GBP8, Beta-1,3-glucan-binding protein (Px_105380183); Integrin7, Integrin (Px_105381998); SP23, Serine Protease (Px_105388679); PGRP1, Peptidoglycan Recognition Protein (Px_105388777); PPO5, Prophenoloxidase (Px_105393465); Cecropin5, Cecropin (Px_105394858); cd-SP1, clip domain serine protease (105395144); Defensin, Defensin (Px_105380306); PPO1, Prophenoloxidase (Px_105386870); Serpin1, Serpin (Px_105392292); Trypsin12, Trypsin-like serine protease (Px_105383571).

24 h, and 36 h), using high-throughput RNA-Seq and DGE analysis.

Dynamics of immunity-related genes exhibited that most of the genes were downregulated following *B. thuringiensis* infection. Our results are in accordance with previous reports, which indicated that the number of downregulated immune related genes was higher, compared to upregulated genes in *P. xylostella* and *Bemisia tabaci* following *Isaria fumosorosea* and *Eretmocerus mundus* infection (Mahadav et al., 2008; Xu et al., 2017).

Recognition of pathogens mediated by pattern recognition molecules is the initial process of defense against intruders, eliciting the innate immune response of insects (Shakeel et al., 2017c). Until now, a variety of pattern recognition molecules have been identified, including PGRPs, β-GBPs, hemolin, scavenger receptors, and lectins (Hultmark, 2003).

In the signal recognition group of this study, most of the PGRPs, β-GBPs, and scavenger receptors were downregulated, except for a few members of the lectin family that were upregulated in response to *B. thuringiensis*. As previously demonstrated, Vip3Aa (a *B. thuringiensis* toxin) also reduced the expression of PGRPs in *Spodoptera litura*, however, contrary to our findings, an increased expression of other pattern recognition molecules was observed (Song et al., 2016). A similar trend of downregulated expression of PGRPs was observed in *P. xylostella* and *Drosophila melanogaster* following *I. fumosorosea* infection and destruxin injection (Pal et al., 2007; Xu et al., 2017). Our results suggest that PRRs like PGRPs, GNBPs, and scavenger receptors may be the target of *B. thuringiensis*, and lectins are responsible for the activation of the immune response of *P. xylostella* to *B. thuringiensis*.

Serine proteases, crucial proteolytic enzymes, play a significant role in a wide range of physiological processes, including digestion, signal transduction, and invertebrate defense responses (Ross et al., 2003). Serine proteases perform the catalytic function through the action of a catalytic triad, which is composed of His, Asp, and Ser amino acid residues (Perona and Craik, 1995). In general, serine proteases exist in the inactive pro-enzyme form and are activated by specific proteolytic cleavage (Ross et al., 2003). In our study, a mixed response of upregulated and downregulated expression of serine proteases was observed after *B. thuringiensis* infection over the time course. Notably, most of the upregulated serine proteases showed a very high expression at the initial stage of infection (6 h). A drastic variation in the gene expression after *B. thuringiensis* feeding may suggest the involvement of candidate genes in the process of protoxin activation or degradation.

Serpins are considered the most effective molecules to inactivate serine proteases when they are no longer in need. Serpins are widely reported inhibitors with documented roles in insect digestion, development, metamorphosis, and are also considered important components of the immune system (Ross et al., 2003). In this study, serpins showed downregulated expression after *B. thuringiensis* infection at different time courses. Consistent with our report, most of the serpins were downregulated when exposed to *I. fumosorosea* infection (Xu et al., 2017), however, an upregulated expression pattern of serpins was observed when *Spodoptera exigua* was challenged with the Vip3Aa toxin of *B. thuringiensis* (Bel et al., 2013).

Signal transduction pathways play a vital role in the proper functioning of the immune system in insects, as they are involved in amplifying the immune response signals and inducing antimicrobial activity (Hillyer, 2016). The signal transduction pathways include Toll, IMD, JNK, and JAK-STAT pathways. It is worth mentioning that in this study, components of all these pathways were identified, and bacterial challenges also resulted in the suppression of immune signal transduction pathways. Components of the Toll pathway such as Spätzle, Myd88, cactus, and toll-interacting protein showed a downregulated expression in response to *B. thuringiensis*. Contrary to our findings, neither the toll-like receptor gene nor the IMD gene was found to be regulated when *S. exigua* and *S. litura* were treated with the Vip3Aa toxin of *B. thuringiensis* (Bel et al., 2013; Hernández-Martínez et al., 2017).

Antimicrobial peptides, evolutionarily conserved short immunity-related proteins, play a significant role in the insect immune system by acting against a wide range of pathogens, including bacteria, viruses, fungi, or parasites (Bulet et al., 2004).

In insects, antimicrobial peptides are induced in specific tissues such as the hemocytes or body fat. In the current era, antimicrobial peptides could become a valuable alternative

to conventional antibiotics, to reduce antimicrobial resistance, as they show a different mechanism of action, when compared with antibiotics (Hancock and Sahl, 2006; Vale et al., 2014).

It is worth mentioning that a significantly downregulated expression of antimicrobial peptides was observed following *B. thuringiensis* infection. Contrary to our findings, an increase in the expression of antimicrobial peptides was observed in *Trichoplusia ni* exposed to *B. thuringiensis* (Tamez-Guerra et al., 2008). However, there are a few reports of reduced expression of antimicrobial peptides in response to pathogens and parasites in *P. xylostella*, *D. melanogaster*, *Locusta migratoria*, *Helicoverpa armigera*, and *Meligethes aeneus* (Pal et al., 2007; Xu et al., 2017). Similar to our results, the expression of antimicrobial peptides such as lysozyme was reduced in *P. xylostella* and *L. migratoria* following fungal infection (Zhang et al., 2015; Xu et al., 2017). Moreover, the expression of antimicrobial peptides like moricin and gloverin was also suppressed in the hemocytes of *H. armigera* after *Escherichia coli* infection (Xiong et al., 2015). The suppression of immune response, especially the reduction in the expression of antimicrobial peptides in the host by entomopathogenic fungi and bacteria, in both previous reports and in this study, would have obvious benefits for the success of pathogenic fungi and bacteria. The reason for the reduced expression of antimicrobial peptides in this study might be the release of other toxins and secondary metabolites along with the main Cry1Ac toxin produced by *B. thuringiensis*, to overcome the immune system of *P. xylostella*. Thus, the ability to reduce the production of antimicrobial peptides is likely to aid fungal and bacterial survival in a variety of insect hosts.

CONCLUSION

In conclusion, this study has addressed the response of the immune system of *P. xylostella* to *B. thuringiensis* exposure at different time points. *B. thuringiensis* infection led to a marked reduction in the response of the immune system of *P. xylostella* as the number of downregulated immune genes was higher at all time points, compared with upregulated genes. In the light of our findings, we speculate that *B. thuringiensis* might have released several other toxins and secondary metabolites along with the main Cry1Ac toxin, in order to overcome the immune system of *P. xylostella*. However, a series of functional validation experiments are to be performed to evaluate the immunity-related genes identified in this study.

AUTHOR CONTRIBUTIONS

FJ and XX conceived and designed the experiments. SL, XX, and ZZ performed the experiments. QZ, JZ, and SL analyzed the data. QZ and JX contributed reagents, materials, and analysis tools. SL, MS, and XX wrote the manuscript. MS, FJ, and XY revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01478/full#supplementary-material

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The *in vivo* dsRNA Cleavage Has Sequence Preference in Insects

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Exogenous dsRNA enters the insect body and can induce the RNAi effect only when it is cleaved into siRNA. However, what kinds of base composition are easier to cut and what kinds of siRNA will be produced *in vivo* is largely unknown. In this study, we found that dsRNA processing into siRNA has sequence preference and regularity in insects. We injected 0.04 mg/g dsRNA into Asian corn borers or cotton bollworms according to their body weight, and then the siRNAs produced *in vivo* were analyzed by RNA-Seq. We discovered that a large number of siRNAs were produced with GGU nucleotide residues at the 5′- and 3′-ends and produced a siRNA peak on the sequence. Once the GGU site is mutated, the number of siRNAs will decrease significantly and the siRNA peak will also lost. However, in the red flour beetle, a member of Coleoptera, dsRNA was cut at more diverse sites, such as AAG, GUG, and GUU; more importantly, these enzyme restriction sites have a high conservation base of A/U. Our discovery regarding dsRNA *in vivo* cleavage preference and regularity will help us understand the RNAi mechanism and its application.

Keywords: insect, RNA interference, *in vivo* dsRNA-processing, siRNA, sequence-specific cleavage

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INTRODUCTION

RNA interference (RNAi) technology is widely used in scientific research as a genetic tool (Boettcher and McManus, 2015; Blake et al., 2017). It is more likely to be used as a new approach in agricultural pest control (Burand and Hunter, 2013; Kim et al., 2015; Joga et al., 2016). RNAi can be triggered by introducing double-stranded RNA (dsRNA), which is processed into effective small interfering RNAs (siRNAs) by the Dicer enzyme. Then, the generated siRNAs are incorporated into the RISC complex with other proteins, enter into the subsequent RNAi pathway, and then cause the gene silencing effect (Fire et al., 1998; Tijsterman and Plasterk, 2004; Winter et al., 2009). Therefore, the Dicer enzyme processing of the dsRNA into siRNAs is the key step in the RNAi pathway; however, it is not clear how dsRNA is recognized and cleavaged by the Dicer enzyme, or what kinds of siRNAs will be produced *in vivo*.

A previous *in vitro* study indicated that the PAZ domain of Dicer is capable of recognizing the 3′-overhang structure and the 5′-phosphate monoester structures of the dsRNA. Dicer selects cleavage sites by measuring a set distance (∼21 nucleotides) from the 3′- or 5′-end to ensure the precise and effective biogenesis of siRNAs. The PAZ domain is crucial for the siRNA production process. Mutations in the PAZ domain can decrease siRNA length fidelity and RNAi silencing activity *in vivo* (Kandasamy and Fukunaga, 2016). The 3′-counting rule (Zhang et al., 2004;

MacRae et al., 2006, 2007) and the 5′-counting rule (Park et al., 2011) have been proposed to explain how Dicer enzymes process dsRNA. In addition to the ends of small hairpin RNAs (shRNAs)/pre-miRNAs, Dicer can recognize the loop/bulge structures for accurate processing. Thus, the loop counting rule has also been proposed (Gu et al., 2012). These results can explain the siRNA's length and the initiating mode when dsRNA is processed by the Dicer enzyme.

In addition, the studies from Vermeulen et al. (2005) indicated that Dicer has sequence preferences when processing dsRNAs. Therefore, Dicer recognizes the preferred nucleotide residues on the dsRNA and then processes them into siRNA. A recent result has confirmed that Dicer-like enzymes have sequence cleavage preferences in *Paramecium* (Hoehener et al., 2018). Besides this, the Mini-III RNase family protein BsMiniIII in *Bacillus subtilis* is capable of cleaving a long dsRNA substrate in an ACCU/AGGU sequence-specific manner (Glow et al., 2015). Different Dicer-like enzymes or Mini-III RNases have different cleavage capacities on nucleotide bases (Glow et al., 2016; Hoehener et al., 2018). These results illustrated that different RNase III protein families have different preference recognition sites. Therefore, we hypothesized that the dsRNA-processing model was based on the RNase III family proteins and may have some regularity. However, our hypothesis is based on the *in vitro* study results. When a dsRNA segment entered an organism in a complex *in vivo* environment, what kinds of siRNA can be processed for the RNAi pathway? And whether its processing mode has sequence preference is largely unknown.

Here, using a high-throughput small RNA sequencing and bioinformatics analysis strategy, we dissected the *in vivo* dsRNA-processing mode. The Asian corn borer (*Ostrinia furnacalis*) and cotton bollworm (*Helicoverpa armigera*) were selected as models of Lepidoptera insects. We discovered the *in vivo* rule of dsRNA processing in insects. dsRNA processing into small RNA is only related to sequence composition and is not related to the sequence length. GGU was the preferred three-nucleotide digestion site in these two Lepidoptera insects. However, in the red flour beetle (*Tribolium castaneum*), a coleopteran insect, the dsRNA was cut at more diverse sites, such as AAG, GUG, and GUU. These results indicated that the dsRNA processing mode is not only related with the sequence composition, but also related to the *in vivo* environment in different organisms. This is probably a major reason for different RNAi efficiencies in different insect species.

MATERIALS AND METHODS

Insect Culturing

The Asian corn borer (*O. furnacalis)* and cotton bollworm (*H. armigera*) eggs were originally obtained from fields in Shanghai, China and reared in the laboratory at 25 ± 1°C and 75% relative humidity under a 14/10 h light/dark photoperiod. The larvae were fed on a modified artificial diet (Wang et al., 2011).

The red flour beetle was obtained from the laboratory of Dr. Ling's at the Key Laboratory of Insect Developmental and Evolutionary Biology at the Shanghai Institute of Plant Physiology and Ecology. They were reared on whole wheat flour containing 5% brewer's yeast at 30°C under a 14/10 h light/dark photoperiod.

dsRNA Preparation

DsRNAs were synthesized using the MEGAscript RNAi Kit (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. T7 promoter sequences were tailed to the 5′ ends of the DNA templates by PCR amplifications. Double-stranded enhanced green fluorescent protein (ds*EGFP*) (GenBank accession no. MF169984) was generated using pPigbacA3EGFP as the template. All the primer sequences are listed in **Supplementary Table 1**. Template DNA and single-stranded RNA were removed from the transcription reaction through DNase and RNase treatments, respectively. dsRNA was purified using MEGAclear columns (Ambion, Austin, TX, United States) and eluted in nuclease free water. dsRNA concentrations were measured using a BioPhotometer (Eppendorf, Hamburg, Germany).

Microinjection and Sample Collection

The fifth-instar larvae of the Asian corn borer (*O. furnacalis*), the third-instar larvae of the cotton bollworm (*H. armigera*), and the fifth-instar larvae of the red flour beetle (*T. castaneum*) were used as experimental materials. Each gram of insect was injected with 0.04 mg dsRNA in the posterior abdominal segment using a capillary needle. Three *O. furnacalis* or *H. armigera* larvae were treated, twelve *T. castaneum* larvae regarded as one treatment were treated, and each treatment was repeated three times. The untreated fifth-instar *O. furnacalis* larvae were regarded as the control group. Four hours after the injections, samples were collected, frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

RNA Isolation and Small RNA Sequencing

Total RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Samples were treated with RNase-free DNaseI (New England BioLabs, Ipswich, MA, United States) for 30 min at 37°C to remove residual DNA prior to small RNA sequencing. Samples were sequenced using an Illumina HiSeq 2000 analyzer at BGI (Shenzhen, China). The sequencing information is listed in **Supplementary Table 2**.

Small RNA Sequencing Analysis

In this research, small RNAs from related treatments were re-mapped onto the dsRNA sequence using local BLASTn (*E*-value $< 10^{-5}$); only one base mismatch was allowed during calculation. The type and number of small RNAs that were processed by dsRNA were calculated and the distribution of small RNAs were subsequently analyzed. The 19–25 nt long small RNAs were used for further analysis. The perl SVG module was used to make a graph, with the x-axis representing the dsRNA sequence and the y-axis representing the depth of sequencing (amount of mapped small RNA).

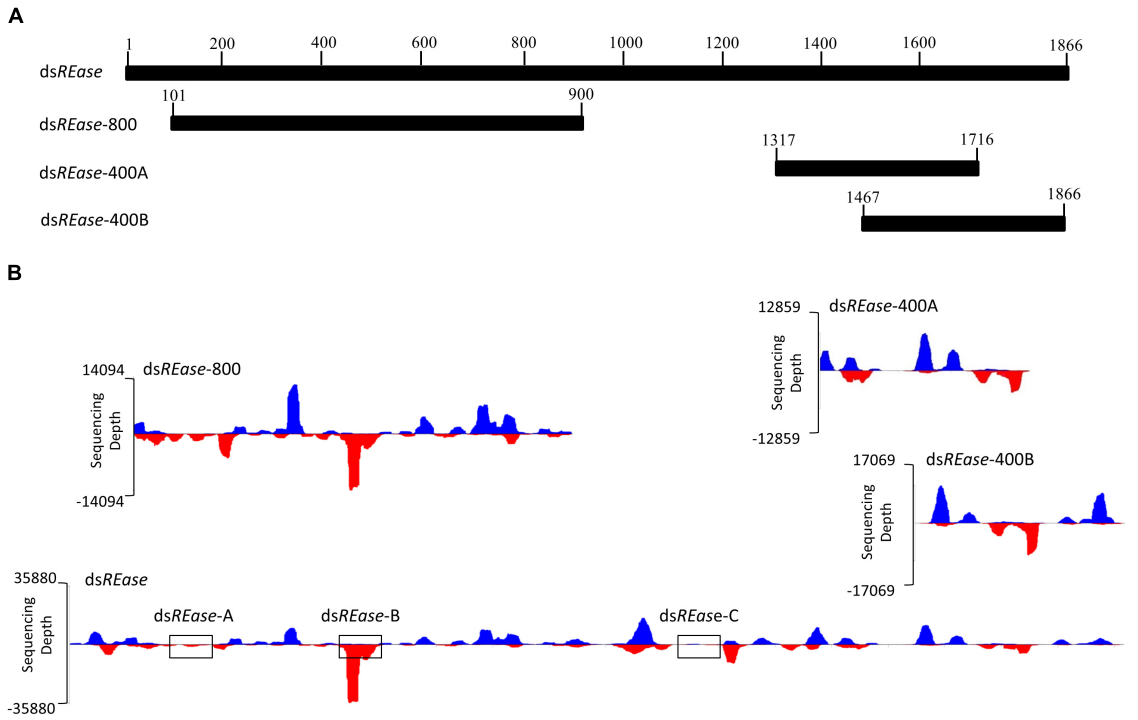

FIGURE 1 | The dsRNA-processing model is closely related to its nucleotide sequence. **(A)** *REase* gene expression levels under different treatments. Fifth instar Asian corn borer larvae were independently injected with 10 μg of ds*REase*, ds*REase*-800, ds*REase*-400A, and ds*REase*-400B. Four hours later, samples were collected and *REase* gene expression levels were determined by qRT-PCR. Compared to the ds*EGFP* treatment, the *REase* gene can be repressed by the four kinds of ds*REase*. **(B)** Processing mode of the four kinds of ds*REase in vivo*. Fifth instar Asian corn borer larvae were independently injected with 10 μg of ds*REase*, ds*REase*-800, ds*REase*-400A, and ds*REase*-400B. Four hours later, RNAs were isolated for small RNA sequencing. Small RNAs of 19–25 nt long were re-mapped on the reference sequences (*x*-axis) to produce the graph. Blue peaks indicate that the small RNAs matched on the sense chain. Red peaks indicate that the small RNAs matched on the anti-sense chain. The *x*-axis represents the *REase* sequence, and the y-axis represents the depth of sequencing (amount of mapped small RNA). The three black boxes of ds*REase*-A, ds*REase*-B, and ds*REase*-C were 100 bp sequences in different position for small RNAs analysis. For the statistical results, please see **Supplementary Figure 3**.

When a small RNA was mapped on the reference dsRNA sequence, the dsRNA cleavage sites were determined. Three nucleotide bases at the front and back of the 5′-end of a small RNA were named as 5′ cleavage site, three nucleotide bases at the front and back of the 3′-end of a small RNA were named as 3′ cleavage site. And then the nucleotide residues of 5′- and 3′-ends cleavage sites were calculated and analyzed.

Real-Time Quantitative PCR (qRT-PCR)

Total RNA was extracted from pools of three surviving dsRNA-treated larvae using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. First-strand cDNA was made from 1 μg of RNA primed by oligo $(dT)_{18}$ using M-MLV reverse transcriptase (Takara, Kyoto, Japan). A qRT-PCR assay that amplified multiple genes was performed using SYBR Premix Ex Taq^{TM} II (Takara). To ensure the qRT-PCR's quality, two or three primer pairs were designed for all the amplification segments, but only one pair was used in the final test. All the primer sequences are listed in **Supplementary Table 1**. Melting-curve analyses were performed for all the primers. To normalize Ct values obtained for each gene, 18S rRNA expression levels were

TABLE 1 | Distributions of 18–42 nt small RNA copy numbers, numbers and percentages after the ds*REase* treatment.

Copy number	Number of each small RNA type	Percent according to copy number	Small RNA number of these kinds RNAs	Percent according to small RNA number
1 10	32360	82.56	76722	18.35
11 100	6168	15.78	187219	44.78
101 500	610	1.56	109255	26.13
501∼1000	29	0.07	18661	4.46
> = 1001	8	0.03	26243	6.28
Total	39193	100	418100	100

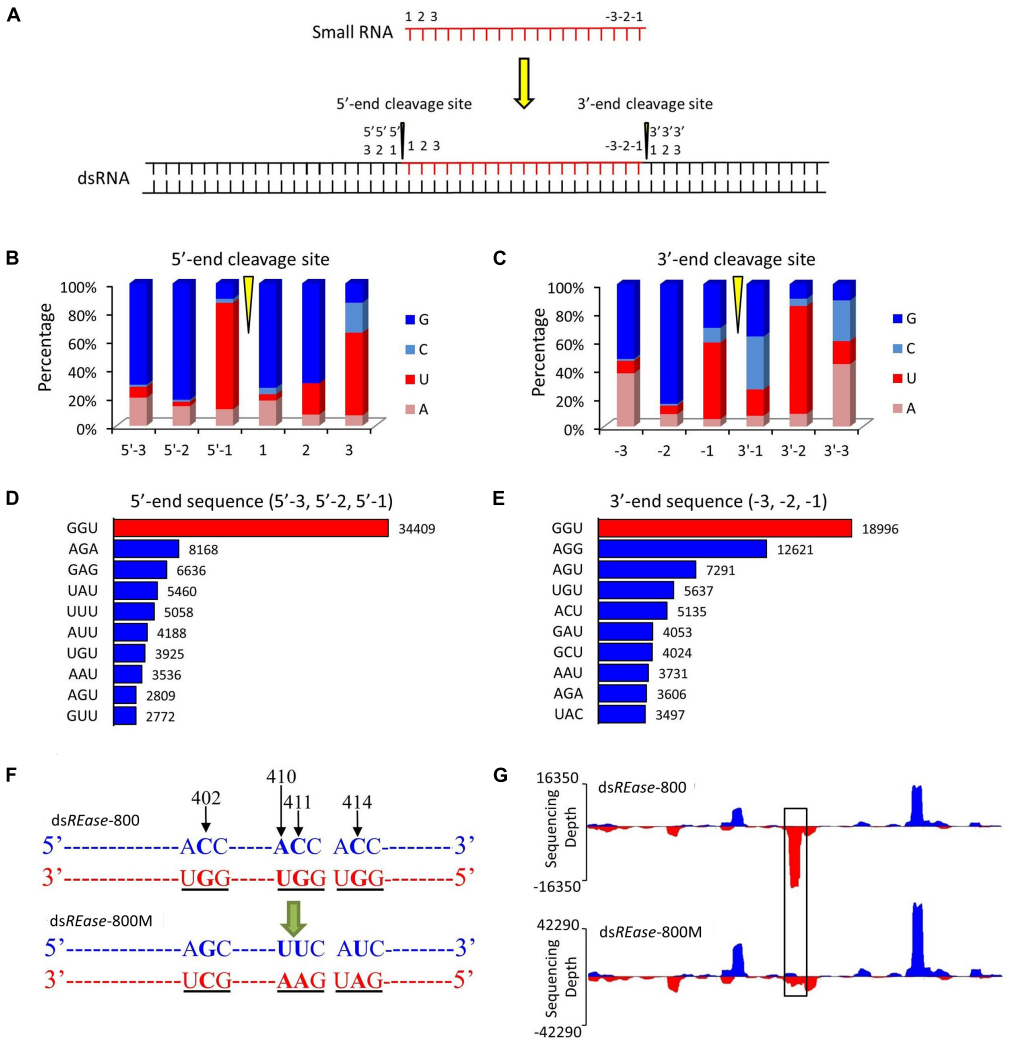

FIGURE 2 | GGU is a major cleavage site for *in vivo* dsRNA-processing into small RNAs. **(A)** Outline of the dsRNA cleavage site analysis. Three nucleotide bases on the 5′- and 3′-ends of a small RNA were labeled as 1, 2, and 3, and -3, -2, and -1, respectively. When one small RNA was mapped on the reference dsRNA sequence, the small RNA cleavage site was revealed. Three nucleotide bases before the 5′-end's cleavage site were labeled as 5′-3, 5′-2, and 5′-1. Similarly, three nucleotide bases after the small RNA's 3′-end cleavage site were named as 3′-1, 3′-2, and 3′-3. **(B,C)** Nucleotide components of 5′- and 3′-end cleavage sites of the top 0.1% of small RNAs that match on the reference sequence. **(D,E)** Three nucleotides components of the top ten 5′- and 3′- end cleavage sites of the top 1.0% of 19–25 nt small RNAs. The nucleotide components of the 5′-end sequence represent the information on sites of 5′-3, 5′-2, and 5′-1, and those of 3′-end sequence represent the information on sites of -3, -2, and -1. **(F)** GGU site's mutant design. One small RNA peak representing an area with three GGU cleavage sites was selected in ds*REase*-800. The site-specific mutations were produced by PCR. The nucleotide site 402, 410, 411, and 414 on the sense chain were mutant from CACC to GUUU. Thus, the three GGU sites on the antisense chain were changed to GCU, GAA, and GAU. **(G)** The small RNA peak was lost when the GGU site was mutant. Four hours later injection with 10 μg ds*REase*-800 with three GGU sites or 10 μg ds*REase*-800M with GGU mutation, total RNA was extracted from the fifth instar Asian corn borer larvae. Then, small RNAs were isolated and sequenced (sequence data are listed in **Supplementary Tables 2**–**5**). The 19–25 nt long small RNAs that matched on the reference sequences were used for graphing.

used (Chapman and Waldenstrom, 2015). The qRT-PCR was carried out using a Mastercycler ep realplex (Eppendorf). All the qPCR assays were repeated three times. The qRT-PCR reactions and data were analyzed according to the methods of Livak and Schmittgen (2001) and Bustin et al. (2009). The qRT-PCR data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to look for treatment effects compared with the untreated control.

RESULTS

Target dsRNA Sequence Composition Determines the Amount of Small RNA

When a dsRNA was introduced into one insect body, what kinds of siRNAs were produced and induced RNA? To analyze this issue, an RNAi efficiency-related nuclease (*REase*) (the full length CDS is 1866 bp) (GenBank accession no. F682492) was selected as a template to synthesize four kinds of dsRNAs. They are

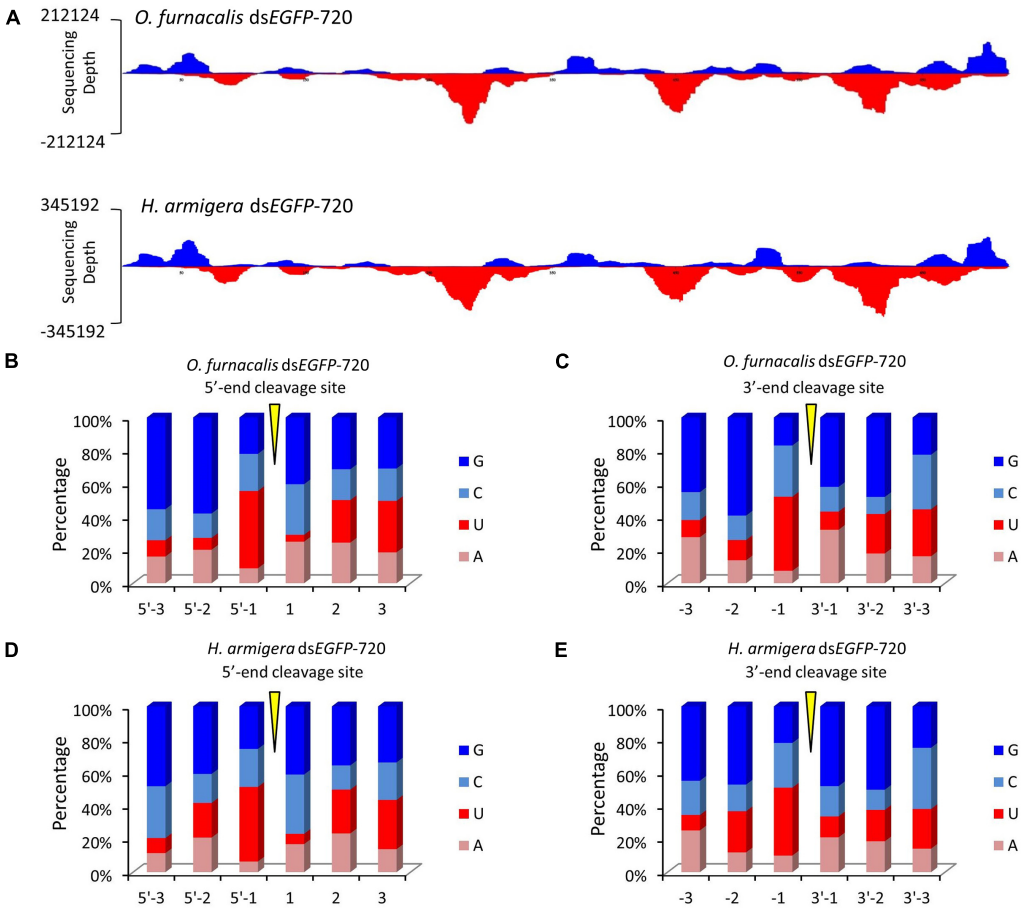

FIGURE 3 | The GGU cleavage site is a universal phenomenon in Lepidoptera. **(A)** Ds*EGFP*-720 revealed similar cleavage modes in the Asian corn borer (*Ostrinia furnacalis*) and in cotton bollworm (*Helicoverpa armigera*) when small RNAs were re-mapped onto the *EGFP* sequence. The *x*-axis represents the *EGFP* sequence, and the *y*-axis represents the depth of sequencing (amount of mapped small RNA). **(B,C)** Nucleotide components of the 5′- and 3′-end cleavage sites of the top 1.0% of small RNAs in the Asian corn borer. **(D,E)** Nucleotide components of the 5′- and 3′-end cleavage sites of top 1.0% of small RNAs in the cotton bollworm.

ds*REase* (1–1866 bp), ds*REase*-800 (101–900 bp), ds*REase*-400A (1317–1716 bp), and ds*REase*-400B (1467–1866 bp) according to different segments (**Figures 1A,B**). Then, each kind of dsRNA was injected into a fifth instar Asian corn borer larva (triplicated). After 4 h, total RNA was extracted for qRT-PCR and small RNA sequencing. The qRT-PCR results indicated that all four kinds of ds*REase* suppressed *REase* gene expression levels compared with the ds*EGFP* treatment (**Supplementary Figure 1**). These results also confirmed that all these four kinds of dsRNA segments can be processed into siRNA *in vivo* and induce target gene RNAi in insect.

To analyze the siRNA produced by these four kinds of dsRNA *in vivo*, small RNAs were sequenced using an Illumina HiSeq 2000 analyzer at BGI (Shenzhen, China). Approximately 90% of the small RNA sequences were 18–30 nt, and 70% were 19–25 nt (**Supplementary Figure 2** and **Supplementary Table 2**). The lengths of these small RNAs conformed to those of siRNAs. Thus, we assumed that most of these small RNAs are siRNAs, which can combine with Argonaute protein, and result in the RNAi effect on the target gene. In this study, 19–25 nt small RNAs were selected and re-mapped on the corresponding dsRNA sequences of ds*REase*, ds*REase*-800, ds*REase*-400A, and ds*REase*-400B (**Supplementary Table 3**). With the *x*-axis representing the dsRNA sequence, and the y-axis representing the depth of sequencing (amount of mapped small RNA) to make a graphing, a very interesting phenomena was revealed (**Figure 1B**). All the same dsRNA sequences were processed into similar small RNA *in vivo* by a consistent processing mode. More important is that the small RNA peak always appeared at the same sequence position among the different treatments (**Figure 1B**). This phenomenon also implied that some dsRNA segments with small RNA peaks will produce large amounts of small RNAs, while other segments without small RNA peaks will only produce a limited amount of small RNAs (**Figure 1B**), the three black boxes of ds*REase*-A, ds*REase*-B, and ds*REase*-C; for statistical results please see **Supplementary Figure 3**. At the same time, the control treatment, which was not injected with exogenous dsRNA, only produced a small number of small RNAs that mapped on corresponding dsRNA sequences (**Supplementary Table 3**). Thus, we confirmed that the mapped small RNAs of

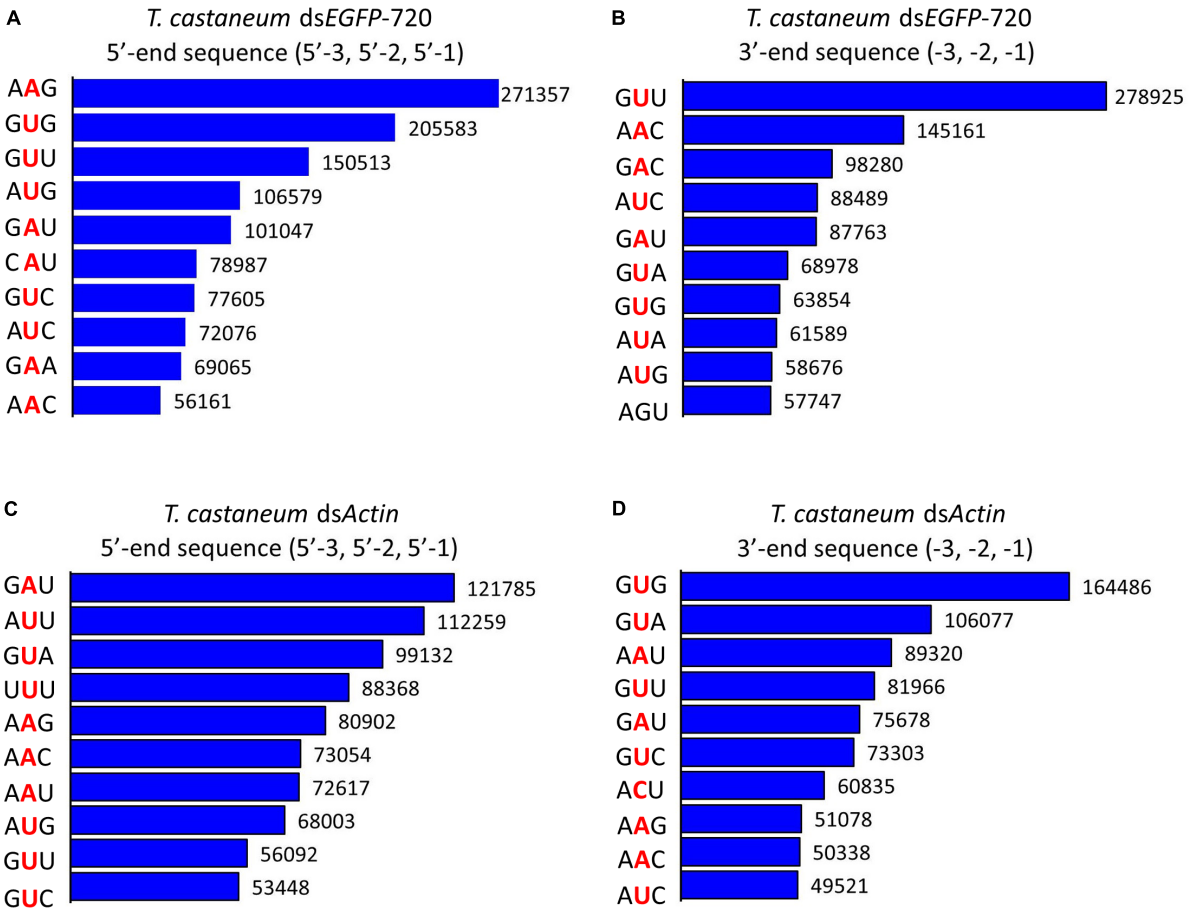

FIGURE 4 | Top 10 three-nucleotide components at the 5′- and 3′-end cleavage sites of the 1.0% of small RNAs in the red flour beetle (*Tribolium castaneum*). **(A,B)** 5′- and 3′-end cleavage sites for ds*EGFP*-720. **(C,D)** 5′- and 3′-end cleavage sites for ds*Actin*.

19–25 nt resulted from the dsRNA were injected into the insects' bodies. Accordingly, we hypothesized that specific nucleotide hot points for digestion may exist when dsRNA is processed into small RNAs *in vivo*.

GGU Is an Enzyme Digestion Hot Point When dsRNA Is Processed Into Small RNAs

To discover the cleavage hot points when ds*REase* was processed into small RNAs, the types and copy numbers of each kind of small RNA that was mapped on the full- length *REase* gene were thoroughly analyzed. The amount of the total mapped small RNAs was 418,100 (**Supplementary Table 3**), which belonged to 39,193 different types of small RNAs (**Supplementary Table 5**). The copy number, amount and percentage of each kind of small RNA are listed in **Table 1**. More than 82% of small RNAs had copy numbers of less than 10, but they accounted for only ∼18% of the total amount of small RNAs. Only 1.66% of small RNAs had copy numbers of more than 100; however, they accounted for more than 36% of the small RNA amount. These results indicated that a large amount of small RNAs came from the same dsRNA fragment and produced a small RNA peak (**Figure 1B** and **Table 1**). Thus, the dsRNA fragments corresponding to the small RNA peak probably exist at a hot point for enzyme cleavage.

To discover the hot point where the dsRNA is cut to produce siRNA, a total of 38,263 19–25 nt small RNAs that had more than 500 copy numbers were selected. They account for just 0.10% of the 19–25 nt small RNA type, but their amount represented 10.74% of the total mapped 19–25 nt small RNAs. Subsequently, through a series of analyses, three nucleotide residues at the front and back of 5′- and 3′-end were analyzed in all 38,263 19–25 nt small RNAs (**Figure 2A**). The statistical analysis suggested that the nucleotide composition of 35,974 (∼94%) small RNAs had cleavage sites of GGU before the 5′-end and on the 3′-end (**Figures 2B,C**). To further confirm this result, the 1.0% small RNA type (representing 30% of the total amount of 19–25 nt small RNAs) was analyzed, and 64% of the small RNAs had a GGU site before the 5′-end and on the 3′-end (**Supplementary Figures 4A,B**). The top 10 three-nucleotide combinations in these positions are shown in **Figures 2D,E**. The GGU sites represent 45 and 28% of all three-nucleotide combinations before the 5′-end and on the 3′-end, respectively.

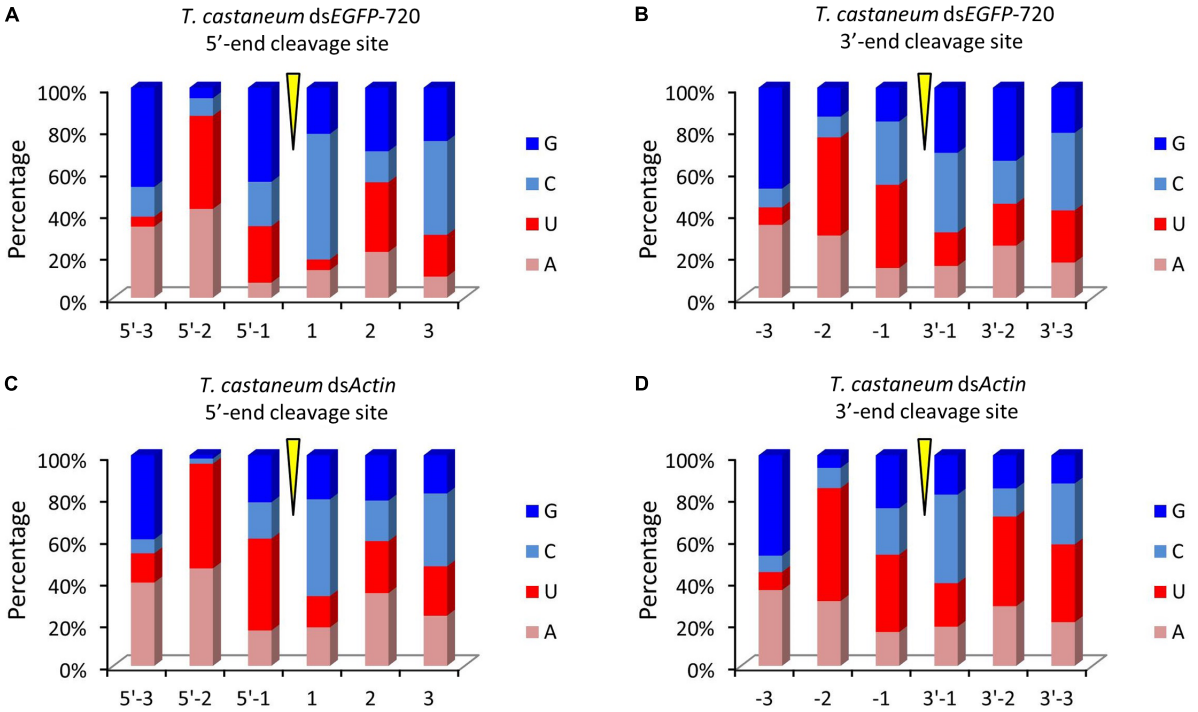

FIGURE 5 | Diversity of small RNA cleavage sites in the red flour beetle. **(A,B)** Nucleotide components of 5′- and 3′-end cleavage sites of the top 1.0% of small RNAs for ds*EGFP*-720 in *Tribolium castaneum*. **(C,D)** Nucleotide components of 5′- and 3′-end cleavage sites of the top 1.0% of small RNAs for ds*Actin* in *T. castaneum*.

We also discovered that most of the GGU sites will produce a small RNA peak (**Supplementary Figure 5**). For confirmation, three GGU sites on the antisense chain of ds*REase*-800 were selected as single base point mutations and the three GGU sites were changed to GCU, GAA, and GAU in ds*REase*-800M (**Figure 2F**, with black underline). Then, ds*REase*-800 and ds*REase*-800M were each separately injected into individual fifth instar Asian corn borer larvae (**Figure 1B**). The small RNA peak was lost when GGU sites were changed into other nucleotides (**Figure 2G**, black box); more importantly, the cleavage mode did not change in other sites on this mutant dsRNA segment. This result further suggested that GGU is an important requirement when dsRNA is being processed into small RNAs *in vivo*.

The GGU Cleavage Site May Be a Universal Phenomenon in Lepidoptera

To explore whether this GGU cleavage site was universal, we selected an exogenous *EGFP* gene to synthesize dsRNA (ds*EGFP*-720). Then, for each gram of insect body weight for Asian corn borer or cotton bollworm larva, ds*EGFP*-720 was injected. The method of small RNA sequencing and re-mapping on the *EGFP* gene sequence was performed as described above (sequencing data are listed in **Supplementary Tables 2–5**). The small RNA mapping results indicated that the two different Lepidoptera insects have similar processing modes for this exogenous dsRNA sequence (**Figure 3A**). The nucleotide analysis of the small RNAs 5′- and 3′-ends also indicated that GGU is a hot point of dsRNA processing to siRNA in both lepidopteron insects (**Figures 3B–E** and **Supplementary Figure 6**).

Major Difference Between the dsRNA-Processing Modes of Coleoptera and Lepidoptera

To further investigate the GGU digestion site in a different insect order, one fifth instar red flour beetle (*T. castaneum*) larva was injected with either ds*EGFP*-720 or ds*Actin* (GenBank accession no. XM_008201747) (0.04 mg dsRNA per gram insect body weight). The sample collection and small RNA sequence analysis were performed as described above (sequencing data are listed in **Supplementary Tables 2–5**).

To our surprise the GGU site was not found among the top 10 three-nucleotide combinations at 5′- and/or 3′-end cleavage sites in *Tribolium*. Instead, AAG, GUU, GAU, and GUG were the major three-nucleotide combinations at those positions (**Figures 4A–D**). Additionally, the site "5′-2" before the small RNA 5′-end contained mainly U and A (ds*EGFP*-720: 86.6%; ds*Actin*: 96.0%). Similarly, the site "-2" on the small RNA 3′-end also contained U and A (ds*EGFP*-720: 76.3%; ds*Actin*: 84.4%) (**Figures 5A–D**). This discovery means that enzyme restriction sites in siRNA processing in Coleoptera insects have a high conservation base of A/U. These results not only indicate the diversity of small RNA cleavage sites in the red flour beetle, but also imply that small RNAs are more easily processed at these kinds of nucleotide sites.

DISCUSSION

In this research, using high-throughput small RNA sequencing and bioinformatics analyses, we discovered that the same dsRNA sequence segments, no matter their lengths, undergo a similar *in vivo* siRNA-processing mode (**Figures 1B**, **3A**). This result also implies that the dsRNA nucleotide sequence determines the siRNA type and amount. In addition, dsRNA produced siRNAs have strong base bias, GGU is the preferred recognition and cleavage sites when dsRNA is processed into siRNA in Lepidoptera (**Figures 2**, **3**). However, the recognition and cleavage sites are more diverse in Coleoptera (**Figures 4**, **5**). These results help to explain why the RNAi efficiency is so difference between these two insect orders (Terenius et al., 2011; Ivashuta et al., 2015; Joga et al., 2016).

Previous *in vitro* studies led to the 3′- and 5′-counting rules for dsRNA-processing models using the Dicer enzyme (Zhang et al., 2004; MacRae et al., 2007; Park et al., 2011). These rules, based on northern blot results, can explain siRNA lengths but are unable to distinguish nucleotide sequences (Gu et al., 2012). Moreover, previous studies usually selected relatively shorter dsRNAs for northern blot analysis, resulting in a limited spectrum of nucleotides sequences. To address these deficiencies, we used small RNA sequencing and bioinformatics analyses to extend the base composition range. We noticed that the small RNA peaks were not always produced at the 5′- or 3′-end, and this was mainly related to the nucleotide sequence components. Sequence cleavage preference had been shown in RNase III family proteins, different Dicer-like enzymes in *Paramecium* have different cleavage preference sites (Hoehener et al., 2018), and BsMiniIII in *B. subtilis* has a strong preference for ACCU/AGGU as a cleavage site (Glow et al., 2016). These results are consistent with our high-throughput sequencing analysis. Although our result is the processing mode of dsRNA *in vivo*, dsRNA is not exclusively performed by RNase III family members. It can also be accomplished by the cooperative actions of several enzymes, such as a specific exo- or endo-ribonuclease. In addition to being cleaved by an RNase III protein family member, dsRNA can also be degraded by some nucleases, such as RNase A and REase (Starega-Roslan et al., 2015; Guan et al., 2018). However, the processing of dsRNA into siRNAs has its own regularity in each species, even in the complicated *in vivo* environment. These result confirmed that the *in vivo* dsRNA processing has some regularities.

The regularity of the *in vivo* siRNA-processing mode will help in designing effective dsRNA segments for RNAi technology. Previous studies failed to discern gene segments when designing dsRNA. A study on *Acyrthosiphon pisum* showed that there was no significant difference when designing dsRNA based on the 5′ or 3′-end for the hunchback gene (Mao and Zeng, 2014). Experiments in *Aedes aegypti* showed that dsRNA was designed based on the 3′-end of the *apoptotic* gene, resulting in a higher mortality rate than those based on the 5′-end (Pridgeon et al., 2008). However, studies on *Litopenaeus vannamei* showed that dsRNA designed based on the 5′-end was more effective against antiviral effects (Loy et al., 2012). These results suggest that the RNAi effect of the 5′ or 3′-end segment as dsRNA templates vary among genes (Scott et al., 2013). According to our finding, for more effective RNAi, segments that easily produce small RNA peaks should be selected as dsRNA targets. GGU is a preferred cleavage site in Lepidoptera (**Figures 2**, **3**). Most small RNA peaks have one or more GGU nucleotide residues (**Supplementary Figure 5**). Once the GGU were mutated, the small RNA peaks were lost (**Figure 2G**). However, in Coleoptera, the siRNA's 5′ or 3′-end nucleotide residues are more diverse and GGU is not among the top 10 nucleotide combinations (**Figures 4**, **5**). Most results indicated that members of Coleopteran are more sensitive to RNAi than those of Lepidoptera insects (Terenius et al., 2011; Ivashuta et al., 2015; Joga et al., 2016). Thus, the difference in RNAi efficiencies between these two insect orders may result from a difference in their genomes' nucleotide compositions, the codon bias of their genes (Behura and Severson, 2012), enzyme-substrate contacts (Glow et al., 2016), RNAi pathway-related gene (Dowling et al., 2016), or various environmental differences that result in differences in dsRNA stability (Spit et al., 2017). Here, we discovered that there was a large difference in the dsRNA's cleavage between Lepidoptera and Coleoptera insects. The dsRNA-processing sites are more diverse in Coleoptera and this insect order is sensitive to RNAi; our discovery supplies new evidence for RNAi efficiency. Discerning these regularities will increase the understanding RNAi mechanisms and aid in the design of effective dsRNAs for *in vitro* studies and applications.

AUTHOR CONTRIBUTIONS

XM and RG designed research. RG and SH performed research. RG and HL analyzed the data. XM and ZS wrote and revised the paper.

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SUPPLEMENTARY MATERIAL

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REVIEW
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RNA Interference in Insects: Protecting Beneficials and Controlling Pests

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Insects constitute the largest and most diverse group of animals on Earth with an equally diverse virome. The main antiviral immune system of these animals is the post-transcriptional gene-silencing mechanism known as RNA(i) interference. Furthermore, this process can be artificially triggered via delivery of gene-specific double-stranded RNA molecules, leading to specific endogenous gene silencing. This is called RNAi technology and has important applications in several fields. In this paper, we review RNAi mechanisms in insects as well as the potential of RNAi technology to contribute to species-specific insecticidal strategies. Regarding this aspect, we cover the range of strategies considered and investigated so far, as well as their limitations and the most promising approaches to overcome them. Additionally, we discuss patterns of viral infection, specifically persistent and acute insect viral infections. In the latter case, we focus on infections affecting economically relevant species. Within this scope, we review the use of insect-specific viruses as bio-insecticides. Last, we discuss RNAi-based strategies to protect beneficial insects from harmful viral infections and their potential practical application. As a whole, this manuscript stresses the impact of insect viruses and RNAi technology in human life, highlighting clear lines of investigation within an exciting and promising field of research.

Keywords: insects, RNA interference, environmental RNAi, systemic RNAi, insecticides, delivery systems, viruses, antiviral immunity

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INTRODUCTION TO CELL-AUTONOMOUS, ENVIRONMENTAL AND SYSTEMIC RNAi IN INSECTS

The discovery of RNA interference (RNAi) constitutes an important milestone in the study of regulatory RNAs (Fire et al., 1998). In this process, small (s)RNA molecules of 18–31 nucleotides (nt) long effectuate a sequence-specific gene silencing response, acting at the post-transcriptional level through cleavage or blockage of longer RNAs containing a matching sequence (Siomi and Siomi, 2009). Based on their origin, biogenesis, structure and role in distinct biological processes, small RNAs are classified in three main cell-autonomous pathways: (1) genome encoded microRNAs (miRNAs), which regulate a multitude of biological processes; (2) PIWI-interacting (pi)RNAs, which silence transcripts derived from selfish genomic elements, such as transposons (Klattenhoff and Theurkauf, 2008); and (3) small interfering (si)RNAs, which defend the organism against invading viruses (Wang et al., 2006). However, recent studies revealed that some level

of functional crosstalk can occur between the different sRNA-mediated pathways. A fascinating example is found in insects, where an important antiviral role of the piRNA pathway has been described in mosquitoes (Keene et al., 2004; Schnettler et al., 2013; Miesen et al., 2015, 2016; Palatini et al., 2017; Varjak et al., 2017).

In insects, the siRNA pathway is activated when double-stranded (ds)RNA molecules, as products of viral replication, are recognized in the cytoplasm and processed into siRNAs of 18-24 nt by the RNase type III enzyme Dicer-2 (Siomi and Siomi, 2009). Cleavage of viral RNA targets is then further exerted by an Argonaute-2 (Ago2) containing 'RNA induced silencing complex' (RISC), which encompasses the siRNA guide strand. Interestingly, this RNA silencing mechanism can also be triggered by artificial administration of gene-specific long dsRNA, a technique that is generally designated as RNAi (Wynant et al., 2014b). This dsRNA treatment can result in functional knockdown effects that in fact can be considered as auto-immune defects, since the siRNA pathway, an antiviral immune defense mechanism of insects, is being misled to target an endogenous transcript of the host. As such, RNAi has become the most widely used reverse genetics research tool in insects and holds great potential to contribute to novel strategies for species-specific control of insect pests and to combat viral infections in disease-vectoring and beneficial insects.

An interesting aspect of the RNAi response in insects is its potential systemic character, also known as systemic (sys)RNAi. Specifically, in some insects administration of dsRNA can result in the generation of an RNAi response throughout the entire body (Turner et al., 2006; Meyering-Vos and Müller, 2007; Bautista et al., 2009; Bolognesi et al., 2012; Wynant et al., 2012; Abd El Halim et al., 2016; Darrington et al., 2017). However, the precise mechanism of both short- and long-distance intercellular transfer of the sysRNAi-signal, as well as the exact nature of this signal, still remain elusive. Different reports indicate that the cellular uptake of dsRNA in insects, also referred to as environmental (env)RNAi, occurs via scavenger receptor-mediated endocytosis both in cultured cells and *in vivo* (Saleh et al., 2006; Wynant et al., 2014c). It is also known that the uptake of naked dsRNA is length-dependent. It occurs efficiently for long dsRNA molecules of around 200–500 base pairs (bp) and even of up to circa 1000 bp. However, for shorter constructs such as siRNAs, this efficiency decreases (Saleh et al., 2006; Huvenne and Smagghe, 2010; Bolognesi et al., 2012; Miller et al., 2012; Wang et al., 2013). Furthermore, it has been shown that lipophorins can adhere to dsRNA fragments in the insect hemolymph, suggesting a possible role of these proteins in either protection, transport, or both, throughout the body (Wynant et al., 2014a). In addition, two main findings have been reported for *Drosophila melanogaster*. First, viral infection of cultured *Drosophila* cells increased the formation of nanotube-like structures through which short-distance transport of dsRNA and RISC components can occur (Karlikow et al., 2016). Second, it has been shown that flies use hemocyte-derived exosome-like vesicles to systemically spread an antiviral siRNA signal in the hemolymph (Tassetto et al., 2017). At present, it is still unclear how all these separate findings might fit together and whether they can be extrapolated to other conditions and species (**Figure 1**).

In this review article, we start by discussing the potential of the RNAi technique to contribute to insect pest control. On this matter, we review several application strategies that have been tried, their limitations and the most promising approaches described in the available scientific literature thus far. On a parallel perspective, we summarize relevant insect viral infections and review the use of viruses as bio-insecticides. Finally, with perspective to the natural antiviral role of the RNAi mechanism, we discuss the potential use of RNAi for protecting beneficial insects from harmful viral diseases.

RNAi-BASED INSECT PEST CONTROL

Despite frequent use of insecticides, approximately 18–20% of the global crop harvest is still lost due to damage caused by pest insects (Sharma et al., 2017). A major underlying cause is insect population resistance against the most commonly used insecticides, posing a persistent challenge to agriculture (Tabashnik et al., 2013; Zhu et al., 2016). Furthermore, the devastating impact of chemical insecticides on the environment and other organisms, such as beneficial insects, can no longer be ignored (Ansari et al., 2014). Taking the previous statements into account, it becomes evident that the current array of insect pest combatting methods is insufficient to secure global food production for the next decades. Finding alternative options to improve plant protection strategies is therefore critical.

In this context, an interesting perspective is represented by the RNAi technique. The potential of this mechanism is inherent in its mode-of-action, namely the subsequent degradation of complementary target mRNA upon entry of specific dsRNA into the cell (Agrawal et al., 2003). Therefore, by delivering dsRNA targeting any endogenous gene transcript to the intended pest organism, expression of this gene can be knocked down at the post-transcriptional level. Thus, through careful selection of an essential target gene, this mechanism can lead to insect mortality. The sequence-specific nature and the possibility to theoretically target any non-conserved, 'lethal' gene, make RNAi an ideal candidate for further application as a species-specific insecticide.

A proof-of-concept study was executed in 2007 by Baum et al. In this research, a transgenic corn crop was genetically engineered to express dsRNA against the V-ATPase A transcript of the Western corn rootworm *Diabrotica virgifera virgifera*. Feeding *D. virgifera virgifera* with this modified plant resulted in larval stunting and in the premature death of the insect. Additionally, dsRNA functioned as a crop protectant as feeding damage to the transgenic corn was greatly reduced (Baum et al., 2007). A similar study was executed for the cotton bollworm *Helicoverpa armigera*. In this research, Mao et al. (2007) showed that plant-mediated expression of dsRNA targeting the cytochrome P450 monooxygenase gene (CYP6AE14) could increase the toxic effects of gossypol, a cotton metabolite that is otherwise tolerated by the cotton bollworm. Silencing of CYP6AE14 led to delayed larval growth when gossypol was supplemented in the diet (Mao et al., 2007). It should be noted that another research has since shown that CYP6AE14 is likely not directly involved in gossypol metabolism but rather plays a more general role in the insect

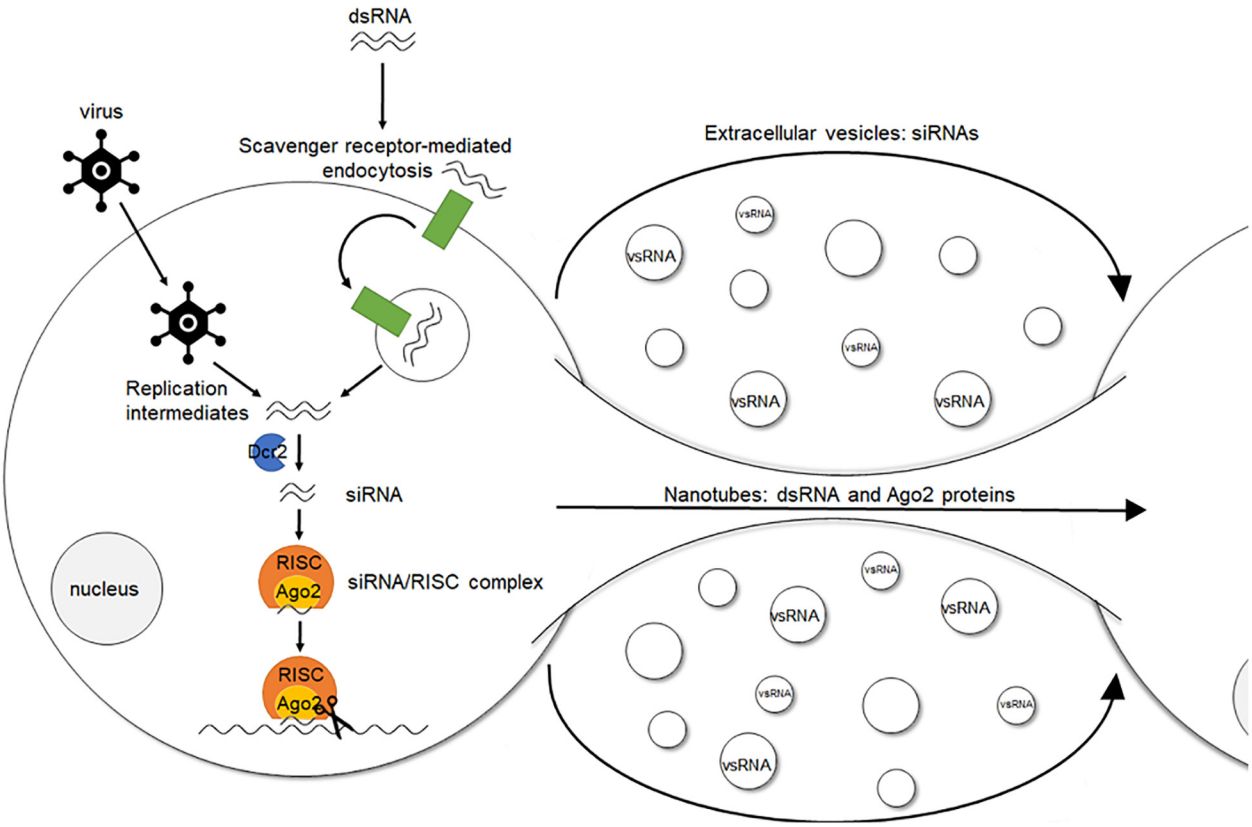

FIGURE 1 | Simplified model of environmental, cell autonomous and systemic (antiviral) RNAi in insects. The siRNA pathway is triggered by dsRNA molecules. These duplexes naturally occur inside the cell during viral replication or can be artificially delivered. In the latter, cellular uptake of dsRNA, i.e., environmental RNAi, occurs via scavenger receptor-mediated endocytosis. Once inside the cell, i.e., cell autonomous RNAi, the dsRNA molecules are recognized in the cytoplasm and processed into siRNAs by Dcr2. Cleavage of viral RNA targets or endogenous transcripts is then further exerted by an Ago2-containing RISC, which encompasses the siRNA guide strand. Regarding antiviral RNAi in *Drosophila*, viral infection increases the formation of nanotube-like structures, through which short-distance transport of dsRNA and RISC components can occur. In addition, hemocyte-derived exosome-like vesicles systemically spread an antiviral RNAi signal (vsRNAs) in the hemolymph. This spread of the RNAi signal to cells in which the RNAi response had not been initiated before is named systemic RNAi. dsRNA, double stranded RNA. Dcr2, Dicer2. siRNA, small interfering RNA. RISC, RNA-induced silencing complex. Ago2, Argonaute2. vsRNA, viral small RNAs.

stress response to ingestion of plant toxins (Krempl et al., 2016). Nonetheless, the research of Mao et al. remains an interesting example of the application potential of dsRNA-mediated plant protection.

This section will continue by reviewing the predominant theories regarding the variable nature of the RNAi response across the class Insecta. Bearing in mind the promising use of RNAi technology as an insecticide, an overview of dsRNA delivery systems is given next. Finally, current use of RNAi-based insecticides will be summarized.

Variable Efficiency of the RNAi Response

Although an RNAi response has been detected at least once in most economically important insect orders, such as Coleoptera, Diptera, Orthoptera, Lepidoptera, and Hemiptera, the efficiency of the induced response may vary between species and even within the same organism (Bellés, 2010; Wynant et al., 2014b; Xu et al., 2016). Whereas some insects, such as the Colorado potato beetle *Leptinotarsa decemlineata,* and the western corn rootworm *D. virgifera virgifera*, are consistently able to generate a systemic RNAi response; other species, such as the tobacco cutworm *Spodoptera litura,* and the silkworm *Bombyx mori*, show a more variable and generally less efficient response (Terenius et al., 2011). The nature of this variable efficiency has been the subject of much speculation and it is likely that a lot remains to be discovered.

Extracellular Nucleases Inhibit the Efficiency of the RNAi Response

Some insect species, such as the desert locust *Schistocerca gregaria,* and the migratory locust *Locusta migratoria*, are able to display an efficient systemic RNAi response after injection of dsRNA into the body cavity (Luo et al., 2012; Wynant et al., 2012). However, when dsRNA is fed to these insects, they appear to be refractory (Luo et al., 2013; Wynant et al., 2014c). As most food sources contain nucleic acids, it follows that nucleases are an integral part of the insect digestive system. In addition, it has been suggested that nucleases in the digestive track may

also exert a function of protection against viruses (Musser et al., 2002). Unfortunately, presence of high nuclease activity can have an inhibitory effect on the RNAi response (Katoch and Thakur, 2012).

Wynant et al. (2014d) showed that a digestive enzyme solution, collected from the midgut of *S. gregaria*, had the ability to quickly degrade dsRNA (i.e., 150 nanograms of dsRNA within 5 min). Subsequently, four different sequences coding for dsRNases were identified from *S. gregaria* transcriptome data (Wynant et al., 2014d). In several other species, such as the pea aphid *Acyrthosiphon pisum*, the tarnished plant bug *Lygus lineolaris*, *B. mori,* and *L. migratoria,* the limited RNAi response after feeding with dsRNA has been linked to the presence of dsRNA-degrading enzymes in the digestive system (Arimatsu et al., 2007; Allen and Walker, 2012; Liu et al., 2012; Luo et al., 2013; Christiaens et al., 2014).

The limiting effect of nucleases is not unique to the digestive tract. In fact, their activity in the hemolymph has been linked to a lowered RNAi efficiency in a number of species (Singh et al., 2017). Whereas dsRNA remained stable in the hemolymph of the RNAi efficient German cockroach *Blattella germanica*, injection of dsRNA into the body cavity of the RNAi refractory tobacco hornworm *Manduca sexta,* led to its swift degradation (Garbutt et al., 2013). Furthermore, in the Asian corn borer *Ostrinia furnacalis,* the nuclease Rease was found to be upregulated in the hemolymph after administration of dsRNA (Guan et al., 2018). Reducing transcript levels of this gene led to a significantly improved RNAi response in this insect. Phylogenetic analysis revealed homologous genes in seven other lepidopteran species, suggesting that this nuclease might be Lepidoptera-specific (Singh et al., 2017).

Surprisingly, the presence of dsRNases has even been recorded in insects which generally show an efficient RNAi response after feeding with dsRNA, such as the Colorado potato beetle *L. decemlineata* (Singh et al., 2017; Spit et al., 2017). Spit et al. (2017) showed that an enzyme solution collected from the gut of this beetle was capable of degrading dsRNA. Additionally, the induced RNAi response after feeding dsRNA could still be significantly increased after the knockdown of two dsRNA degrading enzymes, Ld_dsRNase1 and Ld_dsRNase2 (Spit et al., 2017). Although it has become clear that most insect orders contain species wherein the efficiency of the RNAi response is somehow limited by the presence of dsRNases, not all species appear to be equally affected. It is therefore likely that differences in the activity of dsRNA degrading enzymes contribute to the tissue-, stage- and species-dependent variability in RNAi sensitivity observed in insects (Singh et al., 2017). In this context, it is notable that extracellular nuclease activity will additionally influence dose-dependence of the RNAi response as it conditions the quantity of dsRNA that remains available for uptake. Certainly, it appears that the role of dsRNases in limiting the RNAi response cannot be ignored. Moreover, since reducing transcript levels of dsRNases already led to an improved RNAi sensitivity in some insect species, these nucleases may have to be taken into account when considering future RNAi-based insect pest control strategies (Guan et al., 2018).

Tissue-Dependence of the RNAi Response

The fruit fly *D. melanogaster,* is a well-known example of an insect that is recalcitrant to external administration of dsRNA (Whyard et al., 2009). Remarkably, this is a generalization that does not apply to the whole insect: *D. melanogaster* hemocytes can take up extracellular dsRNA and generate an RNAi response. This sensitivity can also be observed in the hemocyte-derived *D. melanogaster* S2 cell line, a commonly used *in vitro* model for RNAi research (Clemens et al., 2000).

While this inconsistency is striking, the fruit fly is not the only organism in which the sensitivity of the RNAi response appears to be cell type or tissue-dependent. Another striking example presents itself in *S. gregaria*; independent studies in this species have proven that an efficient RNAi response can be induced in various tissues, ranging from the brain to the Malpighian tubules (Badisco et al., 2011; Marchal et al., 2011a,b, 2012; Ott et al., 2012; Van Wielendaele et al., 2012; Lenaerts et al., 2016, 2017a,b). Nevertheless, Wynant et al. (2012) have observed that the ovaries and testes of this locust species showed a lower RNAi efficiency when compared to these other tissues. Further examples can be found in the African malaria mosquito *Anopheles gambiae,* and the yellow fever mosquito *Aedes aegypti*. In the former, a reduced RNAi susceptibility was observed in the salivary glands, whereas in the latter both head and ovarian tissues responded less efficiently than other tissues to external application of dsRNA (Boisson et al., 2006; Telang et al., 2013). Finally, lepidopterans are known to have a variable RNAi susceptibility and tissue-dependency has also been observed for a number of species in this order. Particularly wing-disk and larval epidermal tissues appear to be problematic (Terenius et al., 2011).

Little is known about the exact causes of the tissue-dependency of the RNAi response, and as such they may vary between and within species. Research using the lepidopteran Sf9 and Hv-E6 cell lines showed that dsRNA molecules are unable to escape after endosomal uptake (Shukla et al., 2016). Thus, in these insect cells, the RNAi response is not induced because the dsRNA cannot enter the cytoplasm. In this context, it has been suggested that this inability might contribute to the poor susceptibility and possibly to the tissue-dependency often observed in this insect order (Shukla et al., 2016). Similarly, in *L. migratoria* it was found that a reduced uptake of dsRNA in oocytes and follicle cells resulted in a less efficient ovarian RNAi response (Ren et al., 2014). In *S. gregaria,* the reduced responsiveness of ovaries and testes could be attributed to reduced expression levels of *argonaute-2* and *dicer-2,* two crucial RNAi genes (Wynant et al., 2012). Likewise, in several lepidopteran species it was also suggested that variable expression of core RNAi components might be linked to the inconsistent RNAi response observed in these insects (Terenius et al., 2011; Garbutt and Reynolds, 2012).

Intra-Species Differential Sensitivity and Resistance

To fully understand the problem of variable RNAi efficiency in insects, intraspecies variations also need to be considered. Indeed, some populations of the same species appear to be differentially sensitive to external administration of dsRNA. This was most recently observed in *L. migratoria* by Sugahara et al. (2017). In this research, four different lab strains of the migratory locust

were examined, each originating from a geographically isolated location in Japan. Two of the tested lab strains were found to be very sensitive to injection with dsRNA, while the other two appeared to be completely refractory. Even within the same lab strain, different individuals could respond with different degrees of sensitivity (Sugahara et al., 2017). In a comparable research, three phenotypically different field populations of *D. virgifera virgifera* were given the same dsRNA treatment to see whether they would respond in a similar way. The efficiency of the RNAi treatment varied between the three populations, indicating that the RNAi susceptibility differed for each population (Chu et al., 2014). It has been suggested that a similar phenomenon could be occurring in the red flour beetle, *Tribolium castaneum* (Spit et al., 2017). While some lab strains of this species show a highly sensitive response to feeding with dsRNA, other lab strains appear to be unresponsive to this method of administration (Whyard et al., 2009; Miyata et al., 2014; Abd El Halim et al., 2016; Spit et al., 2017).

The cause for these intraspecies variations in RNAi sensitivity remains uncertain. Sugahara et al. (2017) proposed that in locusts these intraspecies differences could be attributed to a genetic component. They postulate that RNAi sensitivity is regulated by an incompletely dominant gene or several genes that remain to be determined (Sugahara et al., 2017). On the other hand, another theory suggests persistent viral infections as a key-determining factor in the establishment of variable RNAi efficiencies between insect populations of the same or different species. Swevers et al. (2013) hypothesize that these infections could reduce insect RNAi sensitivity through the expression of viral suppressors of RNAi, the saturation of the RNAi machinery by viral siRNAs, or the manipulation of host gene expression. However, this remains speculative as the effect of specific persistent viral infections on the efficiency of the RNAi response in lepidopteran cells remains to be demonstrated (Swevers et al., 2016). Thus, further investigation is necessary to verify this hypothesis.

Taken together, observations regarding the variation of RNAi efficiency suggest that the potential emergence of RNAi-resistance in currently RNAi-sensitive insects is a real possibility. Indeed, in a recent study, an RNAi-resistant insect population was created. Resistance was induced by consistently feeding a field population of the Western corn rootworm with transgenic maize plants expressing *DvSnf7* dsRNA. Khajuria et al. (2018) showed that resistant insects displayed reduced dsRNA uptake from the gut lumen after feeding. Furthermore, the researchers were able to determine that the resistance was not limited to *DvSnf7* dsRNA, since the insects displayed cross-resistance to feeding with several different dsRNAs (Khajuria et al., 2018). Further research into the underlying mechanisms causing the development of resistance in RNAi-sensitive insects could provide important insights into the efficient application of RNAi as an insecticide.

Target Selection and Construct Design

Taking the aforementioned variabilities into account, it is unsurprising that the set-up of RNAi technology experiments requires careful consideration. Selection of the target gene of interest, for instance, is crucial to their success or failure. The ideal target gene should be abundantly transcribed, produce an mRNA with a high turnover rate and translate into a protein with a low half-life (Scott et al., 2013). To become applicable as an insecticide, transcript reduction of the intended target gene must additionally lead to mortality in the insect. Furthermore, off-target effects should always be considered. With regard to this, dsRNA constructs should preferably be chosen in non-conserved regions of the target mRNA to avoid cross-silencing among other species or isoforms of the gene of interest. Correspondingly, research has shown that RNAi can be highly species-specific if the dsRNA construct is well-designed. By targeting the variable 3′-UTR region of the greatly conserved γ-Tubulin transcript, Whyard et al. (2009) showed that a species-specific knockdown could even be achieved in four closely related *Drosophila* species. Similarly, Kumar et al. (2012) could induce very specific transcript reductions for three highly similar CYP genes in the Tobacco hornworm *Manduca sexta*.

Moreover, the length of the dsRNA construct should be contemplated as the optimal length for dsRNA uptake varies from insect to insect (Bolognesi et al., 2012). Research has shown that for most insects this optimum lies between 200 and 520 bp (reviewed by Huvenne and Smagghe, 2010). A last criterium that should be taken into account is the dosage of dsRNA that is administrated. This concentration should be adjusted according to the abundance of target mRNA. As most genes are not stably expressed during the entire life cycle of the insect, temporal expression according to life and developmental stage should be taken into account. In addition, this concentration can be species dependent. In insect species where dsRNAses limit RNAi efficiency in the gut, for instance, an overdose of dsRNA may be required to induce an RNAi signal. This was extensively reviewed by Scott et al. (2013).

RNAi Delivery Systems

Clearly the obstacles of insufficient RNAi sensitivity must be solved before RNAi technology can be further applied as a universal insecticide. An elegant solution is presented by packaging dsRNA in such a way that it is protected against degradation and uptake is facilitated. This may be achieved through the use of delivery systems. Many different strategies have already been proposed in the existing literature and will be discussed here. An important feat to keep in mind is that the specificity of these systems has to be prudently considered. As such, the effect of all proposed delivery strategies on other animals and on human consumption has to be investigated thoroughly before they can be applied as vehicles for insecticidal dsRNA. Regardless, the great potential of these systems is undeniable. An overview of all delivery systems reported so far can be found in **Table 1**.

Micro-Organisms

The bacterial system, in its simplicity, is one of the most successful methods of dsRNA delivery in insects. This system makes use of the genetically modified HT115 bacterial strain, which lacks the dsRNA-degrading bacterial endonuclease RNase III. Furthermore, this strain contains the T7 polymerase gene, controlled by the inducible lac operon. HT115 is often combined

TABLE 1 | Overview of delivery systems used for the successful delivery of dsRNA in several economically important insect orders.

Insect order	Category	Delivery system	Species	Target gene*	Reference
Lepidoptera	Micro-organism	Bacteria	*Spodoptera exigua*	*Chitin synthase A (SeCHSA)*	Tian et al., 2009
			Spodoptera exigua	*Chymotrypsin 2 (SeCHY2)*	Vatanparast and Kim, 2017
			Helicoverpa armigera	*Ultraspiracle protein (USP)*	Yang and Han, 2014
			Sesamia nonagrioides	*Juvenile hormone esterase (SnJHE)*	Kontogiannatos et al., 2013
	Viral	BmNPV	*Sesamia nonagrioides*	*Juvenile hormone esterase (SnJHE)*	Kontogiannatos et al., 2013
		AcMNPV	*Heliothis virescens*	*Juvenile hormone esterase (HvJHE)*	Hajós et al., 1999
		Sindbis Virus	*Bombyx mori*	*Broad-Complex (Br-C)*	Uhlirova et al., 2003
	Nanoparticle	FNP	*Ostrinia furnacalis*	*Chitinase-like gene CHT10*	He et al., 2013
		Guanylated polymers	*Spodoptera exigua*	*Chitin synthase B*	Christiaens et al., 2018
Coleoptera	Micro-organism	Bacteria	*Leptinotarsa decemlineata*	*β-actin (actin), Protein transport protein sec23 (Sec23), Coatomer subunit beta (COPβ)*	Zhu et al., 2011
	Proteinaceous	PTD-DRBD	*Anthonomus grandis*	*Chitin synthase II* (*AgChSII*)	Gillet et al., 2017
Hemiptera	Micro-organism	Bacterial symbiont – *Rhodococcus rhodnii*	*Rhodnius prolixus*	*Nitrophin 1 (NP1), Nitrophin 2 (NP2), Vitellogenin (Vg)*	Whitten et al., 2016
		Bacterial symbiont – *BFo2*	*Frankliniella occidentalis*	*α-Tubulin (Tub)*	Whitten et al., 2016
Diptera	Micro-organism	Yeast symbiont – *Saccharomyces cerevisiae*	*Drosophila suzukii*	*γ-Tubulin 23C (γTub23C))*	Murphy et al., 2016
		Chlamydomonas reinhardtii	*Anopheles stephensi*	*3-hydroxykynurenine transaminase (3-HKT)*	Kumar et al., 2013
		Pichia pastoris	*Aedes aegypti*	*Juvenile hormone acid methyl transferase* (*AeaJHAMT*)	Van Ekert et al., 2014
	Nanoparticles	*Chitosan*	*Aedes aegypti*	*Semaphorin-1a (sema1a)*	Mysore et al., 2013
			Aedes aegypti	*Single-minded (Sim)*	Mysore et al., 2014
			Aedes aegypti	*Vestigial gene (vg)*	Kumar et al., 2016
			Anopheles gambiae	*Chitin synthase 1 (AgCHS1), Chitin synthase 2 (AgCHS2)*	Zhang et al., 2010
	Liposomes	Lipofectamine 2000, Cellfectin, Transfectin, BMRIE-C	*Drosophila melanogaster*	*γ-Tubulin (γ-Tub)*	Whyard et al., 2009
			Drosophila sechellia	*γ-Tubulin (γ-Tub)*	Whyard et al., 2009
			Drosophila yakuba	*γ-Tubulin (γ-Tub)*	Whyard et al., 2009
			Drosophila pseudoobscura	*γ-Tubulin (γ-Tub)*	Whyard et al., 2009
		Lipofectamine 2000	*Drosophila suzukii*	*Alpha-coatomer protein (alpha COP), Ribosomal protein S13 (RPS13), Vacuolar H[+]-ATPase E subunit (Vha26)*	Taning et al., 2016
		Effectene	*Aedes aegypti*	*Inositol-requiring enzyme 1 (Ire-1), X-box binding protein-1 (Xbp-1), Caspase-1 (Cas-1), SREBP cleavage-activating protein (Scap), site-2 protease (S2P)*	Bedoya-Pérez et al., 2013
			Aedes aegypti	*Mitogen-activated protein kinase p38*	Cancino-Rodezno et al., 2010

**All target genes listed gave a more efficient knockdown than similar treatment with naked dsRNA.*

with L4440, a plasmid specifically designed to contain two T7 promoters flanking its multiple cloning site. Transformation of the bacteria with L4440 will lead to expression of dsRNA within the cell. This method was first utilized in the nematode *Caenorhabditis elegans* (Timmons and Fire, 1998; Timmons et al., 2001). Since then it has also been applied to insects, as described below.

Tian et al. (2009) first fed bacteria expressing *Chitin synthase A* (*SeChsA*) dsRNA to the beet armyworm *Spodoptera exigua* in 2009. They found that this delivery method not only lead to an efficient knockdown but also to reduced larval growth and insect death (Tian et al., 2009). Moreover, feeding dsRNA in this way induced sysRNAi as reduced transcript levels were observed in the trachea and epidermis of treated insects (Tian et al., 2009). A similar experiment was performed in *L. decemlineata,* where a knockdown was achieved for several target genes after the insects were fed with dsRNA-expressing bacteria. In addition, the insects showed increased mortality as well as reduced weight gain (Zhu et al., 2011). Finally, bacterial delivery of dsRNA targeting the *ultraspiracle* gene transcript was shown to improve

the RNAi efficiency through feeding in *H. armigera* (Yang and Han, 2014).

The mechanism through which this bacterial system facilitates dsRNA-uptake remains elusive. It is likely, however, that packaging dsRNA in a protective bacterial shell may have a stabilizing effect on the presence of dsRNA in the lumen of the digestive system. With regard to this, pre-treatment of the bacteria was shown to improve release of dsRNA in insects. Specifically, research has shown that sonication improved the efficiency of the induced RNAi response in *S. exigua* (Vatanparast and Kim, 2017). Therefore, it is possible that in this case weakening the bacterial cell wall through pretreatment stimulated dsRNA-uptake (Vatanparast and Kim, 2017). However, there is no concrete evidence for this and it remains to be proven.

The potential pathogenicity of *Escherichia coli* to several insect species implies that beneficial insects could be negatively affected by the use of this delivery system. Therefore, it has been suggested that a more appropriate approach might be achieved by focusing on symbiotic bacteria or yeasts. In the bloodsucking insect *Rhodnius prolixus,* and the western flower thrips *Frankliniella occidentalis,* knockdowns were achieved by delivering genetically engineered symbiotic bacteria capable of expressing dsRNA (Whitten et al., 2016). Furthermore, research suggests that this method of feeding might potentially lead to horizontal transfer of the RNAi signal in *R. prolixus* through symbiont-contaminated feces. More specifically, the eGFP-tagged symbiont could be detected in untreated younger instar insects after they had been fed with feces from treated insects. This implies that, through the use of this symbiont, possibly whole colonies could be targeted with only a minimal amount of bacteria (Whitten et al., 2016). Likewise, in the spotted wing fruit fly, *Drosophila suzukii,* it was found that feeding with a genetically modified symbiotic yeast led to induction of the RNAi response and resulted in reduced larval fitness (Murphy et al., 2016). Finally, it was shown that a knockdown could be achieved by feeding larvae of the mosquito species *Anopheles stephensi*, with a dsRNA delivery system consisting of transgenic microalgae (Kumar et al., 2013). These alternative delivery vehicles were suggested to have a negligible pathogenic impact on non-target insects, making them attractive options for application as ecologically friendly insecticides in the field.

Viruses

Viruses are extremely efficient at infecting cells and thus at delivering nucleic acid material into the intracellular environment. As RNAi is known to play a vital part in insect antiviral immunity (Bronkhorst and Van Rij, 2014), the use of viral delivery systems becomes an intriguing pitch, since the natural path of dsRNA cell entry is simulated. Furthermore, as many viruses have a very specific host range, a high degree of species-specificity could be achieved through careful virus screening and selection (Kolliopoulou et al., 2017). However, despite its many positive facets, viral delivery of dsRNA is still faced with a number of obstacles. Since many viruses have developed counter-measures against the RNAi mechanism, such as viral suppressors of RNAi, it is likely that not all viruses will be equally applicable as a delivery system (Swevers et al., 2013; Kolliopoulou et al., 2017). Some examples of successful experimental use of a viral delivery system are given below.

Kontogiannatos et al. (2013) found that a recombinant BmNPV baculovirus, encoding a juvenile hormone esterase specific hairpin, could induce gene-specific knockdown phenotypic effects in the Mediterranean corn borer, *Sesamia nonagrioides*. Surprisingly, despite careful selection of the viral carrier, the virus itself also seemed to affect the vitality of the insect. Therefore, it is likely that not all observed phenotypic effects could be attributed to the knockdown (Kontogiannatos et al., 2013). In *B. mori,* it was discovered that injection with a recombinant Sindbis virus (SINV) could achieve a knockdown of the transcription factor Broad-Complex (Br-C). Uhlirova et al. (2003) determined that engineering SINV to express an antisense RNA strand for Br-C led to reduced Br-C mRNA levels in this insect. This resulted in decreased rates of larval to pupal molting as well as developmental defects in those larvae that were able to reach adulthood (Uhlirova et al., 2003). It is of interest to mention that recombinant strains of SINV have additionally been used as a viral delivery system in the mosquito, *Aedes aegypti,* as a control measure for the RNAi-induced inhibition of dengue virus (Adelman et al., 2001).

While viral delivery systems show a lot of potential and are generally considered to be among the most efficient methods for dsRNA delivery, their *in vivo* application has not been widely investigated yet (Kolliopoulou et al., 2017). This may be due to the many safety issues that accompany this method of delivery. As not all insect viruses have a specific host range, a biosafety issue that needs to be thoroughly considered is cross-infection of beneficial insects with these highly virulent delivery systems. Furthermore, the ecological implications of releasing transgenic viruses into the field will need to be carefully considered, especially with regard to stability and turn-over time. A last point that will need to be evaluated is the possibility of transgene transfer from recombinant viral vesicles to wild type viruses (Kolliopoulou et al., 2017).

Nanoparticles

In order to increase stability and uptake efficiency, dsRNA can also be incorporated into a nanoparticle. Nanoparticles are polyplex-based delivery systems, consisting of either natural or synthetic polymer subunits. The most utilized nanoparticles are chitosan-derived.

Chitosan is a non-toxic, biodegradable molecule that can be obtained by deacetylation of chitin, one of the most abundant biopolymers in nature that is especially known for its structural function in the exoskeleton of arthropods (Dass and Choong, 2008). Due to its poly-cationic character and many amino groups, chitosan is able to bind dsRNA through electrostatic interaction. Chitosan:dsRNA nanoparticles are thus formed through self-assembly during the binding process. Incorporation of dsRNA into such a nanoparticle complex increases stability and uptake of the dsRNA *in vivo* (Zhang et al., 2010). This method of oral dsRNA delivery appeared to be especially effective in the African malaria mosquito *A. gambiae,* and the yellow fever mosquito *A. aegypti*. In these two species successful application of

chitosan-mediated dsRNA delivery led to a knockdown in various independent experiments (Zhang et al., 2010; Kumar et al., 2013; Mysore et al., 2013, 2014; Zhang X. et al., 2015).

Additionally, nanoparticles can consist of synthetically modified polymers. An interesting example is presented by He et al. (2013), who generated a fluorescent nanoparticle (FNP) to facilitate dsRNA uptake in the Asian corn borer *Ostrinia furnacalis*. FNP consists of a core chromophore, allowing FNP uptake to be observed through fluorescence microscopy, and two outer shell layers that facilitate binding to dsRNA and prevent aggregation in water (He et al., 2013). Complexation of FNP with dsRNA targeting the chitinase-like gene CHT10 caused RNAi silencing after feeding of the Asian corn borer larvae. The treatment resulted in molting defects, reduced larval weight and, eventually, death (He et al., 2013). Finally, Christiaens et al. (2018) developed a series of nanoparticles designed to specifically shield dsRNA from the degrading effects of the highly basic conditions (high pH) that are typical of the lepidopteran gut. In this research, nanoparticle stability in this alkaline environment was enhanced by modifying cationic polymethacrylate derivatives with protective guanidine side groups. Feeding larvae of the beet armyworm S. *exigua* with *chitin synthase B* dsRNA packaged in these pH-stable nanoparticles, led to the swift knockdown of the target gene as well as increased mortality in the experimental insects (Christiaens et al., 2018).

Liposomes

Another means to obtain an increased RNAi efficiency is through the use of lipid-based transfection agents; these vesicles are collectively referred to as liposomes. Liposomes form naturally when transfection agents are brought into an aqueous environment. During this process, the positively charged lipids envelop the negatively charged nucleic acid material, forming compact lipid bilayer particles similar to the phospholipid bilayer of the cell membrane (Dalby et al., 2004). Cell entry of the liposome-encapsulated dsRNA is then achieved through lipofection.

Whyard et al. (2009) first used liposomes to improve the RNAi efficiency in four distinctive drosophilid species: *D. melanogaster*, *D. sechellia*, *D. yakuba,* and *D. pseudoobscura*. By creating liposomes using commercial transfection agents, such as Lipofectamine 2000 and Cellfectin (both available at Invitrogen), the dsRNA-induced mortality was increased (Whyard et al., 2009). Furthermore, mRNA silencing could be improved in the mosquito species *A. aegypti* by feeding it dsRNA packaged in Effectene-liposomes (Cancino-Rodezno et al., 2010; Bedoya-Pérez et al., 2013). Notably, a similar approach also led to liposome-mediated uptake of dsRNA in the tick species *Rhipicephalus haemaphysaloides* (Zhang et al., 2018).

Proteinaceous Delivery Systems

The use of carrier proteins as delivery systems for dsRNA also provides an interesting prospect. Although research within this category remains limited, the best studied protein carriers are represented by the so-called cell-penetrating peptides or CPPs. One of the characterizing traits of these peptides is that they are able to facilitate entry into the intracellular environment while transporting molecular cargo, such as dsRNA. CPPs are short chain cationic peptides that usually consist of 10 – 30 amino acids with a high prevalence of basic residues, such as lysine and arginine (Durzyńska et al., 2015). Although there is still some speculation about the exact cellular mechanisms of CPP-mediated delivery, the most commonly accepted theory is that endocytosis plays an important part (Choi and David, 2014).

To induce an RNAi response through feeding in the Cotton boll weevil *Anthonomus grandis*, a fusion protein was designed containing a peptide transduction domain (PTD) as well as the dsRNA binding domain (DRBD) from the human protein kinase R (Gillet et al., 2017). PTD is an enhanced version of the arginine-rich CPP *trans*-activating transcriptional activator (TAT) of the human Immunodeficiency Virus 1 (HIV-1), engineered to have additional properties that promote endosomal escape of the fusion protein and its cargo into the cytoplasm (Vivès et al., 1997; Wadia et al., 2004). PTD-DRBD, in combination with dsRNA, forms a ribonucleoprotein particle (RNP) that is able to swiftly facilitate uptake in the insect gut. Furthermore, after feeding RNPs to *A. grandis*, Gillet et al. (2017) found that the knockdown for *chitin synthase II* was significantly increased compared to feeding with naked dsRNA.

The CPPs may represent an intriguing solution to the problem of RNAi sensitivity. This category of compounds encompasses a wide diversity of untested candidates and, therefore, many potentially interesting carriers for oral dsRNA delivery remain to be discovered. However, some caution must be exercised as these CPPs belong to a class of very general protein carriers, able of entering mammalian cells as well as arthropod cells (Fawell et al., 1994; Vivès et al., 1997; Wadia et al., 2004; Durzyńska et al., 2015).

Chemical Modifications of Small RNA Oligonucleotides

Although the RNAi response is not effective upon exposure to short dsRNA duplexes such as siRNAs, it is known that chemical modifications of these molecules can improve their stability and uptake (Joga et al., 2016). In fact, feeding of modified siRNAs targeting vital genes can lead to mortality in the diamondback moth, *Plutella xylostella* (Gong et al., 2011, 2013). Furthermore, the use of modified synthetic miRNA inhibitors, antagomirs and agomirs is an interesting approach that requires further investigation (Liu et al., 2014; Li X. et al., 2015; He et al., 2017).

Plastids: A Plant-Based Delivery System

dsRNA delivery through genetically engineered plants has been achieved for many insect species, often resulting in reduced growth and developmental delay (Baum et al., 2007; Mao et al., 2007; Pitino et al., 2011; Kumar et al., 2012). Since plants possess their own RNAi machinery, transgenic dsRNAs produced *in planta* are swiftly diced into siRNAs instead of accumulating (Vazquez et al., 2010). However, efficient uptake of dsRNA in insects requires that administrated duplexes have a minimum length of 60 bp (Bolognesi et al., 2012). Therefore, as insects take up siRNAs much less efficiently than long dsRNAs, the corresponding toxicity of the transgenic plant will also be reduced.

While it is debatable whether plants really can be classified as typical delivery systems, an interesting cross-over presents itself in the research of Zhang J. et al. (2015). In their research, a potato plant was genetically engineered to produce dsRNAs in chloroplasts, a plant organelle that lacks the RNAi pathway thus allowing long dsRNAs to accumulate here. The dsRNA molecules expressed in these transgenic plants targeted β-*actin* and *Shrub*. Feeding larvae of the Colorado potato beetle with leaves from this modified potato plant resulted in 100% RNAi-induced mortality (Zhang J. et al., 2015). Thus, the chloroplasts function as a kind of delivery system within the plant, ensuring that dsRNA of the correct length reaches the target insect. Naturally, this discovery has major implications for the further mode of application of RNAi insecticides in the field.

Current RNAi-Based Insecticides

The RNAi response has been thoroughly researched in the Western corn rootworm (WCR) *D. virgifera virgifera* (Baum et al., 2007; Rangasamy and Siegfried, 2012; Wu et al., 2017, 2018; Camargo et al., 2018). The WCR is a well-known pest insect with a significant economic impact on the maize harvest in the United States, as well as in Europe. In fact, it is estimated that in the United States alone, crop losses due to this plague amount to more than $1 billion annually (Sappington et al., 2006). The WCR has a very sensitive RNAi response to oral administration of dsRNA and many target genes with lethal or detrimental effects have already been identified in this insect (Baum et al., 2007). It is therefore not so surprising that the first RNAi-based insecticides for the control of this insect have already been approved by the United States Environmental Protection Agency (EPA)[1].

The proposed RNAi insecticide, developed by Monsanto and Dow Agrosciences, will be known as SmartStax Pro®. This plant-incorporated protectant (PIP) will employ a pyramid strategy: several different Bt-proteins, as well as dsRNA targeting the WCR *Snf7* gene, will be expressed in this plant (Head et al., 2017). Bt-proteins, also known as crystalline toxins, insert themselves into the gut epithelium of the insect, causing gut paralysis and resulting in the death of the insect (Copping and Menn, 2000). On the other hand, downregulation of Snf7, a gene that plays an essential role in protein trafficking, will also result in mortality (Bolognesi et al., 2012). This combined strategy is designed to lead to the swift death of the insect, while also reducing the chances that insects will develop resistance against this PIP (Head et al., 2017). As RNAi is a budding technology within the field of agriculture, it is likely only a matter of time before SmartStax Pro® and other, yet to be discovered insecticidal strategies, will appear on the market.

INSECT VIRAL INFECTIONS AND RNAi-BASED ANTIVIRAL IMMUNITY

Insects represent the largest group of animals on Earth in terms of biodiversity, with an estimated number of 5.5 million different species (Stork, 2018). This diversity reflects in a matching range of infecting viruses, which in addition to positively or negatively affecting insect populations, can also have a major impact on human well-being (Miller and Ball, 1998; Roossinck, 2011). In this section, important concepts regarding the patterns of viral infection pathogenesis will be addressed. Then, relevant insect disease-causing and persistent viral infections will be reviewed. At last, the use of viruses for insect biological control, as well as the potential use of the RNAi technology to protect beneficial insects from harmful viral infections will be discussed.

Patterns of Viral Infection – From Lethality to Non-pathogenicity

Viral infections can be classified according to their effect on the host, ranging from presenting no obvious harmful symptoms to being highly pathogenic or even lethal. These distinct outcomes exist in a variable range and are generally linked to different levels of viral particle production. Therefore, although this classification is not established beyond doubt, efforts have been made to classify them in three main groups, namely: acute, persistent and latent. Acute infections are characterized by high levels of viral replication and increased viral particle production. Generally, these infections are limited in time; either by the death of the host or by the clearance of the virus by the host immune system. On the other hand, persistent infections are characterized by constant, but relatively low, levels of viral replication and of viral particle production. These infections can manifest themselves for longer periods of time as often an equilibrium is established between the attack and counterattack strategies of the virus-host system. Although some persistent infections have the potential to cause variable levels of pathogenic effects, clear effects on fitness are often not observed. Finally, latent infections consist in the presence of the viral genome in the host cell without actual production of viral particles. The viral genome (in DNA form) can remain latent either as an episome or can be integrated in the host genome as a provirus. During this latency, viruses maintain the potential to resume viral replication and start producing viral particles, a process which is referred to as reactivation (Boldogh et al., 1996; Swevers et al., 2013; Nathanson and González-Scarano, 2016).

Additionally, the terms chronic and slow infection are often used, although mostly in the context of human viral diseases. A chronic infection is generally defined as the outcome of an acute infection in which neither host mortality nor virus clearance occur, meaning a persistent or latent outcome derived from an acute infection. In a slow infection, viral replication and particle production are slow but not constant, increasing overtime (Boldogh et al., 1996; Virgin et al., 2009). **Figure 2** summarizes these different patterns of viral infection.

Acute Viral Infections in Insects – Disease in Beneficials and Control Strategies for Pests

Clear examples of acute viral infections are the ones affecting beneficial insects, such as bees and economically important lepidopteran species. Recently, worrying losses in bee populations

[1]https://www.epa.gov/newsreleases/epa-registers-innovative-tool-control-corn-rootworm

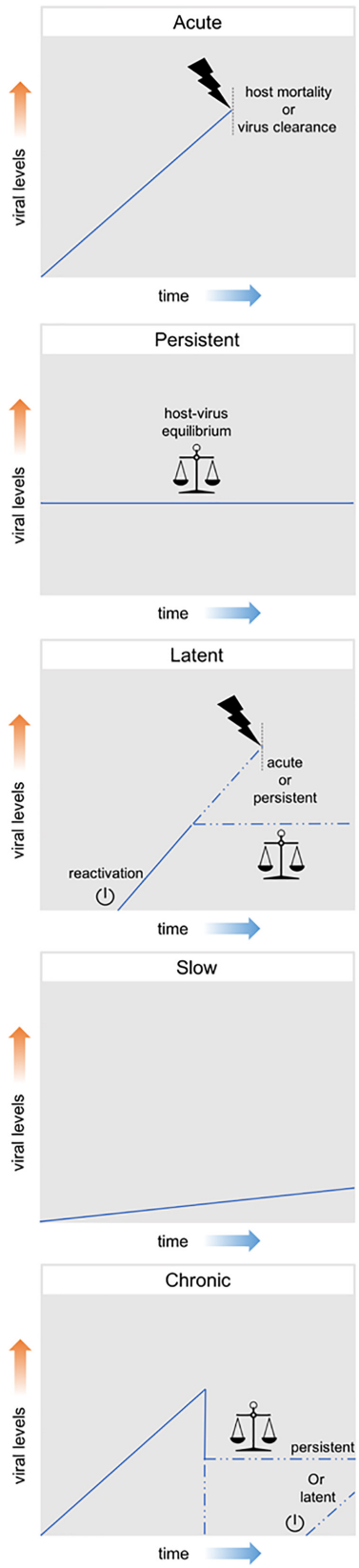

FIGURE 2 | Continued

FIGURE 2 | Patterns of viral infection. Acute infections are represented by a high increase in viral levels and are limited in time either by the death of the host or by the clearance of the virus by the host immune system. Persistent infections consist in constant, but relatively low, viral levels and can manifest themselves for long periods of time. Latent infections consist in the presence of the viral genome in the host cell without actual production of viral particles. During this latency, viruses maintain the potential to resume viral replication and start producing viral particles (reactivation). Chronic infections are generally defined as the outcome of an acute infection in which neither host mortality nor virus clearance occur, meaning a persistent or latent outcome derived from an acute infection. Slow infections are characterized by a slow, but not constant, increasing in viral levels overtime.

have been observed. These are typically associated with environmental pollution, specific pesticides or the presence of parasitic infections (Goulson et al., 2015). However, the impact of diseases caused by different viruses, though often overlooked, cannot be ignored. In fact, several studies have identified multiple harmful viruses infecting honeybees and bumblebees (Bailey et al., 1963, 1964, 1982; Bailey, 1969; Bailey and Woods, 1974, 1977; Benjeddou et al., 2001; Maori et al., 2007; Rana et al., 2011; Granberg et al., 2013; Chen et al., 2014; Meeus et al., 2014; Roberts and Anderson, 2014; Ravoet et al., 2015; Ueira-Vieira et al., 2015; Benaets et al., 2017; Natsopoulou et al., 2017). In addition, in the specific case of honeybees, losses have also been linked to the so-called Colony Collapse Disorder (CCD) which, in general terms, results in the sudden death of colonies. Although the specific causes of this phenomenon are still to be unraveled, it is thought that the aforementioned factors have been correlated with it, including the prevalence of several viral diseases (Brutscher and Flenniken, 2015). Another relevant example can be found in silkworms, whose viral diseases often cause sizeable economic losses to the sericulture industry in Asiatic countries (Sanakal et al., 1996; Jiang et al., 2016; Gani et al., 2017; Chen et al., 2018). **Table 2** presents an overview of viruses with significant impact on bees and silkworms. In addition, viruses affecting economically important shrimps are also contemplated.

In addition to the viruses that cause harmful infections in beneficial insects, others can use insects as vectors to infect other animals or plants – arthropod-borne viruses, or arboviruses. Important examples are the ones responsible for human diseases, transmitted via mosquitoes (e.g., Chikungunya Virus, Dengue Virus, Yellow Fever Virus, West Nile virus, Japanese Encephalitis Virus and Zika Virus) (Ng et al., 2011; Shi et al., 2015; Minkeu and Vernick, 2018); and plant viruses which may be deleterious to crops (Ng and Perry, 2004; Hohn, 2007; Whitfield et al., 2015).

Insect viruses can also have a positive impact on human wellbeing. This is the case for viruses whose hosts are considered pest species. Because of this, insect viruses have long been researched for use as pest control agents. Some examples of viruses as delivery systems for RNAi were already discussed. Until recently, the use of microbial bio-insecticides has remained stagnant. However, increased concern about health and pollution hazards, environmental awareness, increased governmental restrictions on synthetic pesticides, advances in farming technology and other factors are fueling a solid growth

TABLE 2 | Overview of viruses with a significant impact on beneficial insects and on economically relevant shrimps.

Beneficial arthropods	Infecting virus	Genome - Taxonomy (Genus)	Host stage	Symptoms acute stage	Reference
Honey bee					
	Sacbrood Virus	+ssRNA – Iflavirus	Pupae/Adults	Failure to pupate, death	Gisder and Genersch, 2017
	Varroa destructor virus-1	+ssRNA – Iflavirus	Pupae/Adults	Deformed wings, shortened lifespan, Colony collapse	Natsopoulou et al., 2017
	Chronic Bee Paralysis Virus	+ssRNA – Noda/Tombus-virus	Adults	Paralysis, death	Bailey et al., 1963
Honey bee and Bumblebee					
	Black Queen Cell Virus	+ssRNA – Cripavirus	Pre-/Pupae	Decomposed and black pre-/pupae	Bailey and Woods, 1977
	Kashmir Bee Virus	+ssRNA – Dicistrovirus	Adults/pupae	Reduced fecundity, death	Bailey and Woods, 1977; Meeus et al., 2014
	Israeli Acute Paralysis Virus	+ssRNA – Dicistrovirus	Adults	Reduced fecundity, paralysis, darkening, hair loss, death	Chen et al., 2014; Meeus et al., 2014
	Acute Bee Paralysis Virus	+ssRNA – Dicistrovirus	Adults	Paralysis, darkening, hair loss, death	Bailey et al., 1963; de Miranda et al., 2010
	Deformed Wing Virus	+ssRNA – Iflavirus	Pupae/Adults	Deformed wings, shortened lifespan, Colony collapse	Benaets et al., 2017
	Slow Bee Paralysis Virus	+ssRNA – Iflavirus	Pupae/Adults	Paralysis of anterior legs, death	Bailey and Woods, 1974; Niu et al., 2016
Silkworm					
	Bombyx mori Nucleopolyhedrosis virus	dsDNA – Alphabaculovirus	Larvae/Pupae/Adults	Molting failure, hyperactivity, translucent skin, white hemolymph, death	Steinhaus, 1949
	Bombyx mori Cypovirus	dsRNA – Cypovirus	Larvae/Pupae/Adults	Delayed larval growth, failure to pupate	Payne and Rivers, 1976
	Infectious Flacherie Virus	+ssRNA – Iflavirus	Larvae	Flaccidity, retarded growth, death	Himeno et al., 1974; Shimizu, 1975
	Bombyx mori Densovirus	ssDNA – Iteravirus	Larvae	Flaccidity, retarded growth, death	Shimizu, 1975
Penaeid shrimp					
	Taura Syndrome Virus	+ssRNA – Cripavirus	All stages	Lethargy, epithelial necrosis of entire body, death	Hasson et al., 1995
	Infectious Hypodermal and Hematopoietic Necrosis Virus	ssDNA – Brevidensovirus	Juvenile, Adults	Cuticular deformities, impaired growth, death	Kalagayan et al., 1991; Shike et al., 2000; Prasad et al., 2017
	Yellow Head Virus	+ssRNA – Okavirus	All stages	Yellow discoloration, systemic necrosis, death	Sittidilokratna et al., 2008
	White Spot Syndrome Virus	dsDNA – Whispovirus	Juveniles, Adults	White spots, red/yellow discoloration, death	Sudha et al., 1998; Escobedo-Bonilla et al., 2008
	Infectious Myonecrosis Virus	dsRNA – Unclassified	Juveniles, Subadults	White spots in, and necrosis of, skeletal muscles, death	Poulos et al., 2006; Prasad et al., 2017

in the biopesticide market. To be applicable as biopesticides, the viruses must comply with several requirements: they must be specific, highly virulent, and lethal to the targeted pest insect, while preferably also inducing epizootics (Fuxa, 1991). Despite the large diversity in entomopathogenic viruses, commercial research and use is mainly restricted to the family of *Baculoviridae*. Even though baculoviruses can infect arthropods belonging to several insect orders, only Lepidoptera-specific viruses belonging to the genera Nucleopolyhedrosis virus (Alphabaculovirus) and Granulovirus (Betabaculovirus) have been developed into commercial products (Lacey et al., 2015). The widescale use of Baculoviruses can be explained by the already extant knowledge and expertise on this family of viruses as well as their useful characteristics. Baculoviruses display remarkable specificity and infections are highly lethal. During the late stage of infection, Baculoviruses produce occlusion bodies (OBs). These OBs increase resistance to the environment and make baculoviral insecticides easier to store and apply in the field. The major drawbacks of viral biopesticides are: the current absence of practical mass

production systems resulting in high production costs; the (relatively) slow kill rate; short shelf-life and inconsistent field performance (Lacey et al., 2015; Arthurs and Dara, 2018).

To date, the most widely used viral biopesticide is the *H. armigera* NPV, with over 10 manufacturers in China alone and new product registrations occurring on a yearly basis[2]. Other important viral agents are the *S. exigua* NPV, *S. litura* NPV and *Cydia pomonella* GV. A comprehensive list of virus-based commercial insecticide products has been assembled by Lacey et al. (2015) and Arthurs and Dara (2018).

Virome and Insect Persistent Viral Infections

The idea that persistent viral infections are ubiquitous has recently started to emerge. In fact, the word 'virome' is often used nowadays and this field of research has gained a lot of interest in insects. This is due to three main reasons: (1) the advent of genomic and transcriptomic techniques; (2) the growing idea that the microbiome, including the virome, has the potential to interfere (both positively and negatively) in many biological processes; (3) and the establishment of viruses as crucial drivers of evolution. The latter has gained interest not only due to the ability of genetic mobile elements to cause mutagenesis, but also due to their potential capacity of providing hosts with beneficial gene-regulatory machinery (Swevers et al., 2013; Massart et al., 2014; Bikel et al., 2015; Koonin, 2016; Chuong et al., 2017; Nouri et al., 2018).

In addition to the major direct impact of insect viruses on human life, as reviewed above; insect viral infections might play crucial roles on the ecological equilibrium of our planet. Therefore, it is of great interest to understand the mechanisms underlying the establishment and maintenance of insect viromes. In this context, persistent viral infections gain special relevance. Since these do not always cause obvious pathogenesis, their existence is often neglected. However, recently, identification of persistent viruses in insects has become recurring, with several reported cases both *in vivo* and in cultured cells (Katsuma et al., 2005; Habayeb et al., 2006; Li et al., 2007; Wu et al., 2010; Jovel and Schneemann, 2011; Iwanaga et al., 2012; Ma et al., 2014; Suzuki et al., 2015; Swevers et al., 2016; Santos et al., 2018). Interestingly, whether an infection is persistent or acute does not depend only on the virus itself, but also on the host. As demonstrated by several loss-of-function and deep sequencing studies, the role of RNAi in the established equilibrium between the persistent virus and the insect host is clear (Wu et al., 2010; Goic et al., 2013; Zografidis et al., 2015; Petit et al., 2016; Santos et al., 2018). However, the possible role of still unidentified factors has to be considered. A particularly interesting example is the Flock House Virus (FHV), which is known to cause persistent infections in lepidopteran cell lines and acute infections in crickets and flies (Longdon et al., 2012; Swevers et al., 2016). Remarkably, and by still unknown mechanisms, FHV is able to cause the two types of infection in *D. melanogaster* S2 cells (Goic et al., 2013). Further research regarding the diversity of insect viromes and the (RNAi-based) mechanisms involved in persistent-to-acute viral-host interactions would be of great value to understand their influence on several physiological processes; as well as their potential to contribute to efficient strategies to protect beneficial insects from harmful pathogens and to control dangerous pest insects.

RNAi-Based Antiviral Immunity to Protect Beneficial Insects

As discussed in the previous sections, it is clear that a deep understanding of the interactions between insects and their viruses is of great value. In addition, the current demand to control insect viral infections stresses the need to search for original approaches to fight these infections. Since RNAi is the main insect antiviral immune response, it is only logical to think of this mechanism as a potential form to protect beneficial insects against harmful viral infections.

In this context, the use of virus specific dsRNA aiming to trigger the RNAi pathway against viral infections has already been explored in bees. More specifically, delivery of targeted virus dsRNA by injection or feeding has been demonstrated to be effective in protecting honeybees against several relevant viral infections (Maori et al., 2009; Hunter et al., 2010; Liu et al., 2010; Desai et al., 2012; Flenniken and Andino, 2013; Brutscher et al., 2017). In line with these findings, feeding virus-specific dsRNA to bumblebees has been demonstrated to act against the IAPV infection (Piot et al., 2015). Surprisingly, delivery of non-specific dsRNA has revealed to trigger an antiviral response in both honeybees and bumblebees (Flenniken and Andino, 2013; Piot et al., 2015; Brutscher et al., 2017).

Similar approaches have been investigated in lepidopteran insects with promising results. First, transfection or expression of virus-specific dsRNA in cell lines was shown to result in lower viral levels (Valdes et al., 2003; Isobe et al., 2004; Kanginakudru et al., 2007). Then, transgenic *B. mori* silkworms expressing virus-specific dsRNA have been reported to exhibit higher survival rates upon NPV infection on several occasions. In fact, this approach has been demonstrated to be effective in a commercially valuable silkworm strain (Isobe et al., 2004; Kanginakudru et al., 2007; Subbaiah et al., 2013). In addition, a similar strategy has been successfully tested to obtain protection of the silkworm to the *B. mori* cytoplasmic polyhedrosis virus (BmCPV) (Jiang et al., 2017). At last, injection of virus-specific dsRNA has been demonstrated to protect the mealworm beetle, *Tenebrio molitor,* against viral infection as well (Valdes et al., 2003). Notably, comparable approaches have also been successfully applied in two economically relevant crustacean species (Robalino et al., 2004, 2005; Tirasophon et al., 2005; Yodmuang et al., 2006; Attasart et al., 2010; Labreuche et al., 2010; Bartholomay et al., 2012). Recently, *Trichoplusia ni* High Five cells overexpressing key components of the RNAi machinery, namely *B. mori* Dicer2 and Argonaute2, have been reported to present reduced CrPV-induced mortality (Santos et al., 2018). This tactic remains to

[2]https://agrow.agribusinessintelligence.informa.com/AG012300/Biopesticide-development-in-China

TABLE 3 | Summary of the investigated strategies to obtain improved antiviral defense in insects and in economically relevant crustacean species.

Species	*In vivo*/*in vitro*	Virus	Strategy	Outcome	Reference
A. mellifera, the western honeybee	*In vivo*	IAPV	Oral delivery of virus-specific dsRNA	Lower mortality; lower viral transcript levels	Maori et al., 2009
A. mellifera, the western honeybee	*In vivo* - colonies	IAPV	Oral delivery of Remebee-I (a IAPV-specific dsRNA product)	Florida colony: higher bee population per hive; higher adult forager activity; higher hive total weight gain (honey); Pennsylvania colony: higher hive total weight gain (honey); lower Nosema levels.	Hunter et al., 2010
A. mellifera, the western honeybee	*In vivo*	DWV	Oral delivery of virus-specific dsRNA	Lower proportion of adult bees with deformed wings; lower viral transcript levels; adult survival was not affected	Desai et al., 2012
A. mellifera, the western honeybee	*In vivo*	SINV-GFP	Injection of virus-specific and unspecific dsRNA	Lower viral abundance	Flenniken and Andino, 2013
A. mellifera, the western honeybee	*In vivo*	SINV-GFP	Injection of virus-specific and unspecific dsRNA	Lower viral abundance	Brutscher et al., 2017
A. cerana, the eastern honeybee	*In vivo*	CSBV	Oral delivery of virus-specific dsRNA	Lower larvae mortality; lower viral transcript levels	Liu et al., 2010
A. Cerana, the eastern honeybee	*In vivo*	CSBV	Oral delivery of virus-specific dsRNA	Lower larvae mortality; lower viral transcript levels	Zhang et al., 2016
B. terrestris, the bumblebee	*In vivo*	IAPV	Oral delivery of virus-specific and unspecific dsRNA	Lower viral transcript levels in the head	Piot et al., 2015
Tenebrio molitor, the mealworm beetle	*In vivo*	AcNPV-GFP	Injection of virus-specific dsRNA	Lower mortality	Valdes et al., 2003
Spodoptera frugiperda, the fall armyworm	*In vitro* – Sf21 cells	AcNPV-GFP	Transfection with virus-specific dsRNA	Reduced fluorescence; lower viral protein levels; lower viral transcript levels; lower amount of viral particles; reduced cell morphological changes	Valdes et al., 2003
B. mori, the silkworm	*In vitro* – BmN cells	BmNPV	Expression (transient transfection) of virus-specific dsRNA	Lower virus titer in the cell culture medium	Isobe et al., 2004
B. mori, the silkworm	*In vitro* – BmN cells	BmNPV	Expression (constitutive transfection) of virus-specific dsRNA	Lower virus titer in the cell culture medium; lower viral transcript levels in the cells	Isobe et al., 2004
B. mori, the silkworm	*In vivo*	BmNPV	Transgenic animals expressing virus-specific dsRNA	Reduced levels of viral DNA in the hemolymph	Isobe et al., 2004
Spodoptera frugiperda, the fall armyworm	*In vitro* – Sf9 cells	AcNPV	Expression (constitutive transfection) of virus-specific dsRNA	Lower virus titer in the cell culture medium; reduced OBs in the cells	Kanginakudru et al., 2007
B. mori, the silkworm	*In vivo*	BmNPV and GFP-BmNPV	Transgenic animals expressing virus-specific dsRNA	Lower mortality; reduced levels of OBs in the hemolymph; reduced fluorescence; reduced viral protein levels; lower viral transcript levels	Kanginakudru et al., 2007
B. mori, the silkworm	*In vivo*	BmNPV	Transgenic animals expressing single or multiple virus-specific dsRNAs (targeting different genes)	Lower mortality rates; lower levels of OBs in the hemolymph; lower viral DNA levels; reduced viral protein levels	Subbaiah et al., 2013
B. mori, the silkworm	*In vivo*	BmCPV	Transgenic animals expressing single or multiple virus-specific dsRNAs (targeting different genes)	Lower mortality rates; lower viral transcript levels	Jiang et al., 2017
T. ni, the cabbage looper	*In vitro* – High five cells	CrPV	Expression (transient transfection) of BmDicer2 and BmArgonaute2	Reduced cell mortality	Santos et al., 2018
Litopenaeus vannamei, the pacific white leg shrimp	*In vivo*	TSV and WSSV	Injection of unspecific dsRNA	Lower mortality; lower accumulation of viral particles; lower levels of tissue damage.	Robalino et al., 2004
Litopenaeus vannamei, the pacific white leg shrimp	*In vivo*	WSSV	Injection of virus-specific dsRNA	Lower mortality	Robalino et al., 2005

(Continued)

TABLE 3 | Continued

Species	*In vivo/in vitro*	Virus	Strategy	Outcome	Reference
Litopenaeus vannamei, the pacific white leg shrimp	*In vivo*	WSSV	Injection of unspecific dsRNA of multiple sizes (50–200 bp)	Lower mortality	Labreuche et al., 2010
Litopenaeus vannamei, the pacific white leg shrimp	*In vivo*	WSSV	Injection of virus-specific and unspecific dsRNA	Lower mortality	Bartholomay et al., 2012
Penaeus monodon, the Asian tiger shrimp	*In vitro* – Oka cells	YHV	Transfection of virus-specific and unspecific dsRNA	Reduced cytopathic effects; lower viral transcript levels in the cell medium; lower viral protein levels	Tirasophon et al., 2005
Penaeus monodon, the Asian tiger shrimp	*In vivo*	YHV	Injection of virus-specific dsRNA	Lower viral transcript levels; reduced mortality	Yodmuang et al., 2006
Penaeus monodon, the Asian tiger shrimp	*In vivo*	YHV	Injection of unspecific dsRNA	Lower mortality	Yodmuang et al., 2006
Penaeus monodon, the Asian tiger shrimp	*In vivo*	DNV	Injection of virus-specific dsRNA	Lower viral DNA levels	Attasart et al., 2010

IAPV, Israel Acute Paralysis Virus; DWV, Deformed Wing Virus; SINV, Sindbis Virus; CSBV, Chinese Sacbrood Virus; NPV, Nuclear Polyhedrosis Virus; CPV, Cytoplasmic Polyhedrosis Virus; CrPV, Cricket Paralysis Virus; TSV, Taura Syndrome Virus; WSSV, White Spot Syndrome Virus; YHV, Yellow Head Virus; DNV, Densovirus; GFP, Green Fluorescence Protein; OBs, Occlusion Bodies; Ac, Autographa californica; Bm, B. mori.

be tested *in vivo* and with regard to infections by other viruses. However, since RNAi is a broadly-acting antiviral immune mechanism in insects and considering that improvement of the RNAi response is observed in transgenic *B. mori* larvae overexpressing Argonaute2 (Li Z. et al., 2015), this constitutes a promising approach. **Table 3** presents a summary of the investigated strategies to obtain improved antiviral defense in insects, as well as in economically relevant crustacean species.

Considering these potential strategies to control insect viral diseases, the use of genetically engineered insects deserves special attention due to the risks of environmental contamination. In fact, up to date, the release of transgenic insects has been limited to sterile animals, with the aim of reducing pest species populations (Reeves and Phillipson, 2017; Wilke et al., 2018). However, the use of genetically engineered beneficial insects, as would be the eventual goal for various species of bees, would mostly require the maintenance of viable populations and therefore the use of fertile transgenic animals. On the other hand, the breeding of domestic silkworms closely depends on humans and is, therefore, highly controlled. Thus, the use of transgenic moths in sericulture is more feasible and might hold great potential with lower risks compared to the creation of other valuable transgenic species. In this context, it is important to keep in mind that several challenges will need to be overcome before such innovative strains can be obtained. For example, special regard should be payed to the productivity and fitness of such transgenic lines, as well as to the maintenance of the phenotype throughout several generations (Jiang and Xia, 2014). Furthermore, a last consideration should be given to the eventual development of resistance by these viruses. Thus, before actual implementation, approaches to minimize this issue should be contemplated, such as the use of inducible expression systems which would be activated only in the case of viral disease, or the alternate expression of different transgenes.

CONCLUSION AND FUTURE PROSPECTS

It is clear that the impact of RNAi technology and of insect viral infections on human life cannot be underestimated. In fact, in agricultural and industrial contexts, this is likely to become

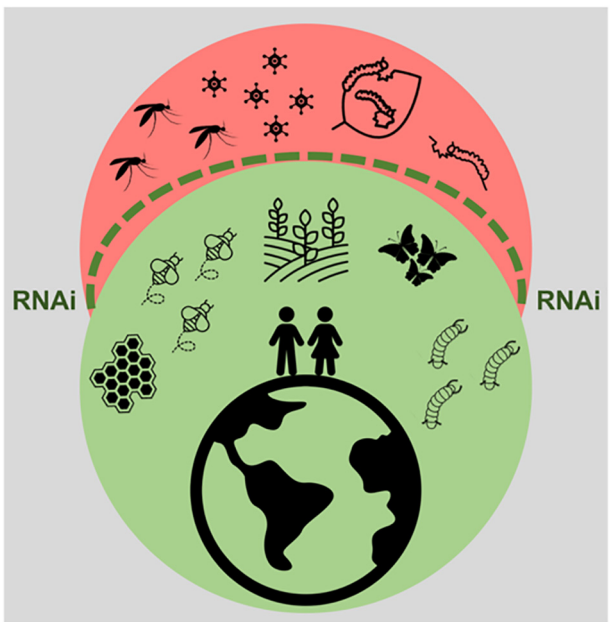

FIGURE 3 | Impact of RNAi technology and of insect viral infections in human life. The RNAi technology, represented by the dashed green line, promises to exert protection against pest insects, such as the ones threatening crop production and the ones constituting vectors for viral diseases. This technology also holds potential to protect beneficial insects from harmful viral infections. In addition, insect viruses constitute important bio-insecticides.

even more prominent in the years to come. In the first place, RNAi technology poses great potential to contribute to highly specific insect control strategies through delivery of dsRNA to pest species. Although the application of this technique has so far been limited by the variable RNAi efficiency amongst economically important insects, delivery systems provide a promising solution. Of these, an incredible wealth of options is available, with encouraging success rates. In the second place, insect baculoviruses form an interesting class of highly species-specific insecticides, which are currently under use. Due to the increasing knowledge on insect virus diversity, the potential use of other viral families cannot be excluded. In addition, the RNAi mechanism shows great potential as a combatant against viruses, which form an undeniable threat to beneficial insects. The efficient delivery of virus-specific dsRNA is a promising approach to protect beneficial insects such as pollinators. In addition, in the case of silkworms, the use of transgenic lines, resistant against such viral infections seems possible in the foreseeable future. This paper reviews the current literature on practical applications based on insect viruses and RNAi, as summarized in **Figure 3**. Although some of the described aspects still need to be thoroughly researched and therefore have to be considered with caution, this is an undeniably exciting field of research, full of potential.

AUTHOR CONTRIBUTIONS

EV, DS, LM, T-WV, and JB conceived the manuscript. EV, DS, LM, and T-WV wrote the parts of the text. EV and DS worked on the structure and prepared the final version of the manuscript. EV, DS, and LM prepared the figures. EV, DS, and T-WV prepared the tables. JB corrected the manuscript and suggested further improvements.

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REVIEW
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Prostaglandins and Other Eicosanoids in Insects: Biosynthesis and Biological Actions

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This essay reviews the discoveries, synthesis, and biological significance of prostaglandins (PGs) and other eicosanoids in insect biology. It presents the most current – and growing – understanding of the insect mechanism of PG biosynthesis, provides an updated treatment of known insect phospholipase A_2 (PLA_2), and details contemporary findings on the biological roles of PGs and other eicosanoids in insect physiology, including reproduction, fluid secretion, hormone actions in fat body, immunity and eicosanoid signaling and cross-talk in immunity. It completes the essay with a prospectus meant to illuminate research opportunities for interested readers. In more detail, cellular and secretory types of PLA_2, similar to those known on the biomedical background, have been identified in insects and their roles in eicosanoid biosynthesis documented. It highlights recent findings showing that eicosanoid biosynthetic pathway in insects is not identical to the solidly established biomedical picture. The relatively low concentrations of arachidonic acid (AA) present in insect phospholipids (PLs) (< 0.1% in some species) indicate that PLA_2 may hydrolyze linoleic acid (LA) as a precursor of eicosanoid biosynthesis. The free LA is desaturated and elongated into AA. Unlike vertebrates, AA is not oxidized by cyclooxygenase, but by a specific peroxidase called peroxinectin to produce PGH_2, which is then isomerized into cell-specific PGs. In particular, PGE_2 synthase recently identified converts PGH_2 into PGE_2. In the cross-talks with other immune mediators, eicosanoids act as downstream signals because any inhibition of eicosanoid signaling leads to significant immunosuppression. Because host immunosuppression favors pathogens and parasitoids, some entomopathogens evolved a PLA_2 inhibitory strategy activity to express their virulence.

Keywords: insects, reproduction, prostaglandins, immunity, hormone signaling, phospholipase A_2

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INTRODUCTION

Prostaglandins (PGs) and other eicosanoids are oxygenated metabolites of three C20 polyunsaturated fatty acids (PUFAs), 20:3n-6, 20:4n-6, and 20:5n-3. Of the three, conversion of 20:4n-6, arachidonic acid (AA), into eicosanoids is the most widely considered pathway. Although 20:5n-3, eicosapentaenoic acid has been detected in terrestrial animals, it occurs in higher proportions of total phospholipid fatty acids in marine and aquatic invertebrates and vertebrates. In this essay we focus on AA metabolism, which is converted into three broad groups of

eicosanoids, PGs, epoxyeicosatrienoic acids and a collection of lipoxygenase (LOX) products, such as hydroxyeicosatrienoic acids and leukotrienes. All three groups of eicosanoids occur in insects.

Eicosanoids are generally biosynthesized within cells. They are exported into circulating blood or, in insects, hemolymph, where they may act in autocrine or paracrine mechanisms through cell surface receptors. Here, we review the three major steps of PG biosynthesis in insects. The first step is the release of PUFAs from membrane phospholipids (PLs) by phospholipase A_2 (PLA_2) (**Figure 1**). The second step marks a major departure from the biomedical background, because genes encoding the cyclooxygenase (COX) responsible for converting C20 PUFAs into PGs do not occur in the known insect genomes. In an alternative insect mechanism, a peroxidase (peroxinectin: Pxt) catalyzes the formation of PGH_2, with the five-membered ring structure that characterizes PGs (Park et al., 2014). The third step depends on cell-specific enzymes that convert PGH_2 into any of several PGs, PGE_2 (Ahmed et al., 2018). Here, we treat new discoveries in insect PG biosynthesis.

Stanley (2000), a monograph covering all invertebrates, and Stanley and Kim (2014) provide detailed chemical structures and outline eicosanoid biosynthetic pathways. We do not repeat the chemical structures in detail here, with the exception of structures of three major eicosanoid groups to facilitate reading without looking up the structures. The purpose of this review is to integrate the new information into a slightly clearer picture of eicosanoid biosynthesis with current transcriptome-based functional studies. In addition, eicosanoid actions in insects are explained in different physiological processes of reproduction, metabolism, and immunity.

DISCOVERY AND EXPANSION OF KNOWN INSECT PLA_2S

PLA_2 was initially discovered from snake venom components (Davidson and Dennis, 1990) and in mammalian systems (Kramer et al., 1989). Later, as non-disulfide bond-containing PLA_2s were recognized, it became necessary to classify PLA_2s into groups (Dennis, 1994). At least 16 PLA_2 groups are now recognized, including five major types: secretory PLA_2s ($sPLA_2$s: Groups I–III, V, IX, X, XI, XII, XIII, XIV, and XV), calcium-dependent intracellular PLA_2 ($cPLA_2$: Group IV), calcium-independent intracellular PLA_2 ($iPLA_2$: Group VI), Lipoprotein-associated PLA_2 ($LpPLA_2$: Groups VII and VIII), and adipose phospholipase A_2 ($AdPLA_2$: Group XVI) (Vasquez et al., 2018). $sPLA_2$ and $LpPLA_2$ are secretory proteins that act on extracellular membrane lipids, while $cPLA_2$ and $iPLA_2$ catalyze hydrolysis of fatty acids from intracellular PLs. However, the localization of $LpPLA_2$ and $AdPLA_2$ remains unclear.

PLA_2 actions include digestion of dietary lipids, remodeling cellular membranes, signal transduction, host immune defenses, and production of various lipid mediators or inactivation of a lipid mediator. There also are non-catalytic PLA_2s that act as ligands by binding to receptors or binding proteins (Triggiani et al., 2005). Here, we briefly introduce general characters of five major types of PLA_2s before discussing various insect PLA_2s.

Classification of PLA_2s

$sPLA_2$s are small enzymes (14–18 kDa) with calcium activation (Schaloske and Dennis, 2006). They contain highly conserved amino acid residues and sequences. All organisms express $sPLA_2$, including viruses (Farr et al., 2005), bacteria (Sato and Frank, 2004), plants (Ståhl et al., 1999), and invertebrates (Kishimura et al., 2000), where they exert various actions.

$iPLA_2$, PNPLA9, or $iPLA_2\beta$, is a calcium-independent PLA_2 that acts in membrane remodeling (Ackermann et al., 1994). The longest variant of $iPLA_2$ has a catalytic dyad of Ser/Asp and is comprised of seven ankyrin repeats, a linker region, and a patatin-like α/β hydrolase catalytic domain (Larsson Forsell et al., 1999).

$cPLA_2$ is classified into Group IVA of the PLA_2 superfamily (Clark et al., 1991). It is an 85 kDa protein and regulated by intracellular calcium. This enzyme is widely distributed in cells throughout most types of human tissues and consists of two functional domains C2 and α/β hydrolase. Calcium-binding to the C2 domain causes translocation of the protein to a PL membrane (Channon and Leslie, 1990). $cPLA_2$ catalyzes AA release from various PLs and has lysophospholipase and *trans*-acylase activities (Reynolds et al., 1991).

Platelet-activating factor (PAF) is a potent PL mediator that plays a major role in clotting and inflammatory pathways (Prescott et al., 2000). $LpPLA_2$ catalyzes the hydrolysis of the *sn-2* fatty acid in PAF or other lipid substrate and is thus called PAF acetyl hydrolase (PAF-AH; Tjoelker et al., 1995; Stafforini et al., 1997).

Group XVI PLA_2 is $AdPLA_2$ abundant in adipose tissue (Duncan et al., 2008) and acts in lipolysis via the production of eicosanoid mediators (Jaworski et al., 2009).

Biochemical and Molecular Characters of Insect PLA_2s

Like vertebrates, PLA_2 activity acts in lipid digestion, metabolism, secretion, reproduction, and immunity in insects (Stanley, 2006a). Three types of PLA_2s are detected in insects (**Table 1**). In lipid digestion, PLA_2 performs two crucial roles by direct hydrolysis of dietary PLs at the *sn-2* position to generate nutritionally essential PUFAs and by providing lysophospholipids as insect "bile salts" that solubilize dietary neutral lipids for digestion by other lipases (Stanley, 2006b). The predatory tiger beetle, *Cicindella circumpicta* expresses a midgut calcium-dependent PLA_2 activity (Uscian et al., 1995). Protein fractionation indicated that the enzyme activity was detected in low molecular weight range (about 22 kDa), suggesting a $sPLA_2$. *Manduca sexta* secretes PLA_2 activity from midgut *in vitro* cultures and catalyzes AA release from PL (Rana et al., 1998; Rana and Stanley, 1999). Larvae of the mosquitoes *Aedes aegypti*, *A. albopictus*, and *Culex quinquefasciatus* express midgut PLA_2 activity (Nor Aliza and Stanley, 1998; Abdul Rahim et al., 2018). The peaks of the enzyme activity followed feeding cycles of the

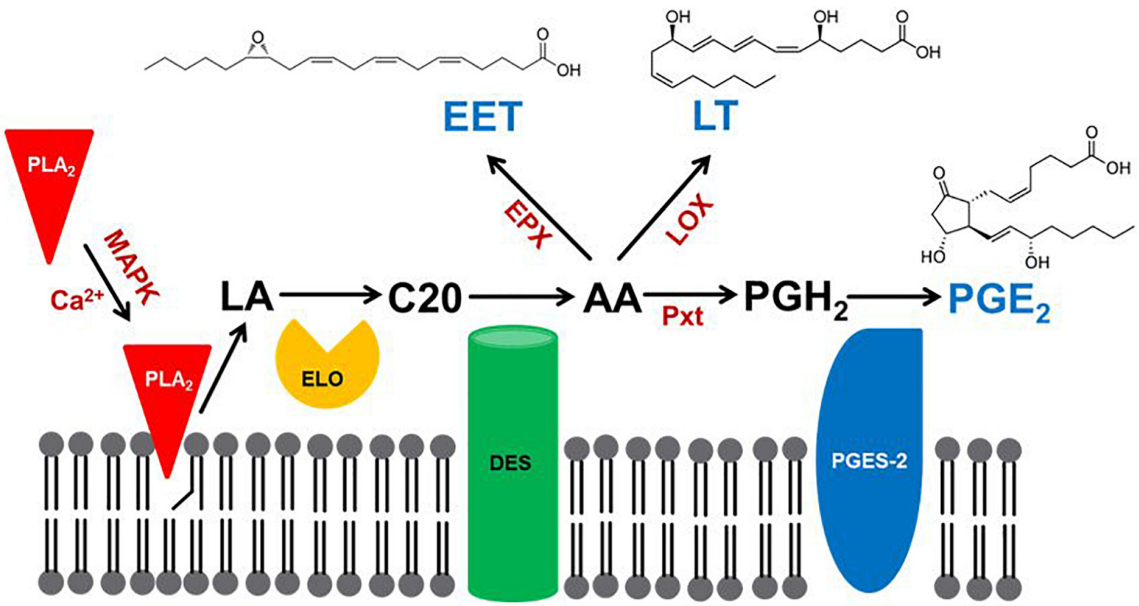

FIGURE 1 | A model for eicosanoid biosynthesis in insects. PLA_2 activated by calcium or mitogen-activating protein kinase (MAPK) catalyzes the hydrolysis of linoleic acid (LA), which is extended in chain length to C20 fatty acid by a specific elongase (ELO). The C20 precursor is oxidized by desaturases (DES) to produce arachidonic acid (AA; Stanley-Samuelson et al., 1988), which is oxygenated by epoxidase (EPX) to produce epoxyeicosatrienoic acid (EET), by lipoxygenase (LOX) to produce leukotriene (LT) or by a specific peroxinectin (Pxt) to produce prostaglandin H_2 (PGH_2). PGH_2 is then isomerized by PGE_2 synthase-2 (PGES-2) to PGE_2.

mosquito larvae. Similar $iPLA_2$-like activity comes from salivary gland of *M. sexta* (Tunaz and Stanley, 2004). Burying beetles, *Nicrophorus marginatus*, inter small mammals as larval food and express a salivary PLA_2 to protect the bodies from decomposition during larval development (Rana et al., 1997). Ryu et al. (2003) characterized a gene encoding a *D. melanogaster* PLA_2, which increased interest in insect PLA_2s.

Recent work by Sadekuzzaman and Kim (2017) using specific PLA_2 inhibitors supports the concept of multiple PLA_2 activities in several tissues of larval *Spodoptera exigua*. Vatanparast et al. (2018) recorded cellular PLA_2 activity in *S. exigua* plasma which is enhanced in response to immune challenge.

All venomous $sPLA_2$s are clustered into the Group III in PLA_2s. Similar $sPLA_2$s were predicted from *Tribolium castaneum* genome (Shrestha et al., 2010). Five $sPLA_2$s encode 173–261 amino acids, in which eight cysteines are conserved. We infer the enzyme is stabilized by formation of four disulfide bonds. All five $sPLA_2$s are expressed in different developmental stages of *T. castaneum*. Among them, four PLA_2s are associated with cellular immune functions. Two $sPLA_2$ genes are encoded and expressed in a hemipteran insect, *R. prolixus* (Defferrari et al., 2014). These are named as Rhopr-PLA2III and Rhopr-PLA2XII because they have Group III and XII-specific active site sequences of "C-C-R-T-H-D-L-C" and "C-C-N-E-H-D-I-C," respectively. Both $sPLA_2$ genes are expressed in most nymphal tissues (especially salivary gland) of *R. prolixus*, in which Rhopr-PLA2XII was more highly expressed than Rhopr-PLA2III.

The first lepidopteran non-venom $sPLA_2$ was identified from *S. exigua* (Vatanparast et al., 2018), which encodes 194 amino acids containing three domains, a signal peptide, a calcium-binding domain, and a catalytic site. This enzyme clusters with other Group III $sPLA_2$s. Though all insect $sPLA_2$s are clustered in Group III, venomous and non-venomous $sPLA_2$s are distinct in amino acid sequences (**Figure 2**). Venomous $sPLA_2$s have more cysteine residues than their non-venomous counterparts, which they may need more stable structures to sustain enzyme activity in external environments (Kim et al., 2018).

As seen in the *Tribolium* and *Spodoptera* systems, $sPLA_2$s are likely to mediate immune responses via AA release because RNA interference (RNAi)-treated larvae exhibited significant immunosuppression and AA treatments rescued the immune responses (Shrestha et al., 2010; Vatanparast et al., 2018). An additional $sPLA_2$ immune function may be its direct antibacterial activity in hemolymph. In mammals, Group IIa $sPLA_2$ is one of the most effective antibacterial agents by hydrolyzing the bacterial membrane PLs (Wu et al., 2010).

Park et al. (2015a) reported an insect $iPLA_2$ in *S. exigua* ($SeiPLA_2A$). $SeiPLA_2A$ encodes a protein with 816 amino acids with a predicted molecular weight of 90.5 kDa. $SeiPLA_2A$ clusters with Group VIA, which is characterized by multiple ankyrin repeats in the N-terminal region with a consensus lipase motif ("GTSTG") in the C-terminal region (Winstead et al., 2000). $SeiPLA_2A$ was localized in cytoplasm by an immunofluorescence assay. $dsSeiPLA_2A$ treatments suppressed gene expression and enzyme activity and led to two pathological phenotypes, loss of cellular immune response and extended larval-to-pupal development.

TABLE 1 | Phospholipase A_2 activities in insects and their predicted PLA_2 types.

Types	Species	Tissues	Enzyme activities[1]	Reference
$sPLA_2$	*Cicindella circumpicta*	Midgut lumen	• Calcium dependency • AA release from PL • Sensitivity to OOPC inhibitor • <22 kDa size	Uscian et al., 1995
	Nicrophorus marginatus	Oral secretion	• Calcium dependency • AA release from PL	Rana et al., 1997
	Cochliomyia hominivorax	Midgut	• Calcium dependency • AA release from PL • Sensitivity to OOPC inhibitor	Nor Aliza et al., 1999
	Manduca sexta	Midgut secretion	• *In vitro* secretion of PLA_2 activity • AA release from PL	Rana and Stanley, 1999
	Drosophila melanogaster	Recombinant protein	• Calcium dependency • AA release from PL • 138 amino acids	Ryu et al., 2003
	Rhodnius prolixus	Plasma	• Calcium dependency • *sn-2* ester bond hydrolysis	Figueiredo et al., 2008
	Tribolium castaneum	Recombinant protein	• BPB sensitivity • *sn-2* ester bond hydrolysis • 173–261 amino acids	Shrestha et al., 2010
	Spodoptera exigua	Plasma	• BPB sensitivity • *sn-2* ester bond hydrolysis	Vatanparast et al., 2018
$iPLA_2$	*Aedes aegypti*	Midgut	• Calcium independency • AA release from PL • Insensitivity to OOPC inhibitor	Nor Aliza and Stanley, 1998
	Manduca sexta	Midgut	• Calcium independency • AA release from PL • Insensitivity to OOPC inhibitor	Rana et al., 1998
		Salivary gland	• Calcium independency • AA release from PL • Sensitivity to OOPC inhibitor	Tunaz and Stanley, 2004
	Rhodnius prolixus	Hemocytes	• Calcium independency • *sn-2* ester bond hydrolysis	Figueiredo et al., 2008
	Spodoptera exigua	All tissues	• BEL sensitivity • *sn-2* ester bond hydrolysis	Sadekuzzaman and Kim, 2017
$cPLA_2$	*Rhodnius prolixus*	Hemocytes	• Calcium dependency • *sn-2* ester bond hydrolysis	Figueiredo et al., 2008
	Spodoptera exigua	All tissues	• MAFP sensitivity • *sn-2* ester bond hydrolysis	Sadekuzzaman and Kim, 2017

[1] *PL, for phospholipid; AA, for arachidonic acid; BPB, for promophenacyl promide; BEL, for bromoenol lactone; MAFP, for methyl arachidonyl fluorophosphates; OOPC, for oleyloxyethylphosphorylcholine.*

Another $iPLA_2$, denoted $SeiPLA_2B$, was identified in *S. exigua* (Sadekuzzaman et al., 2017). This enzyme differs from $SeiPLA_2A$ in several fundamental ways. $SeiPLA_2B$ is a small $iPLA_2$, encoding 336 amino acids with a predicted size of about 36.6 kDa. It lacks ankyrin repeats in the N-terminal region. $SeiPLA_2B$ clusters with Group VIF. Both $SeiPLA_2A$ and $SeiPLA_2B$ are expressed in all developmental stages. The insect $iPLA_2$s are separated into ankyrin and non-ankyrin types (**Figure 3**). An $iPLA_2$ gene was also identified from another lepidopteran insect, *Bombyx mori* (Orville Singh et al., 2016) and it is rich in glycine-histidine repeats. This $iPLA_2$ is highly expressed in fat body and RNAi treatments led to severe abnormal development and mortality.

A molecular signature of vertebrate $cPLA_2$ is the C2 domain, responsible for calcium-dependent translocation of the enzyme to membranes (Nalefski et al., 1998), which has not been recorded in insects. Variation of PLA_2 types were analyzed in *S. exigua* in different developmental stages and tissues (Sadekuzzaman and Kim, 2017). All developmental stages have significant PLA_2 activities. Among larval tissues, hemocytes had higher PLA_2 activities than fat body, gut, or epidermis. Different tissues of fifth instar larvae exhibited variation in susceptibility to inhibitors, with epidermal tissue sensitive to $cPLA_2$ inhibitor alone while other tissues are sensitive to all three inhibitor types. The variation of PLA_2 types in a one species may offer differential mediation of immune functionalities via eicosanoid signaling. In *S. exigua* plasmatocytes, intracellular calcium ion is required for cell spreading, which is inhibited by a calcium chelator (Srikanth et al., 2011). In *M. sexta*, PLA_2 activity in the cytosolic fraction was significantly inhibited by treatment with a $cPLA_2$-specific inhibitor, methyl arachidonyl fluorophosphate

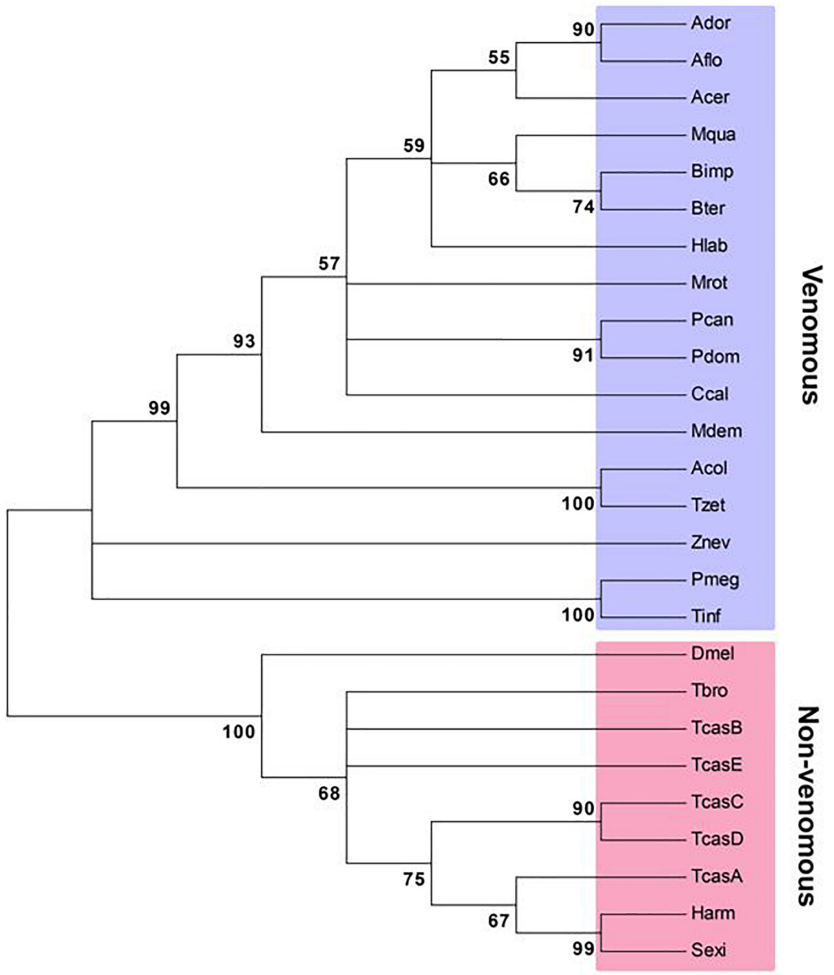

FIGURE 2 | Phylogenetic analysis of venomous and non-venomous $sPLA_2$s. The tree was constructed with Neighbor-joining method using MEGA6.0. Bootstrapping values on branches were obtained with 1,000 repetitions. Amino acid sequences were retrieved from GenBank. Accession numbers are PBC33208.1 for *Apis cerana cerana* (Acer), XP_006621273.1 for *A. dorsata* (Ador), XP_003694784.1 for *A. florea* (Aflo), KYM84159.1 for *Atta colombica* (Acol), XP_003491197.1 for *Bombus impatiens* (Bimp), XP_003400956.1 for *B. terrestris* (Bter), XP_017884585.1 for *Ceratina calcarata* (Ccal), KYM98685.1 for *Cyphomyrmex costatus* (Ccos), KOC68767.1 for *Habropoda laboriosa* (Hlab), XP_003699810.1 for *Megachile rotundata* (Mrot), KOX79218.1 for *Melipona quadrifasciata* (Mqua), JAC85837.1 for *Panstrongylus megistus* (Pmeg), XP_015172342.1 for *Polistes dominula* (Pdom), XP_014602740.1 for *P. canadensis* (Pcan), XP_011150082.1 for *Harpegnathos saltator* (Hsal), XP_008560296.1 for *Microplitis demolitor* (Mdem), NP_001014501.1 for *Drosophila melanogaster* (Dmel), XP_021189466.1 for *Helicoverpa armigera* (Harm), MH061374 for *Spodoptera exigua* (Sexi), JAI14574.1 for *Tabanus bromius* (Tbro), KYQ53077.1 for *Trachymyrmex zeteki* (Tzet), JAS01512.1 for *Triatoma infestans* (Tinf), NP_001139389.1 for *Tribolium castaneum* A (TcasA), NP_001139390.1 for TcasB, NP_001139461.1 for TcasC, NP_001139342.1 for TcasD, XP_966735.2 for TcasE, and XP_021915493.1 for *Zootermopsis nevadensis* (Znev).

(Park et al., 2005). We infer insect $cPLA_2$s occur in a novel molecular form.

Some Entomopathogens Target Insect PLA_2 for Pathogenicity

Eicosanoids transmit non-self recognition to hemocytes and fat body for systemic immune responses (Stanley and Kim, 2014). Blocking eicosanoid biosynthesis would be a highly effective immunosuppressive strategy in entomopathogen-insect interactions (Kim et al., 2018). This pathogenic strategy is used by some entomopathogens. One example is *Trypanosoma rangeli*, which is a mammalian parasite transmitted by the bite of triatomid bugs, *Rhodnius*, and *Triatoma* (Groot, 1952). The parasites develop within the insect hemolymph and then make their way to the salivary glands for the transmission. In *R. prolixus*, *T. rangeli* suppresses hemocyte phagocytosis by suppressing PLA_2 activity to inhibit eicosanoid biosynthesis (Figueiredo et al., 2008). Indeed, the addition of AA prevented the parasite infection. Another example is reported in two genera of entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus* (Kim et al., 2005). These bacteria are symbionts of entomopathogenic nematodes (EPNs) in the Steinernematidae and Heterorhabditidae (Gaugler, 2002; Shapiro-Ilan et al., 2012). After infective juvenile (IJ) nematodes enter host insects, they release symbiotic bacteria into host hemocoel

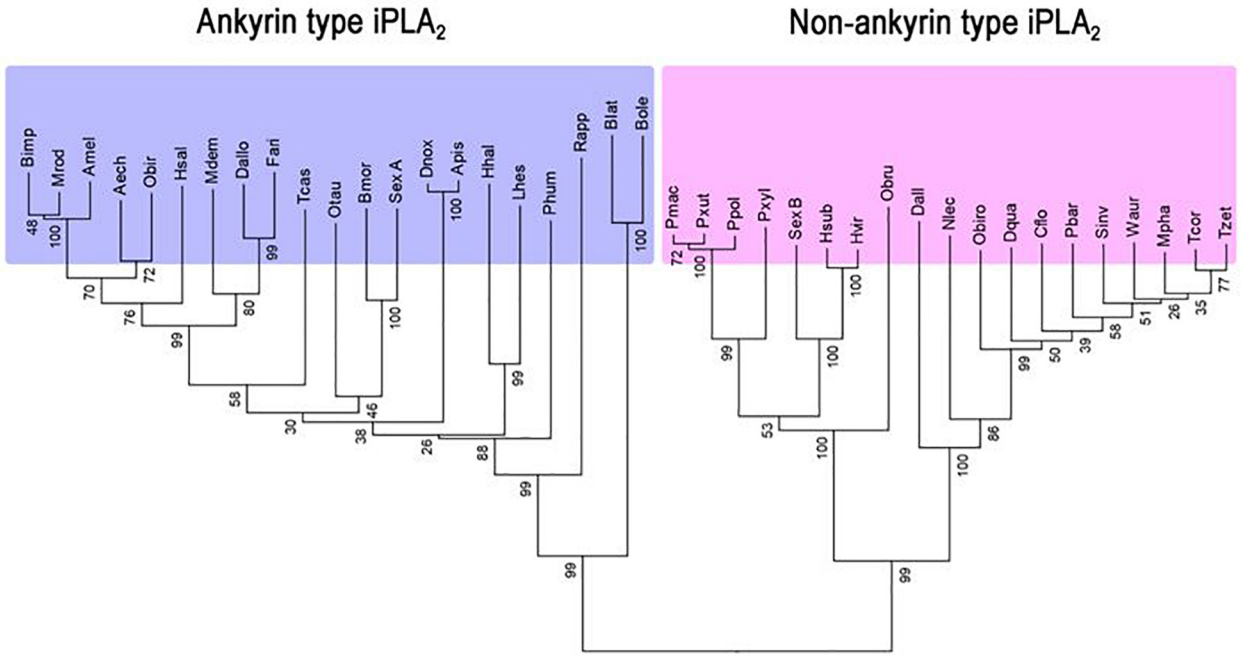

FIGURE 3 | Two groups of insect iPLA$_2$s. Phylogenetic analysis was performed by the Neighbor-joining method using the software package MEGA 6.0. Bootstrap values (expressed as percentage of 1,000 replications) are shown next to the branches. Amino acid sequences were retrieved from GenBank with accession numbers: XP_011063787.1 for *Acromyrmex echinatior* (Aech), XP_006565723 for *Apis mellifera* (Amel), XP_001944054.1 for *Acyrthosiphon pisum* (Apis), XP_004931589.1 for *Bombyx mori* (Bmor), JAI19791.1 for *Bactrocera latifrons* (Blat), XP_014090410.1 for *Bactrocera oleae* (Bole), XP_003492592.1 for *Bombus impatiens* (Bimp), XP_011261757.1 for *Camponotus floridanus* (Cflo), XP_015115454.1 for *Diachasma alloeum* (Dall), XP_015122829.1 for *Diachasma alloeum* (Dallo), XP_014488689.1 for *Dinoponera quadriceps* (Dqua), XP_015368613.1 for *Diuraphis noxia* (Dnox), JAG76826.1 for *Fopius arisanus* (Fari), XP_014284806.1 for *Halyomorpha halys* (Hhal), XP_011144133 for *Harpegnathos saltator* (Hsal), AGG55019.1 for *Heliothis subflexa* (Hsub), AGG55005.1 for *Heliothis virescens* (Hvir), JAQ13976.1 for *Lygus hesperus* (Lhes), XP_003702751.1 for *Megachile rotundata* (Mrod), XP_008559240.1 for *Microplitis demolitor* (Mdem), XP_012538958.1 for *Monomorium pharaonis* (Mpha), XP_015521797.1 for *Neodiprion lecontei* (Nlec), XP_022912622.1 for *Onthophagus taurus* (Otau), XP_011347626.1 for *Ooceraea biroi* (Obir), XP_011340273.1 for *Ooceraea biroi* (Obiro), KOB75232.1 for *Operophtera brumata* (Obru), XP_013147925.1 for *Papilio polytes* (Ppol), XP_014361526.1 for *Papilio machaon* (Pmac), XP_002432031.1 for *Pediculus humanus corporis* (Phum), XP_011641703.1 for *Pogonomyrmex barbatus* (Pbar), XP_013165315.1 for *Papilio xuthus* (Pxut), XP_011550190.1 for *Plutella xylostella* (Pxyl), JAP82800.1 for *Rhipicephalus appendiculatus* (Rapp), XP_011170109.1 for *Solenopsis invicta* (Sinv), XP_018367102.1 for *Trachymyrmex cornetzi* (Tcor), XP_018301493.1 for *Trachymyrmex zeteki* (Tzet), XP_971204.1 for *Tribolium castaneum* (Tcas), XP_011693263.1 for *Wasmannia auropunctata* (Waur), AIN39484.1 for *Spodoptera exigua* A (SexA), and AQW44791.1 for *Spodoptera exigua* B (SexB).

(Forst et al., 1997), which rapidly induces immunosuppression in their hosts (Park and Kim, 2000, 2003). Subsequently, the nematodes develop and reproduce in the insect cadaver (Akhurst, 1980). To induce the host immunosuppression, *Xenorhabdus* and *Photorhabdus* inhibit PLA$_2$ activity to block eicosanoid biosynthesiss (Kim et al., 2005). In pioneering research with *X. nematophila* and their symbiont EPN, *S. carpocapsae*, Park and Kim (2000) injected the bacteria into *S. exigua*. They explored the hypothesis that bacterial factors act to suppress insect immunity by inhibiting eicosanoid biosynthesis. In their first test of the hypothesis, they injected AA into bacterial-infected larvae, which rescued the insect immune responses. They also injected the PLA$_2$ inhibitor, dexamethasone (DEX) which substantially increased the bacterial virulence. This led to another hypothesis that bacterial secretions inhibit PLA$_2$ activity and all downstream biosynthesis of eicosanoids. The authors used a quantifiable, specific immune function, hemocyte nodule formation (nodulation), to monitor the change in immune response after bacterial challenge. Injection of heat-killed *X. nematophila* induced about 57 nodules per larva, compared to the same treatment with live *X. nematophila*, with less than 10 nodules, indicating substantial reduction in the cellular immunity. Injecting AA increased nodulation in the larvae treated with live *X. nematophila*. Therefore, the authors inferred that two genera of entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus* inhibit PLA$_2$ to induce host immunosuppression (Kim et al., 2005). Several commercial sPLA$_2$ preparations from porcine pancreas, honey bee venom, and snake (*Naja mossambica*) venom were strongly inhibited by an organic extract of the *Xenorhabdus* culture broth (Park et al., 2004). To test the bacterial extract on insect sPLA$_2$ activity, an immune-associated sPLA$_2$ from *T. castaneum* was overexpressed, and it was inhibited by the bacterial extract (Shrestha and Kim, 2009). We propose the principle that host nematodes and their symbiotic bacteria suppress insect host immune responses by inhibiting PLA$_2$ activity to optimize their pathogenicity. Ahmed and Kim (2018) supports the idea with their report of a functional correlation between the bacterial virulence and its inhibitory intensity against host PLA$_2$ activity.

Production of multiple PLA_2 inhibitors by the bacteria is more nuanced that first thought because the inhibitors are produced in a sequential pattern during bacterial growth and they exert additional inhibitory activities against different immune responses (Eom et al., 2014). They identified seven bacterial secondary metabolites, in which benzylideneacetone and a dipeptide (pro-tyr) are the most potent to inhibit PLA_2. Though other five bacterial compounds can inhibit PLA_2, they exhibit high inhibitory activities against PO enzyme activity or hemolytic activity to lead to insect immunosuppression (Seo et al., 2012). Because these bacterial secondary metabolites are produced at different bacterial growth phases, we infer that *X. nematophila* sequentially produces them to sequentially and cooperatively inhibit different steps of insect immune responses, including PLA_2 activity.

The entomopathogens also inhibit the direct PLA_2-mediated antibacterial activity. In *S. exigua*, the hemolymph from naïve larvae exhibits high $sPLA_2$ activity, which is further increased in response to bacterial immune challenge (Vatanparast et al., 2018). Thus, we propose that *Xenorhabdus* and *Photorhabdus* bacteria released from host nematodes inhibit $sPLA_2$ in the hemolymph to protect themselves from antibacterial enzyme activity and suppress insect immunity.

BIOLOGICAL SIGNIFICANCE OF EICOSANOIDS IN INSECTS

Eicosanoid and Insect Reproduction

Loher (1979) injected 50 mg PGE_2 into virgin female crickets, *Teleogryllus commodus*, and observed more than fourfold increase in oviposition behavior compared to saline-injected controls. He concluded that PGE_2 is an oviposition stimulant, noting that the PG action site was unknown, possibly via direct action on ovaries or muscles involved in oviposition. We will see that neither was correct.

Loher and his colleagues investigated the point in more detail (Loher et al., 1981). They found about 500 pg PGE_2 in spermathecae from mated, but not virgin females. Spermathecae contained far less PGE_2, about 20 pg/spermatophore. They found that spermatophores and spermathecae from mated, but not virgin, females biosynthesized about 25–35 pmol PGE_2/h/gland and smaller amounts of $PGF_{2\alpha}$. This became the basis of the "enzyme transfer" model, in which a PG biosynthesis activity is transferred to females via spermatophores. Within spermathecae, the transferred enzyme activity converts AA into PGE_2, which is released into hemolymph circulation. The precise target of the PGE_2 remains unknown, although the PGs may interact with a specific receptor located in the terminal abdominal ganglion, the site of the egg-laying behavioral program.

Lange (1984) reported the transfer of PG synthase activity during mating in *Locusta migratoria*. Mating led to a fourfold increase in PG biosynthesis, compared to virgins, in spermathecal preparations. Mating, but not PG treatments, led to substantial increases in egg laying. Similarly, Brenner and Bernasconi (1989) recorded the presence of AA and PG biosynthesis in spermatophores and testes of the hematophagous kissing bug, *Triatoma infestans*. The PG synthase activity is transferred to females during mating because there was PGE_2 synthase activity in spermatophores and a low enzyme activity in spermathecae from mated, but not virgin, bugs. The authors speculated the PGs release egg-laying behavior in *T. infestans*.

PGs release egg-laying behavior in an unknown number of insect species, certainly not all and not even all cricket species. Lee and Loher (1995) reported that treating short-tailed crickets, *Anurogryllus muticus* with PGs did not influence oviposition behavior. Nonetheless, releasing egg-laying behavior is one of several PG actions in insect reproduction.

Machado et al. (2007) investigated the idea that PG signaling acts in follicle development in silk moth, *B. mori*. Incubating follicular epithelial cells in the presence of PG biosynthesis inhibitors, aspirin and, separately, indomethacin, blocked transition from follicle development to choriogenesis. They suggested the PGs act in follicle homeostatic physiology, rather than signaling a more specific developmental step.

Tootle and Spradling (2008) used *in vitro* follicle cultures prepared from *D. melanogaster* to show that stage 10B egg chamber maturation is inhibited in a dose-related manner by the presence of aspirin or the selective COX-2 inhibitor, NS-398. Treating follicles with PGH_2 partially rescued development. Noting that mammalian COXs may have evolved from heme-dependent peroxidases, the authors identified a *Drosophila* peroxidase, Pxt, which produces PGs in a COX-like manner. They also advanced thinking about PG actions beyond general homeostasis to identification of a specific PG action in the actin cytoskeleton within ovarian follicles (Spracklen et al., 2014).

Tootle and her colleagues found more than 150 genes are expressed in specific stages during the final day of follicle development (Tootle et al., 2011), including known and new genes encoding egg shell proteins. Mutations in the *Drosophila Pxt* and RNAi treatments lead to mis-timed appearance of transcripts encoding egg shell proteins and defective egg shells.

The biological significance of the work on *Drosophila* follicle development lies in *Drosophila* as a model of insect and mammalian molecular processes, which teaches that these molecular processes are very basic biological events. They likely occur in most, if not all, animals. Here, we pose this as a recurrent theme, indicating that some PG actions recorded in insects are fundamental actions in virtually all insects, and likely arthropod, species.

PG Actions in Cockroach Fat Body

Steele and his colleagues investigated the biology of hypertrehalocemic hormones (HTH-I and -II). Their model was composed of disaggregated trophocytes prepared by treating fat bodies isolated from the cockroach, *Periplaneta americana*, with collagenase. HTH treatments led to increased concentrations of free fatty acids in the trophocytes. Treatments with the LOX inhibitor nordihydroguairaretic acid (NDGA) and COX-inhibitor (indomethacin: INDO) inhibited the release of free fatty acids. The authors inferred the free fatty acids, or their metabolites, act in synthesis and release of trehalose from trophocytes (Ali and Steele, 1997c). They later suggested the increased free fatty acid concentrations are regulated by

PLA_2 and COX activities (Ali and Steele, 1997a). This is the first recognition that PG and other eicosanoid signaling mediate HTH actions. In direct testing of the idea that PGs act in trehalose synthesis in the isolated trophocytes, they treated separate preparations with HTH, 18:0, 18-1n-9, 18:2n-6, or AA, all of which created similar increases in trehalose synthesis. They also reported that HTH-I treatments led to increased biosynthesis of 20:3n-6 and 20:4n-6, which was blocked by INDO treatments and that treatments with $PGF_{2\alpha}$, but not PGE_2, led to dose-related increases in trehalose efflux from the trophocytes (Ali and Steele, 1997b). The sugar efflux was inhibited by the COX inhibitors, indomethacin and diclofenac. A LOX inhibitor, NDGA and two PLA_2 inhibitors, mepacrine and 4′-bromophenacyl bromide (BPB), similarly led to decreased sugar efflux from HTH-I-treated fat body. Again, the authors inferred eicosanoids act in trehalose synthesis and efflux (Ali et al., 1998).

Sun and Steele (2002) reported that HTH-I and –II treatments substantially increased PLA_2 activity in membrane-enriched trophocyte preparations. The hormone effect, tested with HTH-II, was dose-dependent up to about 20 pmol/ml. Treating trophocytes with the PLA_2 inhibitor, BPB, over the range 0 to 1,000 μM, inhibited PLA_2 activity. The fat body PLA_2 activity may result from a cytosolic PLA_2 because HTH-II treatment led to translocation of the PLA_2 activity from the cytosol to the membrane fraction. This indicates Ca^{2+} is needed for translocation to the membrane and that the PLA_2 *per se* is Ca^{2+}-independent. Their work documents PGs actions in homeostatic hormone signaling.

Eicosanoids and Insect Immunity

Stanley-Samuelson et al. (1991) posed the hypothesis that eicosanoids mediate insect immune responses to bacterial infection. They tested the hypothesis in a series of simple experiments based on treating tobacco hornworms, *M. sexta*, with an inhibitor of eicosanoid biosynthesis, DEX, and ethanol for controls and separately injecting them with a red-pigmented strain of the bacterium *Serratia marcescens*. They withdrew hemolymph samples over a 60-min time course, and recovered no bacteria in hemolymph from controls and increasing numbers of bacterial colonies from the DEX-treated insects. The DEX treatments led to dose-dependent decreases in insect survival, which were reversed in insects treated with AA. In light of the short timeframes of their experiments, the authors surmised that eicosanoid metabolism mediates some or all of the early immune responses in insects. These experiments opened a new research corridor on biochemical signaling in insect immunity.

Nodule formation of hemocytes is a cellular immune response to bacterial and other microbial infection (Dunn and Drake, 1983). Miller et al. (1994) reported that PGs and LOX products mediate formation of hemocyte microaggregates and melanotic nodules following *S. marcescens* infections. Hemocytes migrate toward sites of infection and wounding, where they act in host defense. Merchant et al. (2008) reported that eicosanoids mediate hemocyte migration. Phagocytosis is another cellular immune response by engulfing and secondary killing of invading microbes by phagocytic cells. PGE_2 stimulates phagocytosis in the greater wax moth, *Galleria mellonella* (Mandato et al., 1997), the beet armyworm, *S. exigua* (Shrestha and Kim, 2007) and the bug *Rhodnius prolixus* (Figueiredo et al., 2008). The secondary killing of engulfed microbes is driven by reactive oxygen species (ROS). Park et al. (2015b) demonstrated that eicosanoids mediate ROS production by activating NADPH-dependent oxidase (NOX), as seen also in vertebrates. We infer that both phases of phagocytosis, the engulfment and secondary killing of bacteria are mediated by eicosanoids. Upon infection by parasitoid eggs or EPNs, insects form several hemocyte layers around the relatively large size of pathogens to prevent oxygen or nutrient supply (Strand, 2008). Carton et al. (2002) showed that the hemocytic encapsulation is mediated by eicosanoids in *D. melanogaster* exposed to the endoparasitoid wasp, *Leptopilina boulardi*. Thus, eicosanoids are key mediators of insect cellular immunity (Stanley and Kim, 2014; Kim et al., 2018).

Humoral immune responses in insects include quinone melanization by phenoloxidase (PO) and killing microbes by antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). In the *S. exigua* model, PGE_2 mediates release of inactive prophenoloxidase (PPO) from specific hemocytes (oenocytoids) into hemolymph by activating oenocytoid cell lysis (OCL) through a specific membrane receptor (Bos et al., 2004) that is expressed solely in oenocytoids in all life stages. Inhibiting expression of the *S. exigua* PGE_2 receptor led to reduced OCL and PO activity (Shrestha and Kim, 2008; Shrestha et al., 2011). PPO is activated into PO by enzymes in hemolymph, which initiates melanization, a key step in both humoral and cellular immune responses, and also in wound-healing response (Bidla et al., 2005). Indeed, a treatment of eicosanoid biosynthesis inhibitor (EBI) significantly suppressed clot formation around wounds of *Drosophila* larvae (Hyršl et al., 2011). EBI treatment inhibits expression of two AMP genes of *B. mori* against bacterial challenge (Morishima et al., 1997). In *Drosophila*, EBI specifically inhibits expression of AMP genes in IMD signal pathway (Yajima et al., 2003). In contrast, eicosanoids may mediate expression of AMP genes in both Toll/IMD pathways in the Oriental fruit fly, *Bactrocera dorsalis* (Li et al., 2017). In the fruit fly, a PLA_2 gene is linked with immune responses. Its RNAi treatment led to reduced gene expression of MyD88 and Relish along with suppressive expression of defensin (Toll pathway marker) and diptericin (IMD pathway marker). Similarly, both Toll/IMD signal pathways are controlled by EBI treatment in *S. exigua*, which led to significant suppression of AMP biosynthesis (Hwang et al., 2013). Thus, eicosanoids also mediate humoral immune responses in insects.

Eicosanoids mediating insect immune responses exhibit functional cross-talks with other immune mediators. Upon non-self recognition, immune mediators propagate the recognition signal to nearby immune effectors, hemocytes and fat body (Gillespie et al., 1997). These immune mediators include cytokines (small protein molecules, 5–20 kDa) such as the insect cytokine, plasmatocyte-spreading peptide (PSP; Clark et al., 1997), biogenic monoamines, nitric oxide (NO), and eicosanoids (Kim et al., 2018). Recent reports indicate that there is substantial cross-talk among immune mediators, in which eicosanoids play a crucial role in mediating most downstream signal (**Figure 4**).

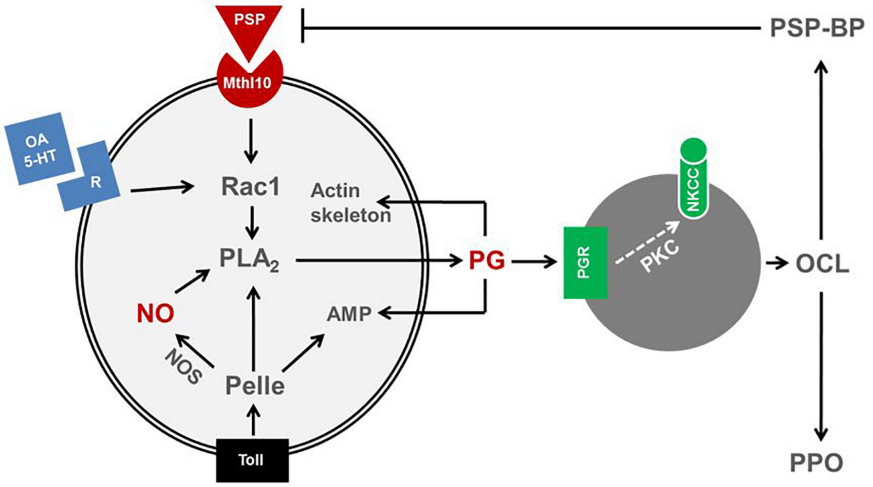

FIGURE 4 | Cross-talk among immune mediators in insects. A cytokine, plasmatocyte-spreading peptide (PSP) binds to its receptor, methuselah 10 (Mthl10) activates a small G protein, Rac1, which is also activated by biogenic monoamines, octopamine (OA) or 5-hydroxytryptamine (5-HT). Rac1 activates PLA_2 to produce prostaglandin (PG). PLA_2 is also activated by a protein kinase, Pelle, which is activated by Toll receptor. The Toll pathway also induces nitric oxide synthase (NOS) or antimicrobial peptide (AMP) genes. NOS synthesizes nitric oxide (NO) and activates PLA_2. The activated PLA_2 is involved in PG biosynthesis. PG triggers oenocytoid cell lysis (OCL) and release PSP-binding protein (PSP-BP) and prophenoloxidase (PPO). OCL is induced by sodium-potassium-chloride cotransporter (NKCC) via protein kinase C (PKC). PSP-BP facilitates PSP degradation. PG also mediates cytoskeletal rearrangement and AMP production.

Octopamine (OA) and serotonin (5-hydroxytryptophan: 5-HT) are biogenic monoamines that stimulate phagocytosis and nodulation in insects via the small G protein, Rac1 (Baines et al., 1992; Kim et al., 2009; Kim and Kim, 2010) through specific cell surface receptors (Dunphy and Downer, 1994; Qi et al., 2016). Phentolamine (an OA receptor antagonist) and ketanserin (a 5-HT receptor antagonist) suppress cellular immune responses of *S. exigua* in a competitive manner, and their inhibitory effects are reversed by an addition of AA (Kim et al., 2009). Eicosanoids are the downstream signals of the monoamines probably by increasing intracellular calcium concentrations as seen in the forest tent caterpillar moth, *Malacosoma disstria* (Jahagirdar et al., 1987) and by subsequently translocating $cPLA_2$ to its substrate PLs (Six and Dennis, 2000). Indeed, a PLA_2 of *T. castaneum* associated with immunity was translocated from cytosol to membrane in response to bacterial challenge (Shrestha et al., 2010).

The insect cytokine, PSP, is expressed as a proPSP in hemocytes and fat body (Clark et al., 1997) and cleaved into a 23 residue PSP that mediates plasmatocyte-spreading behavior in some plasmatocyte subpopulations (Clark et al., 1998). PSP is a member of the ENF peptide family which includes growth-blocking peptide (GBP) and paralytic peptides (PPs; Skinner et al., 1991). PSP induces cell-spreading via an approximately 190 kDa receptor (Clark et al., 2004), identified in *Drosophila* (Sung et al., 2017) as a Methuselah-like receptor-10 (Mthl10), for GBP. PSP mediates hemocyte-spreading behavior via cross-talk with other immune mediators (Kim et al., 2018). The effects of silencing the gene encoding proPSP were reversed by PSP or AA treatments (Srikanth et al., 2011). The PSP-stimulated hemocyte-spreading was impaired by inhibiting eicosanoid biosynthesis. Activation of eicosanoid biosynthesis by PSP or biogenic monoamines follows receptor-driven activation of Rac1. A Rac1 gene (*SeRac1*) that acts in cytoskeleton functions (Kim and Kim, 2010) was identified in *S. exigua* hemocytes (Park et al., 2013). Bacterial challenge up-regulated *SeRac1* expression (by >37-fold) and silencing *SeRac1* inhibited PSP- or biogenic monoamine-mediated hemocyte-spreading behavior. Injection of PGE_2 into *SeRac1*-silenced larvae rescued the influence of these immune mediators on hemocyte-spreading. PSP and biogenic amines increased PLA_2 activity, but not in hemocytes from *SeRac1*-silenced larvae. Therefore, we inferred that Rac1 transduces PSP and biogenic monoamine signaling by activating PLA_2 activity, which leads to eicosanoid biosynthesis. PSP and eicosanoids mediate PPO activation via eicosanoids (Park and Kim, 2014). OCL is required for the release of PPO into plasma, where it is activated (Jiang and Kanost, 2000). In *S. exigua*, PO is activated by PGs, which mediate OCL to release PPO (Shrestha and Kim, 2008). PSP induces PPO activation in *S. exigua* (Park and Kim, 2014), suggesting that PG acts downstream of PSP for PPO activation. Injection of PGE_2 to the larvae treated with DEX rescued the PPO activation. Park et al. (2013) reported that Rac1 facilitates cross-talk between PSP and eicosanoids. In *S. exigua* Rac1 activates PLA_2 for PG biosynthesis. The PPO induction period by PGE_2 treatment was significantly reduced in Rac1-silenced larvae. This reduction of PPO activation by PSP silencing is explained by the absence of endogenous PSP to sustain PLA_2 activation for PG biosynthesis. Thus, PSP requires PGE_2 as a downstream mediator of PPO activation.

Cross-talk between PSP and eicosanoids acts in down-regulation of PPO activation during later infection stages (Park and Kim, 2014). A specific PSP-binding protein (PSP-BP) terminates the PSP activation of PO because RNAi silencing of PSP-BP extended the PPO activation period

(Park and Kim, 2014). This explains how eicosanoids mediate both activation and inactivation of PPO.

NO is a small, membrane-permeable signal molecule that acts in nervous and immune systems in insects and vertebrates (Rivero, 2006). NO is synthesized from L-arginine by NO synthase (NOS), which in mammals exists in three forms (Colasanti et al., 2002). NO mediates immunity in mosquitoes, defending them from malarial parasites (Dimopoulos et al., 1998; Luckhart et al., 1998). In *M. sexta*, RNAi suppressed *NOS* expression showed that NO is directly associated with immunity (Eleftherianos et al., 2009). Cross-talk between cytokine and NO signaling induces AMP gene expression in *B. mori*, where a PSP-like cytokine elevates NO concentration by inducing *NOS* expression (Ishii et al., 2013). Sadekuzzaman et al. (2018) showed that bacterial injection increased NO concentrations in larval hemocytes and fat body and that silencing a *S. exigua* nitric oxide synthase (*SeNOS*) gene suppressed NO concentrations. The silencing of *SeNOS* expression and, separately, injecting L-NAME (a specific NOS inhibitor) led to reduced PLA_2 activities in hemocytes and fat body relative to controls. Injecting a NO donor, S-nitroso-N-acetyl-DL-penicillamine, increased PLA_2 activity in a dose-dependent manner. Eicosanoids did not influence NO concentrations in immune challenged larvae, from which it can be inferred that eicosanoid signaling is downstream to NO signaling.

NO treatments alone led to AMP induction because injection of an NO analog, SNAP, without bacterial challenge induced AMP gene expression (Sadekuzzaman and Kim, 2018). There is an additional line of cross-talk between the Toll/IMD pathways and NO signaling because RNAi of Toll or IMD signal components led to reduced levels of NO by inhibiting NOS expression in *S. exigua* (Sadekuzzaman and Kim, 2018). We infer that Toll/IMD signaling triggers NO signaling, which activates PLA_2 to synthesize eicosanoids. In addition, a recent study (Shafeeq et al., 2018) showed that two Toll signal components (MyD88 and Pelle) activate PLA_2 in *S. exigua*, suggesting a direct cross-talk between Toll and eicosanoid signal pathways.

PROSPECTUS

Prostaglandins and other eicosanoids make up a fundamental signaling system in insect biology. We described their actions at the whole animal, cellular and molecular levels of biological organization. These points mark valuable new knowledge on insect biology. So far, the idea that eicosanoids mediate cellular immune reactions has been confirmed in 29 or so insect species from seven orders (Stanley et al., 2012). Broader testing is necessary to develop the general principle that eicosanoids mediate insect immune functions. Similarly, intracellular cross-talk among immune signal moieties has been investigated in one lepidopteran species, *S. exigua*, which opens questions and hypotheses on the mechanisms of PG actions in insects generally. The overall picture is a broad outline of eicosanoid actions, each of which is an open field of meaningful research.

The eicosanoid signaling system may be a valuable target in applied entomology. Park and Kim (2000) first recognized the pathogenic mechanisms of bacteria in the genera *Photorhabdus* and *Xenorhabdus*, target insect immune reactions by blocking PLA_2s in their insect hosts. Similarly, *T. rangeli* protects itself from immune actions of its host, *R. prolixus* (Figueiredo et al., 2008). We infer that host PLA_2s are such potent targets that at least two bacterial genera and a eukaryotic parasite in the phylum Euglenozoa evolved mechanisms to down-regulate host immunity by blocking eicosanoid signaling via PLA_2s. We identified several genes that were silenced to inhibit insect immunity. We put these genes forward as potential targets that can lead to functional limitations in pest insect immune reactions to microbial and/or parasitic invasions. On the idea that virtually all pest insects become infected during their life cycles in crop plants (Tunaz and Stanley, 2009), targeted inhibition of insect immunity has potential for development into a novel insect management technology.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Transgenerational Developmental Effects of Immune Priming in the Red Flour Beetle *Tribolium castaneum*

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Immune priming, the increased chance to survive a secondary encounter with a pathogen, has been described for many invertebrate species, which lack the classical adaptive immune system of vertebrates. Priming can be specific even for closely related bacterial strains, last up to the entire lifespan of an individual, and in some species, it can also be transferred to the offspring and is then called transgenerational immune priming (TGIP). In the red flour beetle *Tribolium castaneum,* a pest of stored grains, TGIP has even been shown to be transferred paternally after injection of adult beetles with heat-killed *Bacillus thuringiensis*. Here we studied whether TGIP in *T. castaneum* is also transferred to the second filial generation, whether it can also occur after oral and injection priming of larvae and whether it has effects on offspring development. We found that paternal priming with *B. thuringiensis* does not only protect the first but also the second offspring generation. Also, fitness costs of the immune priming became apparent, when the first filial generation produced fewer offspring. Furthermore, we used two different routes of exposure to prime larvae, either by injecting them with heat-killed bacteria or orally feeding them *B. thuringiensis* spore culture supernatant. Neither of the parental larval priming methods led to any direct benefits regarding offspring resistance. However, the injections slowed down development of the injected individuals, while oral priming with both a pathogenic and a non-pathogenic strain of *B. thuringiensis* delayed offspring development. The long-lasting transgenerational nature of immune priming and its impact on offspring development indicate that potentially underlying epigenetic modifications might be stable over several generations. Therefore, this form of phenotypic plasticity might impact pest control and should be considered when using products of bacterial origin against insects.

Keywords: innate immunity, immune priming, transgenerational effects, *Tribolium castaneum*, *Bacillus thuringiensis*, host parasite co-evolution, bacterial infection, oral infection

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INTRODUCTION

Over the last decade a wealth of new evidence has been put forward to demonstrate that invertebrate immune systems can possess forms of immune memory and are sometimes capable of highly specific responses (Contreras-Garduño et al., 2016; Milutinović and Kurtz, 2016; Cooper and Eleftherianos, 2017). The phenomenon enabling a stronger and faster immune response upon

secondary infection has been termed immune priming and shows parallels in memory and specificity to trained immunity of vertebrates (Little and Kraaijeveld, 2004; Kurtz, 2005; Netea et al., 2011; Kurtz and Armitage, 2017; Melillo et al., 2018). The trigger, specificity and duration of the priming can be extremely diverse. Immune priming can be achieved by introducing a sublethal dose of the parasite, an incapacitated, e.g., heat killed agent or using only specific molecules from the original pathogen, e.g., lipopolysaccharides (Contreras-Garduño et al., 2016; Milutinović and Kurtz, 2016). Also, the route how the elicitor is introduced can vary, similar to differences in the route of infection in nature. For experiments involving priming, the priming agent is most commonly introduced via septic wounding and deposition into the haemocoel or orally via feeding (Milutinović and Kurtz, 2016). Furthermore, also abiotic factors, e.g., thermal exposure have been shown to prompt this phenomenon (Wojda and Taszłow, 2013; Eggert et al., 2015).

Additionally, the duration of immune priming effects differs dramatically. In some cases, protection lasts across different life stages, and throughout the entire life span of an individual (Pham et al., 2007; Thomas and Rudolf, 2010; Khan et al., 2016). In some cases, the immune priming is even transferred to the offspring generation (Milutinović and Kurtz, 2016; Dhinaut et al., 2018; Roth et al., 2018). This transgenerational immune priming (TGIP) can occur through either parent. While for the maternal side, the direct transfer of bacterial particles bound to egg-yolk protein vitellogenin has been shown to be involved in certain systems (Salmela et al., 2015), the detailed mechanistic underpinnings of immune priming in general and paternal TGIP in particular still remain to be discovered (Milutinović et al., 2016).

As with any other immune response also the fitness costs of immune priming including those for storing the information have to be considered. These costs are not constraint to a direct reduction in fertility but can also become visible in delayed development or smaller body mass if the priming occurs before the organism reaches maturity. Furthermore, negative effects might only become visible in the offspring generation. In the Coleopteran, *Tenebrio molitor*, maternal priming prolonged offspring larval development (Zanchi et al., 2011) and the strength of this effect depended on the Gram type of the bacteria used for priming (Dhinaut et al., 2018). Immune priming beneficial to the mother can even increase offspring susceptibility to the same parasite (Vantaux et al., 2014). These are all factors demonstrating the complexity of immune priming and showing that this term probably covers several distinct phenomena (Pradeu and Du Pasquier, 2018). It makes predicting host-parasite co-evolution and the emergence of resistance against bacterial pesticides much more difficult if we consider that several forms of immune priming can occur in the same species across different life stages and generations with different consequences.

Immune priming has been studied intensively in the red flour beetle *Tribolium castaneum*, which is a widely abundant pest of stored grains. In this beetle, immune priming has been demonstrated in different life stages, i.e., larvae and adults, as well as within and across generations (Milutinović et al., 2016). In this species, TGIP can occur via both parents (Roth et al., 2010). Previously, mainly two different routes of priming and infection have been used with the beetle. Oral infections with spores only work in larvae and the protective benefits of priming with the supernatant of the spore culture have so far only been studied within generation, mostly even within life stage (Milutinović et al., 2014; Futo et al., 2017; Greenwood et al., 2017). Therefore, the effectiveness of the priming was only confirmed for a few days after exposure. The other priming and infection method uses vegetative cells, which are heat-killed for the priming and are directly introduced into the body cavity via septic wounding (Khan et al., 2016; Milutinović et al., 2016; Tate et al., 2017). In this case, immune priming of adults can be transferred to their offspring and a protection against infection can still be observed in the adults of the offspring generation (Roth et al., 2010; Eggert et al., 2014). But, these different priming techniques and routes of infection lead to different responses as is evident in differential gene expression and immune system activity (Behrens et al., 2014). The pathogen used in most studies of priming in *T. castaneum* is the entomopathogenic and endospore forming bacterium *Bacillus thuringiensis* (Jurat-Fuentes and Jackson, 2012). Proteins from *B. thuringiensis*, so-called Cry toxins are widely used for their insecticidal activity in transgenic crops (Pardo-López et al., 2013; Lacey et al., 2015). Therefore, the study of immune priming in this host parasite model system does not only advance basic research and our understanding of the invertebrate immune system but is also helpful for applied approaches and improving insect control strategies.

With our study we shed further light on the different forms of immune priming against *B. thuringiensis* that can be observed in *T. castaneum*. We here investigated the transgenerational effects caused by three different types of priming, i.e., priming by injection of larvae and male adults and oral priming of larvae by monitoring the development, fitness and survival of bacterial infection (challenge). As paternal TGIP so far has only been tested in the first offspring generation (Roth et al., 2010), we here expanded the experimental time frame to include the adult F_2 generation, investigating whether TGIP is a multigenerational phenomenon extended to more than one subsequent generation. Studies on larval priming have been mainly focused on within generation immune benefits (Milutinović et al., 2016). We therefore here wanted to investigate whether larval TGIP via the oral or septic wounding infection route exists and whether the offspring is affected in a different way by parental treatment.

MATERIALS AND METHODS

Model Organisms

Beetles were derived from a population originally collected in the wild in Croatia in 2010 (Milutinović et al., 2013). Until the start of the experiment, beetles were kept in populations of more than 2,000 individuals in plastic boxes with heat sterilized (75°C) organic wheat flour (type 550) enriched with 5% brewer's yeast. Standard breeding conditions were set at 70% humidity and 30°C with a 12 h light/dark cycle.

In all priming treatments and infections, different entomopathogenic gram-positive *B. thuringiensis* strains

were used. *B. thuringiensis* and its Cry toxins are widely used as insecticides and together with *T. castaneum* form a well-established system to study host parasite co-evolution (Roth et al., 2009; Contreras et al., 2013; Milutinović et al., 2013; Pardo-López et al., 2013). For the different priming methods, we used the *B. thuringiensis* strains, which proved most effective in previous studies (Roth et al., 2010; Milutinović et al., 2014). For priming and challenge by injection we used vegetative cells from *B. thuringiensis* (*Bt*) strain DSM 2046 (German Collection of Microorganisms and Cell Cultures, DSMZ). For the treatments concerning priming and infection by oral uptake, spores and supernatant from *Bt morrisoni bv. tenebrionis* spore cultures (*Btt*, Bacillus Genetic Stock Center, Ohio State University, Ohio, United States) were used. Additionally, *Bt407cry*$^{-}$ (*Bt407*, kindly provided by Dr. Christina Nielsen-Leroux, Institute National de la Recherche Agronomique, La Minière, 78285 Guyancourt Cedex, France) served as a negative control in the oral priming experiment, as it does not produce Cry toxins and does not lead to immune priming nor mortality upon ingestion (Milutinović et al., 2013, 2014).

Paternal Transgenerational Immune Priming of Adults

In this experiment we wanted to investigate, whether paternal TGIP persists past the first filial (F_1) generation (Roth et al., 2010; Eggert et al., 2014) and therefore provides survival advantages upon *Bt* infection to the second filial (F_2) generation. Additionally, we measured the fertility of the primed males and their offspring to determine potential costs of TGIP. For an overview of the experimental design see **Figure 1**.

Injection Priming of the Parental (P_0) Generation

To set up the P_0 generation for this experiment around 2000 beetles from a general stock population were put into a plastic box containing 250 g of flour with yeast. After an oviposition period of 24 h the adults were sieved off and put into a new box for a second 24 h oviposition period. When the offspring had reached the pupal stage, their sex was determined, and all beetles were kept individually from here on onward.

For the priming injections one week after eclosion, 60 male adults were either injected with heat-killed *Bt* suspended in PBS at a concentration of $1*10^9$ cells per ml (injection priming), PBS only to control for the wounding (priming control) or left naïve. The priming suspension was directly injected into the dorsal vessel by dorsally puncturing the epidermis between head and pronotum in a flat angle to minimize tissue damage. Heat-killed *Bt* were produced from an overnight culture as previously described (Roth et al., 2009; Ferro et al., 2017). A nanoinjector (Drummond Nanoject II) was used for this procedure with the injection volume set to 18.4 nl (∼20,000 cells per injection in the *Bt* treatment). Survival after the priming procedure was recorded 24 h later.

Mating and Fitness of P_0 and F_1 Generation

Single mating pairs with naïve, virgin females were set up (n = 39–57). Mating pairs were kept in plastic vials containing 6 g of flour and left to lay eggs for two consecutive 3-day long oviposition periods. Thirteen days after the end of the respective oviposition period, larvae were counted for each pair and individualized into 96 well plates with flour. For the analysis, data from both oviposition periods were combined.

The sex of the offspring was determined when they had reached the pupal stage. One female and one male offspring from each single pair formed a new mating pair to produce the F_2 generation, leading to mating of full siblings (n = 29–53). Mating, oviposition and individualization of offspring larvae were carried out in the same way as described for the parental generation with the exception of the oviposition periods being shortened to 24 h. The fertility of F_1 pairs was recorded as live larvae 12 days post oviposition (dpo).

Bacterial Injection Challenge of Adults of F_1 and F_2 Generation

The priming of adult males of *T. castaneum* with heat-killed *Bt* leads to an increased survival rate in their adult offspring when infected with a potentially lethal dose of the same bacteria (Eggert et al., 2014). Whether this phenomenon is also transferred to subsequent generations has so far not been investigated. We therefore exposed individuals of the F_1 and F_2 generation to a bacterial challenge after the P_0 generation had received a priming treatment. Bacteria were cultured, washed and their concentration in PBS adjusted as for the priming procedure without the heat-killing step (2.2.1). One week after eclosion animals of both sexes were injected with a volume of 18.4 nl. The injection either contained *Bt* cells at a concentration 10^7 vegetative bacterial cells per ml (∼200 cells per injection) in PBS (injection challenge) or only PBS as a control (injection control) and was performed in the same manner as described for priming (2.2.1). A second control consisted of a naïve group that received no injections. In the F_1 generation, three adult siblings from each family were used, one for each challenge treatment (n = 31–44). This was the same for the F_2 generation, but here the challenge was performed on adults originating from two consecutive ovipositions of the same families (oviposition 1: n = 16–42, oviposition 2: n = 24–45). Injections were carried out in the same manner as for the priming treatment (2.2.1). Afterward, the beetles were kept in individual glass vials and their survival was recorded 24 h post challenge.

Transgenerational Effects of Immune Priming in Larvae

Within generation immune priming of *T. castaneum* larvae with *B. thuringiensis* can be achieved by two different exposure routes: first, septic priming can be achieved by the introduction of heat-killed vegetative cells into the hemolymph, which can be done by pricking the cuticle with a needle that was dipped into a suspension of heat-killed bacteria or by injection of heat-killed bacteria in the body cavity. Second, oral priming can be achieved by oral ingestion of spore culture supernatant (Behrens et al., 2014; Ferro et al., 2017). For this, the supernatant derived from a centrifuged *B. thuringiensis* spore culture is sterile filtered (0.2 μm) and then used for the preparation of the priming diet (Milutinović et al., 2014). It is so far unknown, which bacteria-derived components remain in the filtered supernatant

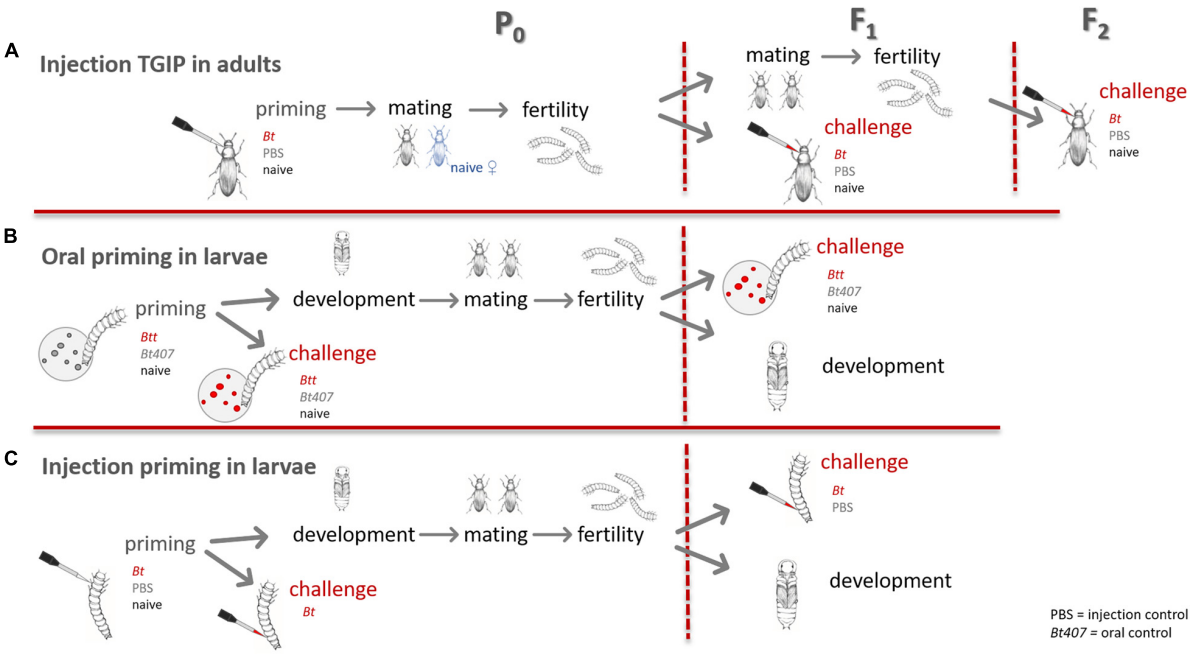

FIGURE 1 | Overview of the experimental design. **(A)** TGIP by injection in adults. **(B)** Oral immune priming of larvae. **(C)** Injection immune priming of larvae.

and might elicit the immune priming. Here, we investigated the costs and transgenerational effects of the two different larval priming methods.

Oral Immune Priming of Larvae

For the culturing and sporulation of *B. thuringiensis tenebrionis* we followed the method given in Milutinović et al. (2013). Milutinović et al. (2014) describe the methodology to orally prime larvae with *Btt* spore supernatant. In short, for the oral priming the spore supernatant is provided to the beetle by mixing with flour and PBS, pipetting the mixture into a 96 well plate and letting the diet dry to form flour disks. In addition to the *Btt* treatment (oral priming), *Bt407* was used as a negative control (priming control) because the supernatant from its spore culture does not provide a priming effect (Milutinović et al., 2014). As a third group a naive control was included with pure PBS to produce the flour disks (naïve).

The P_0 generation originated from approximately 1000 beetles from our stock population ovipositing for 24 h. Larvae of the P_0 generation were exposed to the priming diets 14 dpo for 24 h ($n = 320$). After the priming, a subgroup of the primed larvae was transferred onto naïve flour disks, on which they remained until the oral challenge or were used in producing the F_1 generation.

Oral Immune Challenge of Larvae

The within generation challenge was performed to confirm successful priming. The challenge took place 19 dpo, i.e., 5 days after the exposure to the priming diet, in a full factorial design. Besides the challenge diet of *Btt* spores (oral challenge), two controls were included using either *Bt407* spores, which are not infective to the beetles (challenge control) or flour disks prepared with pure PBS (naïve) ($n = 40$). The same bacteria culturing sporulation assay as for the oral priming was used (Milutinović et al., 2014). The spore concentration was adjusted to $5*10^9$ spores per mL. Larvae stayed on their respective flour disks for the rest of the experiment. Survival after challenge was recorded daily for the next 8 days.

Costs of Oral Immune Priming in Larvae

To identify potential costs of the oral immune priming, we monitored the development of the larvae until adulthood for the three priming treatment groups (oral priming, priming control, and naïve). In the P_0 generation, pupation rates were checked 23 dpo and the proportion of eclosed adults was recorded 27 dpo. In a subgroup of treated larvae, the sex of the individuals was determined during pupal stage and once they had reached sexual maturity (5 days post eclosion) single mating pairs were formed within each priming treatment ($n = 57$–66). Pairs were allowed to mate and produce eggs for two consecutive 24 h oviposition periods. Afterward, the adults were sieved off and offspring larvae were counted 14 dpo to estimate fertility. For the analysis, data from both oviposition periods were combined. To determine whether the oral immune priming produced any costs, which only become visible in the F_1 generation, the development of a subgroup of this larvae was monitored. The offspring larvae were individualized 14 dpo and kept in lose flour the entire time. They were checked for pupation between 19 and 23 dpo and their eclosion rates were noted 28 dpo.

Oral Immune Challenge of F_1 Generation Larvae

Furthermore, we wanted to know whether the oral immune priming of larvae can also be transferred to the F_1 generation, as

has been observed in the priming of adult *T. castaneum* (Roth et al., 2010; Tate et al., 2017). To answer this, a subgroup of the F_1 generation was orally challenged as well. This group was produced by the mating of single pairs, with individuals coming from the same priming group. One individual from each mating pair was used for each of the three challenge treatments (n = 71–76). The challenge was conducted in a similar manner as for the P_0 generation, but without the naïve control. Instead it included two different spore concentrations to counteract the possibility of too high or too low mortality rates. The spore concentration was set to either $1*10^{10}$ spores per ml (high dose) or $5*10^9$ spores per ml (low dose). Larvae were put on naïve flour disks at 14 dpo to ensure similar development as in the P_0 generation and to avoid early pupation, as the development in lose flour is considerably faster than on flour disks. The challenge took place 19 dpo and again survival was monitored for 8 days.

Injection Immune Priming of Larvae

Priming by injection with heat killed *Bt* cells (injection priming) took place 14 dpo. The larvae for this experiment came from a 24 h oviposition of ∼1000 beetles from our stock population. The procedure also included an injection control in which only PBS was used and a naïve group (n = 244). Heat-killed priming bacteria were produced as described above (2.2.1). Priming injections had a volume of 18.4 nl and were placed in a flat-angle laterally under the epidermis of the third-last segment using a nanoinjector (Drummond Nanoject II). The bacterial concentration was adjusted to $1*10^9$ cells per ml (∼20,000 cells per larvae). After the injection, larvae were kept individually in 96 well plates containing flour.

Injection Immune Challenge of Larvae

We performed a within life stage injection challenge to confirm the success of the priming. During the bacterial challenge 19 dpo, i.e., 5 days post priming a subgroup of the animals was injected with 18.4 nl of either vegetative *Bt* cells at a concentration of $1*10^7$ cells per ml suspended in PBS (injection challenge) or only PBS (injection control) (n = 48). Challenge injections were placed in the dorsal vessel at a flat angle dorsally under the epidermis of the first thoracic segment to minimize tissue damage. After the challenge injection, larvae were continued to be kept individually, and their survival was checked 7 days later.

Costs of Injection Immune Priming in Larvae

Also, for the injection priming of larvae, we wanted to test whether the treatment was costly and impacted the development. We therefore checked the proportion of pupae in a subgroup of the P_0 generation (n = 196) 23 dpo and the proportion of eclosed adults in the F_1 generation (n = 72–103) 27 dpo. The F_1 generation was produced from single mating pairs within a priming treatment and offspring larvae were individualized 14 dpo, i.e., the age their parents had been primed.

Injection Immune Challenge of F_1 Generation Larvae

Injection challenge was performed on a subgroup of the F_1 generation larvae, to discover whether a priming benefit and increased protection is transferred to the offspring. The F_1 generation was produced from single mating pairs within the same priming group, which produced eggs for two consecutive 24 h periods (n = 96). The challenge procedure was the same as in the P_0 generation. Larvae were injected 19 dpo with 18.4 nl of either vegetative *Bt* cells at a concentration of $1*10^7$ cells per ml suspended in PBS or only PBS. Again, survival was measured after 7 days.

Statistical Analysis

All statistical analyses were performed in R (R Development Core Team, 2008) using RStudio (R Studio Team, 2015). Additional packages utilized included: MASS (Venables and Ripley, 2002), lme4 (Bates et al., 2015), multcomp (Hothorn et al., 2008), and survival (Therneau and Grambsch, 2000). Data concerning larval survival and development until pupation were tested in a Cox proportional hazard analysis, after it had been ensured that the assumption of hazards being proportional over time had been fulfilled. When this was not the case, generalized linear mixed effects models (GLMM) with a binomial distribution and experimental block as random factor were applied on data for one specific time point for pupation rates. This method was also used to examine eclosion rate. Tukey honest difference (THD) was applied *post hoc* to determine significant differences between individual treatment groups, while adjusting the p-values for multiple testing. X^2-tests were used to analyze survival after injection challenge in cases for which random factors did not apply.

RESULTS

TGIP by Injection of Adults Is Transmitted to the F_2 Generation

In *T. castaneum*, immune priming by injection of heat-killed bacteria into adults has been shown to provide a survival benefit upon bacterial challenge to their offspring (i.e., F_1 generation) when they had become adults themselves (Roth et al., 2010). This effect was observed for both mothers and fathers. Focusing on the paternal priming route, we here investigated whether such trans-generational immune priming (TGIP) is also transferred further, to the F_2 generation. Before challenging the F_2 generation, we first wanted to confirm successful TGIP in the adults of the F_1 generation. However, in the F_1 generation, we observed an unusually high death rate in the control beetles that were injected with buffer only (i.e., challenge control) instead of the bacterial challenge (i.e., injection challenge). In consequence, we did not observe any significant differences in mortality between those beetles, regardless of paternal priming (N = 232, X^2 = 0.707, p = 0.4; **Figure 2A**). However, within the beetles that received an injection challenge, there was a tendency toward TGIP, as we observed a trend toward increased survival in the paternally injection primed group compared to the priming control (N = 69, X^2 = 3.401, p = 0.065; **Figure 2A**). As expected, there was no such difference between the priming treatments in the challenge control (N = 119, X^2 = 0.473, p = 0.78; **Figure 2A**).

We then tested whether TGIP is also passed on to the successive generation. The challenge of the adult F_2 generation proved to be effective, as significantly more beetles died after

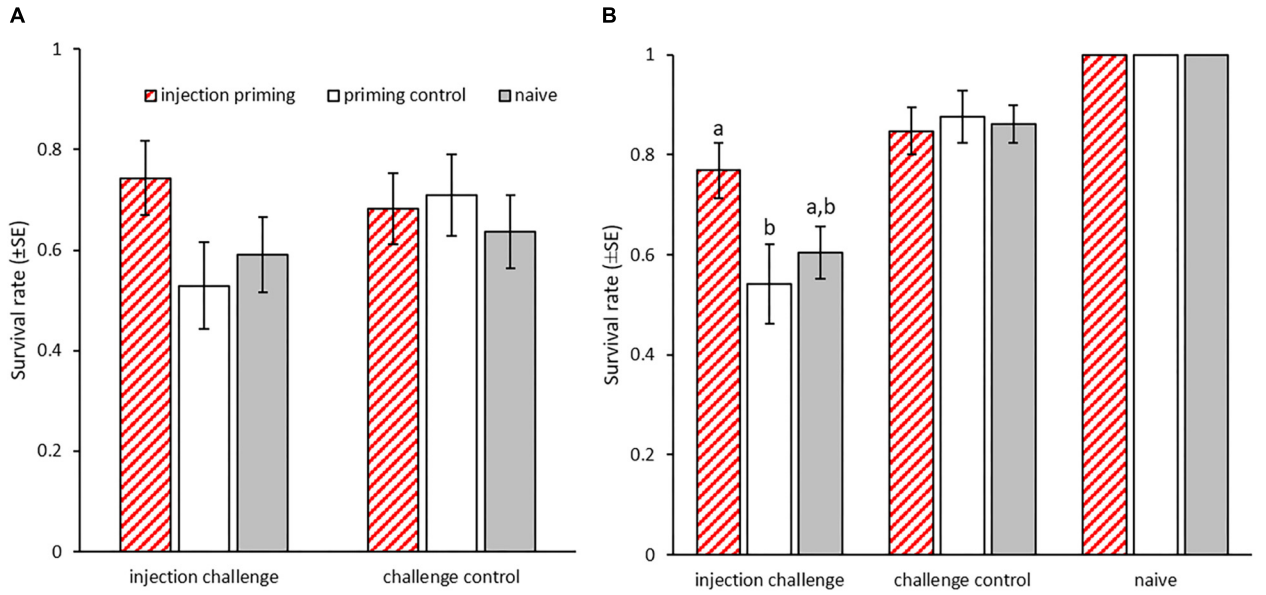

FIGURE 2 | Survival of bacterial injection challenge after paternal TGIP. Male adults were primed by injection with heat-killed *B. thuringiensis*. **(A)** Survival rates 24 h after injection challenge with live *B. thuringiensis* of the adult F_1 generation according to paternal priming (n = 31–44). **(B)** Survival rates 24 h after injection challenge with live *B. thuringiensis* of the adult F2 generation according to grand-paternal priming (two experimental blocks: n = 16–42 and n = 24–45). Different letters indicate significant differences at $p < 0.05$.

injection with live bacteria (injection challenge) than of those that received control injections (challenge control) (GLMM: df = 1, $X^2 = 23$, $p < 0.001$; **Figure 2B**). Furthermore, offspring, whose grandfathers had received injection priming with heat-killed bacteria survived significantly better than those from the priming control group (GLMM: df = 2, $X^2 = 7.3$, $p < 0.05$; THD: $z = -2.492$, $p < 0.05$; further comparisons: injection priming vs. naïve: $z = -2.090$, $p = 0.09$; priming control vs. naïve: $z = -0.827$, $p = 0.68$; **Figure 2B**). Therefore, the previously described TGIP in *T. castaneum* is transmitted past the first offspring generation at a comparable strength to the F_2 generation.

We investigated possible costs of paternal TGIP by counting live offspring 2 weeks after mating as a measure of reproductive success in the P_0 and F_1 generations. We could not observe any effect of paternal priming treatment on fertility for the P_0 (GLM: df = 2, $X^2 = 3.399$, $p = 0.18$; **Figure 3A**) nor the F_1 generation (GLM: df = 2, $X^2 = 7.19$, $p < 0.05$; THD: priming control $z = -0.527$, $p = 0.86$; naïve $z = 2.014$, $p = 0.11$, **Figure 3B**). However, the paternal priming control treatment significantly reduced fertility in the F_1 generation and led to significantly less F_2 larvae compared to the naïve control (THD: $z = -2.381$, $p < 0.05$; **Figure 3B**). Therefore, paternal septic wounding, but not the paternal bacterial priming itself, reduces the fitness of the F_1 generation.

Transgenerational Effects of Priming in Larvae

T. castaneum larvae can be either primed orally by feeding on filtered spore culture supernatant or through the direct introduction of heat-killed bacteria into the hemolymph by pricking or injection (Milutinović et al., 2014; Ferro et al., 2017). Both of these larval routes of priming have so far only been investigated within the same generation. We therefore here investigated whether any protection is transferred to larvae of the F_1 generation. We further asked whether there are any costs associated with such larval priming.

Larval Priming Does Not Affect Fertility

Neither oral nor injection priming of larvae with spore supernatant or heat-killed bacteria, respectively, significantly affected fertility compared to the control groups or the naïve individuals (GLM: df = 4, $X^2 = 2.11$, $p = 0.71$, **Supplementary Figure S1**).

Oral Priming Affects Development Differently in Treated (P_0) and Offspring (F_1) Generation

We monitored larval development after oral priming at 14 dpo to discover potential additional costs and benefits of this treatment besides changes in survival rate upon infection. In the treated P_0 generation, there were significant differences in the pupation rates 21 to 25 dpo (**Figure 4A**). Larvae treated with *Bt407* supernatant (priming control), a bacterial strain that has been shown to not cause any immune priming (Milutinović et al., 2014) reached pupation faster than the *Btt* primed group (oral priming) ($z = -2.906$, $p = 0.0102$). There was also a trend toward earlier pupation of the priming control larvae compared to the naïve control ($z = -2.28$, $p = 0.059$), while the orally primed group and naïve control did not differ ($z = -0.875$, $p = 0.65$). Additionally, there were differences in time until adult eclosion (GLMM: df = 2, $X^2 = 17.52$, $p < 0.001$; **Figure 4B**). At 28 dpo significantly more pupae from the priming control had

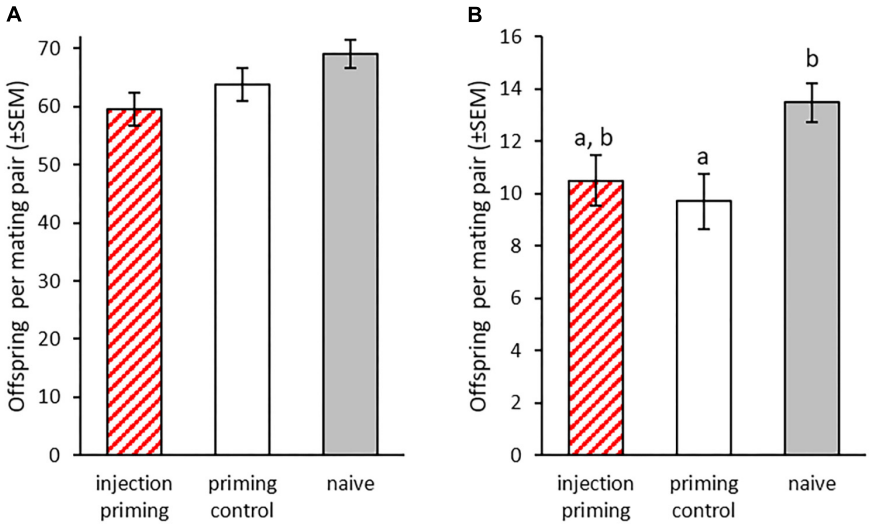

FIGURE 3 | Fertility after injection priming with *B. thuringiensis* in adult males. **(A)** Mean offspring produced by injection-primed males within 6 days in single pair matings (n = 39–57) and **(B)** mean offspring produced by the offspring of injection-primed males within 48 h in single pair matings (n = 29–53). Different letters indicate significant differences at $p < 0.05$.

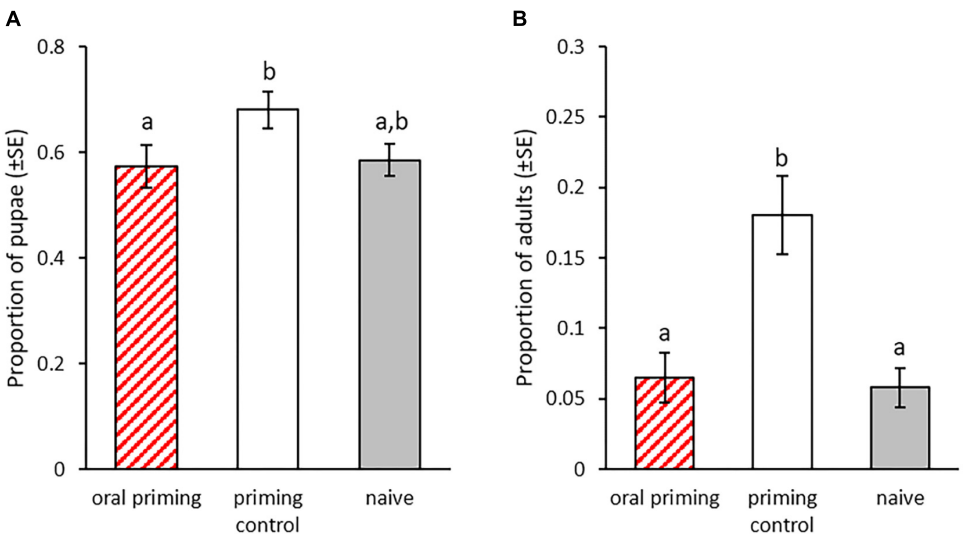

FIGURE 4 | Development after oral priming during the larval stage. Priming with spore culture supernatant took place at 14 dpo for 24 h (n = 196–280). **(A)** Proportion of pupated individuals 23 dpo for nine replicates. **(B)** Proportion of eclosed adults 28 dpo for nine replicates. Different letters indicate significant differences at $p < 0.05$.

eclosed than from the orally primed group ($z = 2.98$, $p = 0.008$) and the naïve control ($z = 3.802$, $p < 0.001$). Again, there was no difference between the orally primed and naïve control ($z = 0.569$, $p = 0.84$).

We also observed the development in the F_1 generation to see if this was influenced by the parental oral priming. Larvae, whose parents were exposed to spore culture supernatant from *Btt* or *Bt407* (oral priming and priming control) developed significantly slower than offspring of the naïve control (GLMM: df = 2, $X^2 = 16.14$, $p < 0.001$; *Bt407*: $z = 3.83$, $p = 0.002$; *Btt*: $z = 3.832$, $p < 0.001$, **Figure 5A**). We found a similar effect for the development until adult eclosion, which on average was reached earliest by the naïve group (GLMM: df = 2, $X^2 = 14.17$, $p < 0.001$; *Bt407*: $z = -3.213$, $p = 0.004$; *Btt*: $z = -3.199$, $p = 0.004$; **Figure 5B**).

No Survival Benefits of Oral Priming for the F_1 Generation

To test whether the exposure to spore supernatants led to a trans-generational priming effect, i.e., increased offspring survival upon oral challenge, larvae of the primed P_0 and the F_1 generation were orally exposed to spores. In the primed P_0 generation, the

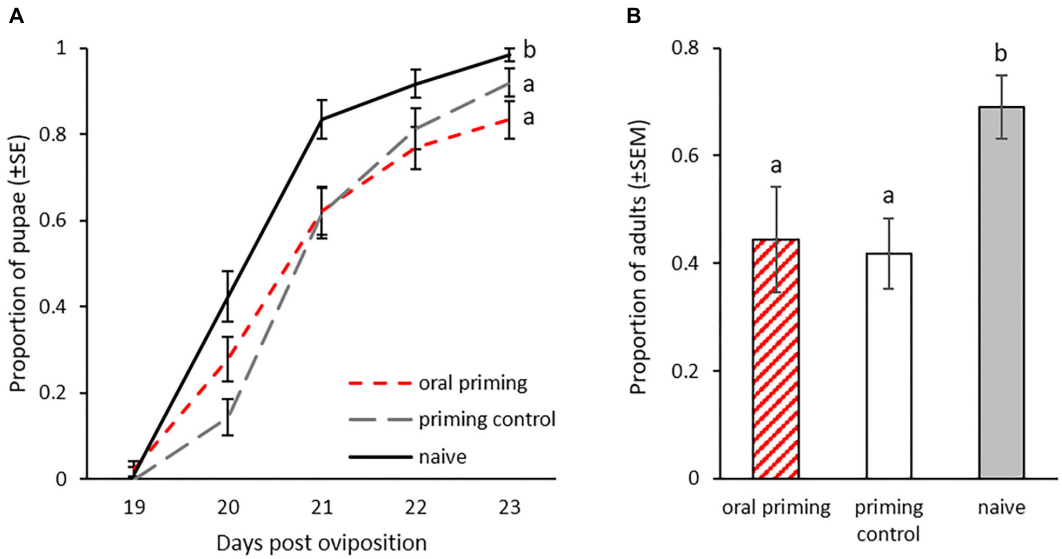

FIGURE 5 | Offspring development after parental oral priming during the larval stage. Priming with spore culture supernatant took place in the P_0 generation at 14 dpo for 24 h. Mating pairs were formed within the treatment groups. F_1 generation larvae were individualized 14 dpo (n = 70–75). **(A)** Pupation rate of F_1 generation (19–23 dpo). **(B)** Proportion of eclosed adults 28 dpo in the F_1 for three replicates. Different letters indicate significant differences at $p < 0.05$.

challenge with *Btt* spores (oral challenge) killed the larvae at a significantly higher rate than the exposure to spores of *Bt407* (challenge control) (df = 1, $X^2 = 12.76$, $p < 0.001$; **Supplementary Figure S2**). This, however was regardless of priming treatment, which did not lead to any significant differences (df = 2, $X^2 = 0.63$, $p = 0.73$; **Supplementary Figure S2**). This might be attributed to the here overall relatively low mortality rate after challenge with only 10.8% of all exposed larvae dying. This probably was caused by the rearing of larvae in lose flour instead of flour disks for the period between priming and challenge, because of which many larvae might have already had reached a pre-pupal stage and stopped feeding.

Although mortality was higher, results for the offspring generation were similar (**Supplementary Figure S3**). Again, the oral challenge proved to cause significant mortality at high (df = 1, $X^2 = 96.63$, $p < 0.001$) and low concentration of spores (df = 1, $X^2 = 47.1$, $p < 0.001$). Furthermore, survival depended on *Btt* spore concentration as the higher dose led to significantly higher mortality (df = 1, $X^2 = 10.85$, $p < 0.001$). However, no effect of parental oral priming was observed (df = 2, $X^2 = 0.69$, $p = 0.71$; **Supplementary Figure S3**).

Transgenerational Effects of Injection Priming in Larvae

In this part of the experiment we investigated potential effects of priming of larvae by injection with heat-killed bacteria. We monitored the development of the larvae after injection priming and the development of their offspring. Nine days after the priming, significantly less individuals from the priming control had pupated compared to the naïve control ($X^2 = 8.466$, $p = 0.003$, **Figure 6A**). The addition of heat-killed bacteria to the injection reduced this effect, resulting in only a trend toward later pupation in the injection priming treatment compared to the naïve control ($X^2 = 3.74$, $p = 0.053$, **Figure 6A**). There was no significant difference in the pupation rate between the injection primed individuals and the priming control ($X^2 = 1$, $p = 0.317$, **Figure 6A**). In the F_1 generation we did not observe any effect of parental priming on the developmental speed, as the eclosion rate was similar for all treatment groups at 27 dpo (GLMM: df = 2, $X^2 = 4.62$, $p = 0.1$, **Figure 6B**).

We challenged the parental and offspring generation by injecting a potentially lethal dose of *Bt* at 19 dpo, i.e., 5 days after the priming procedures for the parental generation, when all individuals were still in the larval stage. As the majority of mortality occurred within 24 h of the bacterial injection, we did not use survival curves in the analysis, but instead used the survival rate differences after 7 days for our analysis.

In the P_0 generation priming did not lead to differential survival after the injection challenge, which caused between 23 and 27% mortality (df = 2, $X^2 = 0.291$, $p = 0.86$). Finally, in the larvae of the F_1 generation, the bacterial injection challenge caused significantly higher mortality than the challenge control (GLMM: df = 1, $X^2 = 244$, $p < 0.001$, **Supplementary Figure S4**). However, also in this case parental priming did not significantly increase survival as there were no significant differences in mortality rates between the parentally primed group and the two controls (GLMM: df = 2, $X^2 = 0.037$, $p = 0.98$, **Supplementary Figure S4**).

DISCUSSION

Tribolium castaneum is one of the rare species for which not only maternal but also paternal TGIP has been observed (Roth et al., 2010, 2018). It is therefore important to further

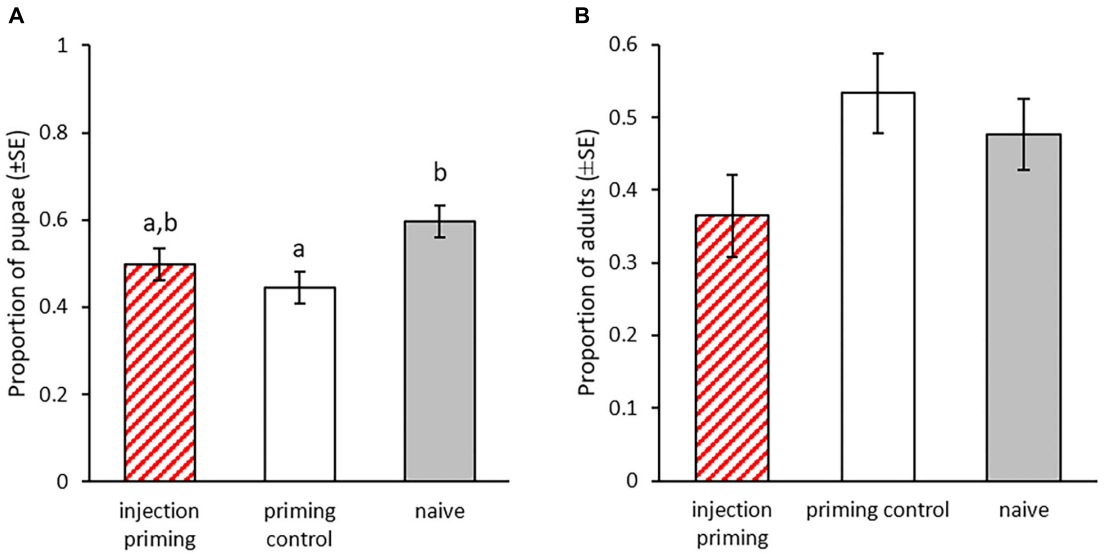

FIGURE 6 | Development after parental injection priming during the larval stage. Priming with injection of heat-killed bacteria took place in the P_0 generation 15 dpo. F1 was produced from mating pairs within the treatment groups. **(A)** Proportion of pupae for the P_0 generation 23 dpo, i.e., 8 days post injection-priming (n = 196). **(B)** Proportion of eclosed adults for the F_1 generation (parental injection-priming) 27 dpo for two experimental blocks (n = 72–103). Different letters indicate significant differences at $p < 0.05$.

study this phenomenon. One of the major open questions regarding paternal TGIP is, whether it is effective in more than one subsequent generation and can be considered to be multigenerational. Additionally, it is important to understand what the costs of TGIP are and if these are also transferred to later generations. We therefore carried out bacterial priming and challenge experiments across three generations using adult beetles.

We found that offspring of primed grandfathers survived a bacterial challenge significantly better than offspring of grandfathers, which had received a priming control injection. Thus, paternal TGIP is persistent for multiple generations at least until the F_2 generation. Astonishingly, the survival advantage of the F_2 generation was at a similar level as observed in previous experiments for the direct offspring (Eggert et al., 2014). We therefore did not see any dilution effect of this phenomenon over subsequent generations. Furthermore, we witnessed indirect costs, not of TGIP itself, but of the wounding procedure during the injection. These fitness costs became only visible after two generations, when the offspring of fathers from the injection control group sired significantly less offspring. In the present experiment, in contrast to previous studies (Roth et al., 2010; Eggert et al., 2014), we were unable to detect a significant priming effect in the adult F_1 offspring after paternal priming. This was likely due to an unusually high mortality in the injection control, maybe caused by a bacterial contamination in the injection buffer that was used for all treatments, thereby reducing a potential effect of priming.

Few studies have investigated the effects of TGIP beyond the first offspring generation. It has been shown that viral silencing agents derived from an RNAi response can be inherited non-genetically from either parent and passed on for several generations (Rechavi et al., 2011). In parthenogenetic *Artemia*, maternal exposure to bacteria provided the offspring with a survival benefit of bacterial infection for all three tested offspring generations (Norouzitallab et al., 2015). Multigenerational effects of paternal TGIP have been described in the pipefish, where due to male pregnancy contact between father and offspring is much more pronounced than in our system (Beemelmanns and Roth, 2017). Although, we are as of today unaware of the mechanisms behind paternal TGIP against bacteria, we can assume that its multigenerational nature will strongly impact the evolution of resistance and tolerance, depending on the costs, benefits, and specificity of TGIP and the prevalence of and therefore chances of repeated exposure to a parasite.

In the second part of this study, we investigated the transgenerational impact of immune priming via two different infection routes in larvae, for which within life stage immune priming has been previously demonstrated (Roth et al., 2009; Milutinović et al., 2014). Additionally to the survival after bacterial challenge, we monitored fitness costs of larval priming, becoming apparent as either directly reduced fertility or by slowing down developmental speed of the treated individual or its offspring. As any form of immunity, also immune priming comes at a cost for the organism (Schmid-Hempel, 2005; Freitak et al., 2009; Sadd and Schmid-Hempel, 2009). While in mosquitos a trade-off between immune priming and egg production has been observed (Contreras-Garduño et al., 2014), we did not find any effects of priming on fertility. Similar numbers of live offspring were produced across all treatments for both priming methods. But we estimated fertility only from a short 48h reproduction period and do not know how the immune priming might affect lifetime reproductive success. Also, we provided the beetle with *ad libitum* food throughout the experiment,

whereas limiting resources can be necessary for uncovering trade-offs with immunity (Moret and Schmid-Hempel, 2000; Kutzer and Armitage, 2016).

However, the oral priming of larvae led to differential speed in their development. Larvae, which had received the priming control diet containing the supernatant from the *Bt407* culture reached pupation considerably faster and emerged as adults earlier. In contrast, the treatment with *Btt* did not lead to differential developmental time compared to the naïve larvae. The same effect was observed previously by Milutinović et al. (2014). It is possible that the supernatant from the *Bt407* control culture contained some nutrients that were transferred to the priming diet and helped the larvae to speed up their development. The supernatant from the treatment *Btt* culture might not contain these nutrients, due to differences in the bacteria. Alternatively, the necessity to mount an immune and priming response, brought on by the exposure to the priming diet might mitigate the potential effect of the additional nutrients.

In the offspring generation, development was strongly affected by parental larval treatment. Both, offspring from the *Btt* primed group and the *Bt407* priming control took longer to pupate and also emerge as adults. This is interesting because although *Bt407* does neither provide an immune priming (Milutinović et al., 2014) nor is able to kill larvae upon ingestion (Milutinović et al., 2013), larvae feeding on its spore supernatant still suffer these fitness costs. These results are in concordance with observations in the mealworm beetle, where maternal priming prolonged larval development, while paternal priming led to a reduction in larval body mass (Zanchi et al., 2011). For the injection priming, we only observed within generation effects on the development. Here the wounding by the injection was sufficient to cause the effect, because larval development was slowed down in the injection of heat-killed bacteria as well as in the injection control treatment compared to the naïve group. Similar delays in development and increased time until pupation after tissue damage were observed in *D. melanogaster* (Halme et al., 2010). In the fly and potentially also the beetle, tissue damage interferes with endocrine signals, which are essential for the progression of development (Halme et al., 2010). In the offspring generation, time until adult emergence was not affected by parental priming. So far, we have no data regarding the development until pupation in this case.

Increased developmental time during the larval and pupal stage can be considered a fitness cost. Longer time spend during the larval stage is costly as it increases several risks. During the larval stage the risk of infection is higher as only larvae can be infected orally with certain bacteria, including *Btt*. Also, there is a higher risk of cannibalism, which happens regularly among larva (Ichikawa and Kurauchi, 2009) and at high densities smaller larvae might be less able to secure sufficient food (Koella and Boëte, 2002). Therefore, prolonged development should decrease probability of survival and delay the start of reproduction. In this experiment we were unable to confirm within-generation immune priming for either of the two used infection methods. This can likely be attributed to the low overall mortality rates following the challenge, which is a problem occasionally encountered in such experiments (see also Tate et al., 2017). However, both within-generation priming methods have been shown to work consistently in our lab (Milutinović et al., 2014; Ferro et al., 2017; Futo et al., 2017).

We did not find any evidence of larval TGIP with the oral nor the injection protocol. For larval priming by septic wounding with a pricking needle, it was observed that TGIP in larvae only occurred in populations, which do not demonstrate within life stage immune priming (Khan et al., 2016), implying that they are incapable of developing and maintaining both forms of immune protection. As beetles from our population have repeatedly been shown to possess larval within life stage priming ability, this is a possible explanation for the absence of larval TGIP.

In the present study, we did not directly address the question of potential mechanisms underlying immune priming. Nevertheless, our results indicate that such mechanisms should enable reactions that can be transferred not only within the organism (i.e., systemic reactions) but even across generations up to the F_2. This might be helpful for narrowing down targets for further in-depth studies from the large range of candidate genes and mechanisms identified in *T. castaneum* (Ferro et al., 2017; Greenwood et al., 2017; Tate et al., 2017; Schulz et al., 2018) and other insect species (e.g., Castro-Vargas et al., 2017; Tassetto et al., 2017; Cime-Castillo et al., 2018; Mondotte et al., 2018). On a more cautionary note, our study also further supports the view that immune priming comprises a multitude of phenomena that might be based on diverse mechanisms among and even within species (for review see Contreras-Garduño et al., 2016; Milutinović and Kurtz, 2016).

In conclusion, we observed that the life stage and route of priming determine the effects on the next generation. We found that the parental priming can be transferred to the F_2 generation, but can also impact offspring development. This demonstrates long-term costs of immune priming that are paid by subsequent generations. As this might have fitness consequences, further experimental research might focus on the evolution of immune priming. This will help to clarify under which circumstances immune priming is favored over the evolution of resistance or tolerance (Tidbury et al., 2012; Tate, 2017). Such knowledge will also be helpful to understand the evolutionary consequences of pest control methods that make use of bacterial products such as toxins derived from *B. thuringiensis*, as these may lead to priming in natural populations of pest organisms.

DATA AVAILABILITY

The raw data generated for this study can be found in the **Supplementary Material (Supplementary Data Sheet)**.

AUTHOR CONTRIBUTIONS

NS, MS, KF, and NK performed the experimental work. NS performed the statistical analysis and drafted the manuscript.

All authors contributed to conception, design of the study, manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00098/full#supplementary-material

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Epigenetic Mechanisms Are Involved in Sex-Specific *Trans*-Generational Immune Priming in the Lepidopteran Model Host *Manduca sexta*

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Parents invest in their offspring by transmitting acquired resistance against pathogens that only the parents have encountered, a phenomenon known as *trans*-generational immune priming (TGIP). Examples of TGIP are widespread in the animal kingdom. Female vertebrates achieve TGIP by passing antibodies to their offspring, but the mechanisms of sex-specific TGIP in invertebrates are unclear despite increasing evidence suggesting that both male-specific and female-specific TGIP occurs in insects. We used the tobacco hornworm (*Manduca sexta*) to investigate sex-specific TGIP in insects because it is a model host for the analysis of insect immunity and the complete genome sequence is available. We found that feeding larvae with non-pathogenic *Escherichia coli* or the entomopathogen *Serratia entomophila* triggered immune responses in the infected host associated with shifts in both DNA methylation and histone acetylation. Maternal TGIP was mediated by the translocation of bacterial structures from the gut lumen to the eggs, resulting in the microbe-specific transcriptional reprogramming of genes encoding immunity-related effector molecules and enzymes involved in the regulation of histone acetylation as well as DNA methylation in larvae of the F1 generation. The third-instar F1 larvae displayed sex-specific differences in the expression profiles of immunity-related genes and DNA methylation. We observed crosstalk between histone acetylation and DNA methylation, which mediated sex-specific immune responses in the F1 generation derived from parents exposed to a bacterial challenge. Multiple routes for TGIP seem to exist in *M. sexta* and – partially sex-specific – effects in the offspring depend on the microbial exposure history of their parents. Crucially, the entomopathogen *S. entomophila* appears to be capable of interfering with TGIP in the host.

Keywords: epigenetics, innate immunity, *trans*-generational immune priming, pathogens, *Manduca sexta*, *Serratia entomophila*

INTRODUCTION

Parents can invest in their offspring by preparing them to cope with pathogens or parasites that only the parents have encountered. The transfer of immunity from parents to offspring is known as *trans*-generational immune priming (TGIP) and has been reported in a wide range of animals, including arthropods (Little et al., 2003; Sadd et al., 2005; Dubuffet et al., 2015; Milutinović et al., 2016). The mechanisms underlying TGIP and the specificity of the resulting immune responses have been investigated in insects such as the bumblebee (*Bombus* spp.), the mealworm beetle (*Tenebrio molitor*), the red flour beetle (*Tribolium castaneum*), and lepidopterans such as the greater wax moth (*Galleria mellonella*) and the tobacco hornworm (*Manduca sexta*) (Dubuffet et al., 2015; Trauer-Kizilelma and Hilker, 2015b; Milutinović et al., 2016; Vilcinskas, 2016; Castro-Vargas et al., 2017; Rosengaus et al., 2017).

Mechanisms of parental investment in the form of TGIP differ between vertebrates and invertebrates and between sexes (Hasselquist and Nilsson, 2009; Roth et al., 2010; Herren et al., 2013; Eggert et al., 2014; Freitak et al., 2014; Knorr et al., 2015; Salmela et al., 2015), supporting Bateman's principle that males gain fitness by increasing their mating success whereas females increase fitness through longevity because their reproductive effort is much higher (Rolff, 2002). The maternal transfer of immunity in vertebrates is realized by antibodies, which are provided by the mother during gestation and (in mammals) during lactation (Hasselquist and Nilsson, 2009). However, the mechanisms of maternal TGIP in insects were unclear until a recent report revealed that bacteria taken up with the diet can translocate from the larval gut to the hemocoel and are ultimately deposited in the developing eggs (Freitak et al., 2014), apparently by binding to egg-yolk proteins (Salmela et al., 2015). The *trans*-generational transmission of bacteria via yolk proteins has also been observed in *Drosophila melanogaster* (Herren et al., 2013). The transfer of bacteria or fragments thereof from mothers to eggs explains at least in part the specificity of maternal TGIP in *G. mellonella* and *T. castaneum* (Freitak et al., 2014; Knorr et al., 2015).

Paternal TGIP is also observed in insects, but the immunological protection is less specific than that conferred by maternal TGIP and the mechanism is unclear (Roth et al., 2010; Eggert et al., 2014). Current concepts in evolutionary biology postulate that environmental stimuli such as stress and pathogens can be translated into heritable phenotypic alterations by epigenetic mechanisms (Nestler, 2016). Therefore, epigenetic mechanisms may explain how fathers can also translate information about the pathogens they have encountered (environmental stimuli) into heritable adaptations of the offspring immune system (phenotypic alteration) without any genetic changes (Vilcinskas, 2016). There is also a large body of evidence indicating that pathogens influence epigenetic gene regulation in their insect hosts (Mukherjee et al., 2017; Vilcinskas, 2017).

We investigated the potential epigenetic basis of TGIP in insects using the tobacco hornworm *Manduca sexta* because male and female larvae are easy to distinguish morphologically, it is widely used as a lepidopteran model to study innate immunity (Jiang et al., 2010), and the complete genome sequence was published recently (Kanost et al., 2016). To mimic natural oral infections and to determine the pathogen-specificity of any TGIP we observed, we supplemented the larval diet with either non-pathogenic *Escherichia coli* or with the entomopathogen *Serratia entomophila*, both of which are known to translocate from the midgut into the hemocoel in *G. mellonella* (Freitak et al., 2014). We tracked bacteria added to the diet to determine whether they were transferred from the gut lumen to the eggs. We also monitored the infected larvae for evidence of an immune response in the host by looking for shifts in complex parameters, specifically developmental timing. We analyzed the expression profiles of selected immunity-related effector genes in F0 and F1 male and female larvae. To determine whether epigenetic mechanisms were suitable to analyze sex-specific TGIP effects we observed, we compared total DNA methylation and histone acetylation in the same cohorts. DNA methylation involves the addition of a methyl group to cytidine residues in the dinucleotide sequence CpG to form 5-methylcytidine, which retains the base-pairing capacity of the unmodified nucleotide but modifies its interaction with regulatory proteins (Vilcinskas, 2017) and seems to be associated with stably expressed genes, related to basic housekeeping in lepidopterans (Jones et al., 2018). *De novo* methylation is established by DNA methyltransferase 3 (DNMT3) and is maintained by the maintenance methyltransferase DNMT1. However, some insect taxa including the Lepidoptera have lost DNMT3 (Bewick et al., 2017). We therefore focused our analysis on DNMT1 and 2 and the methyl-CpG-binding domain protein (MBD). Similarly, the core histone proteins that combine with DNA to form chromatin can be modified to control the density of packing, with the removal of acetyl groups by histone deacetylases (HDACs) causing transcriptional repression due to the tighter packing and lack of access to the DNA and the addition of acetyl groups by histone acetyltransferases (HATs) having the opposite effect (Marks et al., 2003). Accordingly, we also looked at the relationship between histone modification and the expression of HATs and HDACs in both generations.

MATERIALS AND METHODS

Insect Rearing and Diets

Manduca sexta eggs were collected from the in-house stock population for hatching and the larvae were maintained at 26°C, with 30% humidity and a 16-h photoperiod. We separated male larvae from female according to their dark spot in the posterior portion (Stewart et al., 1970). The larvae were reared on a standard artificial *M. sexta* diet (Bell and Joachim, 1976) drenched in overnight bacterial cultures of *E. coli* (9.5×10^7 cfu/meal) or *S. entomophila* (1.5×10^8 cfu/meal), or without bacteria as a control. Additionally, a group of larvae was reared on artificial diet drenched with 100 µl fluorescent BioParticles® consisting of a mixture 1 mg/ml of chemically and heat-killed *E. coli* strain K-12 labeled with Texas Red® (Molecular Probes) per 1 g of diet (Freitak et al., 2014). Larvae were fed *ad libitum*

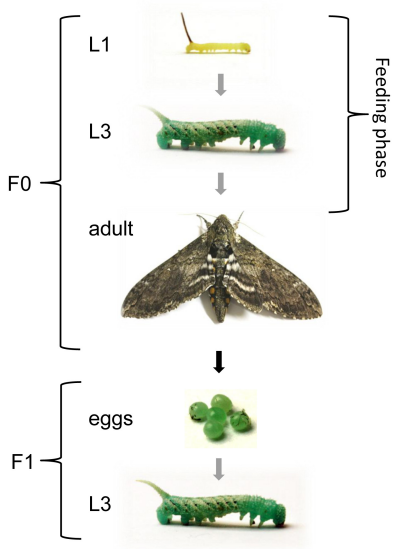

FIGURE 1 | Experimental design to investigate TGIP in *M. sexta*. Larvae were fed on diets supplemented with *E. coli*, *S. entomophila* or fluorescence-labeled bacteria (Bioparticles) starting at the first instar and continuing until the first sampling point at the third instar. Except for the first instar, all displayed stages correspond to sampling points. Gray arrows indicate stage progression, the black arrow indicates generational progression, and the white double arrow indicates the duration of the feeding regimes. The life cycle proceeds as follows: five larval molts, one pupal molt, one imaginal molt, (embryonic) egg stage.

and food was replaced when needed or at least three times per week to ensure a steady supply of bacteria. Feeding was continued until the F0 larvae were removed for dissection at the third-instar stage, or throughout development in the case of specimens that were used to provide offspring for TGIP analysis. Parental development was monitored daily. After pupation, male and female specimens (2:1 ratio) were transferred to flight cages so they could begin mating after eclosion and wing maturation (one cage per treatment). Oviposited eggs were then counted daily for 10 consecutive days after initial oviposition on the provided substrates, i.e., tobacco plants (*Nicotiana tabacum*) and laboratory paper lining the cage walls. Eggs were allowed to hatch and F1 larvae were reared on an uncontaminated artificial diet until they were 1-day-old third-instars for the analysis of gene expression, histone H3 acetylation and DNA methylation (**Figure 1**). This experiment was repeated twice.

Maternal Transfer of Bacteria

Manduca sexta third-instar larvae reared on a diet drenched with fluorescent BioParticles® were embedded in Tissue-Tek® OCT compound (Plano), flash-frozen in liquid nitrogen and stored at −80°C prior to cryostat sectioning. Abdomens, dissected ovaries and oviposited eggs from adult females were treated in a similar fashion. We prepared 10-µm cross-sections on a Leica CM 1850 Cryostat and viewed them under a Leica DM 5000 B fluorescence microscope with the N3 filter for Texas Red and the A4, L5 and Y5 filters as the negative control. Differential interference contrast (DIC) and bright field (BF) images were also captured to provide structural information. Fluorescent photomicrographs were acquired by overlaying the N3 and L5 filter cube images onto the DIC or BF images using the Leica LAS AF Lite image processing platform to optimize the fluorescence visualization.

Identification of Sequences of Putative Epigenetic Regulatory Genes and Immunity-Related Genes

To identify putative *M. sexta* epigenetic regulators (e.g., HATs, HDACs and DNMTs), as well as immunity-related genes (e.g., encoding gloverin and lysozymes), we identified predicted and annotated *M. sexta* proteins based on the published genome sequence (Kanost et al., 2016). To confirm the annotated protein identities, the predicted amino acid sequences were used as queries for BLAST searches (using BLASTp with default parameters) against the NCBI nr database. Sequences with existing annotations matching the *M. sexta* sequences and with more than 55% amino acid sequence similarity to queries were collected for further analysis. All protein sequences were aligned in Geneious (vR10, Biomatters Ltd.) using MUSCLE with default settings, inspected for regions of high-quality alignment and refined manually. During this step, candidates were also scrutinized for the presence of conserved amino acid patterns.

RNA Isolation and Quantitative Real-Time PCR

Midguts dissected from third-instar larvae (F0, F1) were homogenized in liquid nitrogen and total RNA was isolated using the PeqLab peqGOLD MicroSpin total RNA Kit. Sample quantity and purity were assessed using a NanoDrop spectrophotometer (PeqLab). If appropriate, samples were purified using RNeasy MinElute columns (Qiagen). First-strand cDNA was synthesized using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with 500 ng of DNA-free total RNA as the template and a 3:1 mixture of random hexamers and oligo-dT18 primers. Primers for real-time PCR were designed using Primer3 and available primer pairs were selected based on the lowest number of potential self-annealing structures and primer loops. Gene-specific primers are listed in **Supplementary Table 1**. The ribosomal protein L3 gene (*RPL3*) (Koenig et al., 2015) was used for normalization. Quantitative real-time PCR was conducted using an Applied Biosystems® StepOnePlus™ Real-Time PCR System on 96-well plates with the SensiMix™ SYBR® No-ROX Kit as the reporter mix. Each assay was repeated using three biological replicates (each representing pooled RNA from five third-instar larval midguts per sex) and two technical replicates. Fold changes in gene expression were calculated out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Analysis of Histone Acetylation

Midguts were dissected from male and female third-instar larvae (F0, F1), flash frozen in liquid nitrogen, homogenized and stored at −80°C. Global levels of lysine-specific histone H3 acetylation were determined using the EpiQuik Global Histone H3 Acetylation Assay Kit (Epigentek Group Inc.) according to

the manufacturer's protocol. Fold changes of relative histone acetylation were calculated for treatment groups exposed to bacteria against the corresponding control groups.

Preparation of DNA for Methylation Analysis

DNA was extracted from two replicates of five F0/F1 third-instar female/male larvae representing the control, *E. coli* and *S. entomophila* treatment groups using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). For each sample, the larvae were homogenized in liquid nitrogen and 150–200 μg of the resulting powder was used for DNA isolation, with a final elution volume of 100 μl. The DNA was precipitated by adding 10 μl 3 M sodium acetate (Carl Roth) and 200 μl ice-cold 100% (v/v) ethanol (Carl Roth), incubating at −20°C for at least for 2 h, and centrifuging at 4°C at 13,000 × *g* in a microfuge for 15 min (Sambrook and Russell, 2000). The pellet was washed with 20 μl ice-cold 70% (v/v) ethanol in Ambion nuclease-free water (Thermo Fisher Scientific) and dried at room temperature for 15 min before dissolving in 50 μl nuclease-free water on ice for 30 min. The DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). If the A_{260}/A_{230} ratio was less than 1.5, the DNA was purified using the NucleoSpin® gDNA Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions.

Global Analysis of DNA Methylation by LC-MS

DNA samples (1 or 2 μg) were digested with Degradase Plus (Zymo Research) at a ratio of 5 U/μg in a final volume of 25 μl overnight at 37°C, then diluted to 100 μl by adding 75 μl 0.1% (v/v) formic acid (ROTIPURAN®, Carl Roth) in ultrapure water (Milli-Q® Advantage A10 water purification system, Merck Millipore) (Capuano et al., 2014). Calibration curves were prepared by dissolving 2′-deoxycytidine (dC, Sigma-Aldrich) and 5-methyl-2′-deoxycytidine (5mdC, Cayman Chemical) in nuclease-free water on ice, each to a final concentration of 1 mg/ml. The nucleoside stock solutions were diluted with 0.5% (v/v) formic acid in ultrapure water to yield 1, 2.5, 5, 10, 100, 250, 500, 1000, and 2000 pg/μl dC/^{5m}dC-standard solutions. The analysis of genomic DNA was carried out by injecting 5-μl digested DNA samples and standard solutions into an UltiMate 3000 HPLC system (Dionex) followed by quantification in an amazon EDT ion trap mass spectrometer (Bruker Daltonics). Components were separated on a reversed-phase column (Kinetex C18, 2.6 μm, 50 × 2.1 mm, 100 Å, Phenomenex) under isocratic conditions [0.1% (v/v) formic acid (ROTIPURAN) and 5% (v/v) acetonitrile (ROTISOLV, Carl Roth) in ultrapure water] at a flow rate of 150 μl/min and 30°C. Cytidine residues were quantified by multiple reaction monitoring (MRM) after positive electrospray ionization using the following ion source parameters: 1.0 bar nebulizer pressure, 8 l/min drying gas, 200°C drying temperature, 4500 V capillary power and 500 V end-plate offset. Ionization and MRM conditions were optimized for fragmentation reactions for mass/charge ratios 228.1→112.0 (for dC) and 242.1→126.1 (for 5mdC). The data were analyzed using Compass Data Analysis v4.2 (Bruker Daltonics). Fold changes in relative global DNA methylation levels were calculated for treatment groups exposed to bacteria against the corresponding control groups.

Statistical Analysis

We used the mean value for the parental and filial generations for every biological sample for males and females, as a control for natural cross-generational effects. We used the function *Summarize* of the R package FSA v0.8.17 (Ogle, 2016) to calculate means, medians, standard deviations, and standard errors of the mean for all experiments. The quantitative PCR results, global histone acetylation, and global DNA methylation data were analyzed for differences between sexes, treatments, and generations using R v3.2 as previously described (Gegner et al., 2018) (R script, **Supplementary File 1**). To test for differences in developmental times between treatment groups and controls, a Kruskal–Wallis multiple comparison test was applied with Bonferroni adjustment of *p*-values by using the function *dunnTest* in the package FSA v0.8.17.

RESULTS

To assess the potential molecular basis of TGIP in the parental generation, larvae were reared on a diet supplemented with microbes and we analyzed the expression of immunity-related genes as well as the levels of histone acetylation and DNA methylation. We also looked at developmental characteristics to determine the impact of TGIP on life history traits. In parallel, a group of larvae received a diet supplemented with fluorescent particles allowing us to visually monitor the uptake and fate of ingested microbes. In the F1 generation, the fate of the fluorescent particles was traced until the egg stage. In addition, gene expression, histone acetylation and DNA methylation were analyzed in the third larval instar (**Figure 1**).

Diets Supplemented With Bacteria Affect Development

We supplemented larval diets with either the pathogen *S. entomophila* or the non-pathogenic bacterial species *E. coli* and monitored development compared to a control group fed on an uncontaminated diet. As shown before for other species, bacterial exposure delayed larval development significantly, i.e., by approximately 2 days in larvae exposed to *E. coli* and 4 days in larvae exposed to *S. entomophila* compared to untreated controls ($p < 0.001$). The larvae infected with pathogenic bacteria took 2 days longer to pupate than larvae exposed to the non-pathogenic bacteria ($p < 0.001$) (**Figure 2** and **Supplementary Tables 2**, **3**).

Bacteria Can Be Transferred From Mothers to Offspring

We monitored the transfer of bacteria from mothers to their offspring using non-viable *E. coli* labeled with the fluorescent dye Texas Red. These bacteria were added to the larval diet

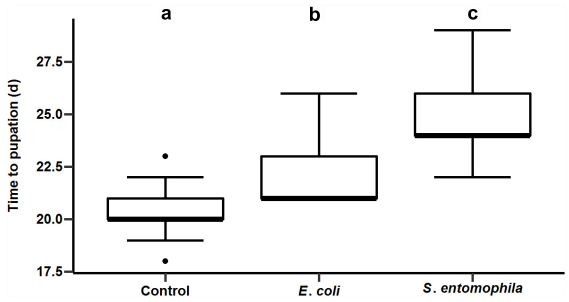

FIGURE 2 | Impact of orally administered bacteria on larval development. The developmental time until pupation of 49 uninfected control larvae, 49 larvae infected with *E. coli*, and 41 larvae infected with *S. entomophila*. Boxplots show median, interquartile, and total regions. Significant differences between groups are depicted by different letters (a–c).

and visualized by fluorescence microscopy in cryosections of third-instar F0 larvae (**Figures 3A–D**), ovaries of adult females derived from these larvae (**Figure 3E**), and oviposited F1 eggs (**Figure 3F**). Using this approach, we determined that the labeled bacteria can translocate from the midgut lumen into the hemocoel, where they attach to the fat body (**Figure 3**). The translocated bacteria are then deposited in the ovaries and taken up into the developing eggs. The labeled bacteria were associated with the follicle epithelium, the ovariole wall and the vitelline membrane. The translocated gut-derived bacteria were ultimately detected in the laid F1 eggs among yolk proteins and lipids (**Figure 3**).

TGIP Affects the Expression of Several Immunity-Related Genes in a Sex-Specific Manner

To determine whether TGIP influences the expression of immunity-related effector genes, namely those encoding gloverin, lysozyme isoforms 1 and 3, and pro-phenoloxidase 2 (PPO2), F0 third-instar larvae were fed on diets supplemented with either *S. entomophila* or *E. coli* (**Figure 4** and **Supplementary Table 4**). In both groups challenged with bacteria, gloverin gene expression was unaffected in the parental generation (**Figure 4A**). However, the lysozyme 3 gene was significantly upregulated in fathers ($p < 0.001$) and mothers ($p < 0.05$) fed on diets supplemented with *S. entomophila*, but downregulated in mothers fed on diets supplemented with *E. coli* ($p < 0.01$) (**Figure 4C**). In the latter group, *PPO2* was also significantly upregulated ($p < 0.001$) (**Figure 4D**). Remarkably, we observed sex-specific changes in gloverin expression ($p < 0.05$) in third-instar F1 larvae from parents challenged with *E. coli*, whereas there was no sex-specific difference in third-instar F1 larvae from parents challenged with *S. entomophila* or in larvae from parents fed on the uncontaminated control diet. Gloverin was upregulated in both sexes ($p < 0.001$) but this was more pronounced in males ($p < 0.05$). Both lysozyme isoforms were also specifically upregulated in F1 males (lysozyme 1, $p < 0.05$; lysozyme 3, $p < 0.001$) (**Figures 4B,C**) and so was *PPO2* ($p < 0.05$). For all of these transcripts, male larvae

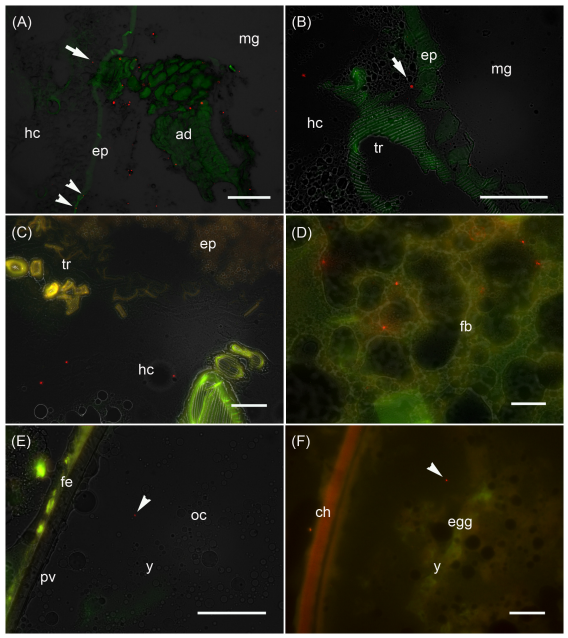

FIGURE 3 | Analysis of the maternal transfer of bacteria in *M. sexta* by fluorescence microscopy Cryosection of third-instar larva **(A–D)**, adult ovary **(E)** and oviposited egg **(F)**. **(A)** Cross-sections of midgut region (md) after the uptake of artificial diet (ad) supplemented with *E. coli* Bioparticles labeled with Texas Red (red spots). Translocated Bioparticles are also detectable in the midgut epithelium (ep, arrowheads) and in the hemocoel (hc, arrow). Scale bar = 100 μm. **(B)** Magnification of the midgut region with adjacent tracheole (tr) shows Bioparticles immediately beneath the midgut epithelium (arrow). Scale bar = 100 μm. **(C)** Cross-sections of the midgut region reveal numerous Bioparticles distributed in the hemocoel. Scale bar = 50 μm. **(D)** Fluorescent bacteria are also abundant in the fat body tissue (fb). Scale bar = 50 μm. **(E)** Female reproductive system dissected from adults reared on artificial diet containing Bioparticles during earlier larval stages. Fluorescent bacteria (arrowhead) are located in the yolk mass (y) of a developing oocyte (oc) which is surrounded by a thin perivitelline membrane (pv) and the follicle epithelium (fe). Scale bar = 100 μm. **(F)** Ovipositioned egg (egg) with an enclosing chorion (ch) contains bacterial probes (arrowhead) in the yolk mass (y). Scale bar = 50 μm.

originating from parents challenged with *E. coli* showed stronger upregulation than males stemming from parents challenged with *S. entomophila* (gloverin, $p < 0.01$; lysozyme 1, $p < 0.01$; lysozyme 3, $p < 0.001$; PPO2, $p < 0.01$). The latter group either showed no differences compared to the control cohort or the genes were slightly downregulated (lysozyme 1, $p < 0.05$; lysozyme 3, $p < 0.01$). In female offspring of parents challenged with *E. coli*, gloverin and lysozyme 1 were significantly upregulated compared to the control cohort (gloverin, $p < 0.001$; lysozyme 1, $p < 0.05$) but the other genes were not. Furthermore, there was no difference in expression between female offspring of parents exposed to the two different species of bacteria.

TGIP Also Influences Histone Acetylation/Deacetylation

The potential epigenetic basis of TGIP was investigated by monitoring the larvae fed on contaminated and uncontaminated

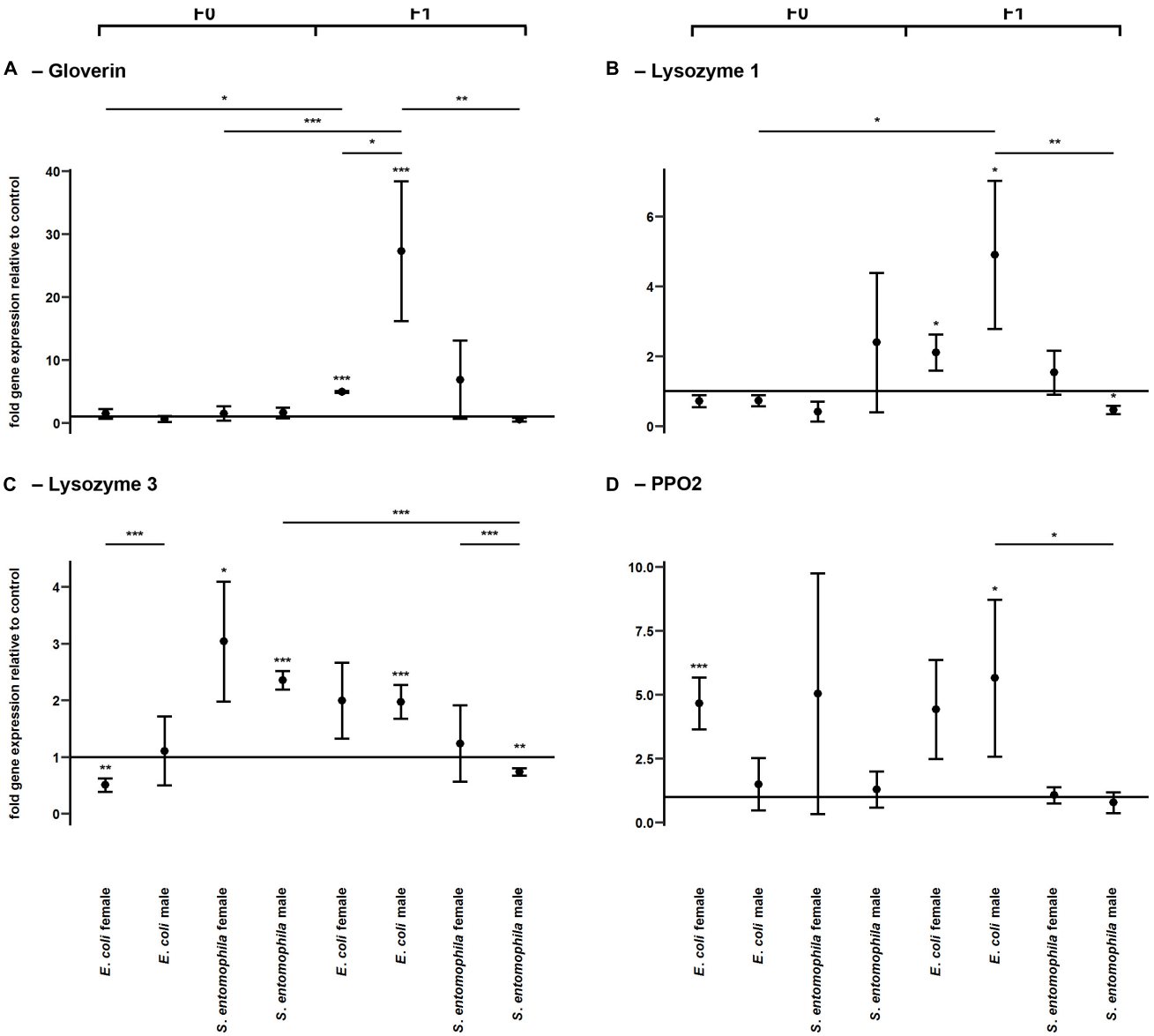

FIGURE 4 | Differential expression of immunity-related genes. Relative expression levels of immunity-related genes in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** gloverin, **(B)** lysozyme 1, **(C)** lysozyme 3, and **(D)** pro-phenoloxidase 2. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the 60S RNA housekeeping gene. Results represent the mean values of three independent biological replicates and error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

diets for the expression profiles of representative genes encoding either HATs (HAT enoki and HAT chameau) or HDACs (HDAC4, HDAC6, SAP18, and SAP130), and comparing the expression profiles of the same genes in the F1 larvae (**Figure 5** and **Supplementary Table 6**). Accordingly, we found that HAT enoki was upregulated in fathers challenged with *E. coli* ($p < 0.01$) and HAT chameau was downregulated in female offspring of parents challenged with *S. entomophila* ($p < 0.001$) (**Figures 5A,B**). For SAP18, we detected a significant treatment-specific difference between the mothers in the different treatment groups, with higher expression in the *E. coli* group ($p < 0.05$) (**Figure 5E**). Fathers challenged with *E. coli* displayed a reduced capacity for deacetylation. We observed the significant downregulation of HDAC6 ($p < 0.05$) and SAP130 ($p < 0.001$) (**Figures 5D,F**). In the F1 generation, female offspring of parents challenged with *S. entomophila* showed a reduced capacity for deacetylation, with a significant downregulation of HDAC4 ($p < 0.05$) and SAP130 ($p < 0.001$) (**Figures 5C,F**). SAP130 was also significantly downregulated in female larvae whose parents were exposed to *E. coli* ($p < 0.001$), but there was a less significant reduction compared with female larvae whose parents were exposed to *S. entomophila* ($p < 0.001$).

Surprisingly, these differences in HATs and HDACs were only partially reflected by the actual relative histone acetylation levels. Mothers exposed to *S. entomophila* displayed a slight but significant reduction in global histone H3 acetylation compared to the control ($p < 0.05$). Male F1 larvae from these mothers displayed significantly higher levels histone H3 acetylation than their counterparts whose parents were not exposed to bacteria ($p < 0.001$) and also when compared to their female peers ($p < 0.001$) (**Figure 5G** and **Supplementary Table 6**).

Impact of TGIP on DNA Methylation

DNA methylation was assessed by monitoring the larvae fed on contaminated and uncontaminated diets for the expression profiles of representative genes encoding DNMT1, DNMT2, and MBD (**Figure 6** and **Supplementary Table 9**). The expression profiles of the same genes were determined in F1 larvae for comparison. There was a sex-specific difference for both *DNMT1* ($p < 0.01$) and *MBD* ($p < 0.05$) expression in the *E. coli* group. These genes were significantly upregulated in mothers compared to fathers (**Figures 6A,C**). Interestingly, *DNMT1* also was significantly upregulated in mothers exposed to *E. coli* relative to those challenged with *S. entomophila* ($p < 0.05$). There also was a sex-specific difference in the expression of *DNMT1* ($p < 0.001$) and *DNMT2* ($p < 0.01$) in F1 larvae of parents challenged with *E. coli*. These enzymes were significantly more upregulated in males than in females, and also with respect to F1 male larvae from mothers challenged with *S. entomophila* (*DNMT1*, $p < 0.001$; *DNMT2*, $p < 0.05$) (**Figures 6A,B**). This treatment-specific difference in gene expression was also observed for MBD across both sexes (males, $p < 0.01$; females, $p < 0.01$).

The relative global DNA methylation levels ranged between $0.09 \pm 0.13\%$ and $1.26 \pm 0.08\%$ (**Supplementary Table 11**). For the *E. coli* treatment group, we observed significantly reduced levels of DNA methylation in both sexes (males, $p < 0.001$; females, $p < 0.01$) but no such effect was found in the *S. entomophila* treatment group. Interestingly, DNA methylation also was significantly lower in fathers challenged exposed to *E. coli* compared to those treated with *S. entomophila* ($p < 0.001$). On the other hand, in the F1 generation, male and female offspring of both parents in both treatment groups displayed significantly reduced DNA methylation levels compared to controls (*E. coli* group males and females, $p < 0.001$; *S. entomophila* group males, $p < 0.001$; *S. entomophila* group females, $p < 0.01$). In larvae stemming from parents challenged with *S. entomophila* there also was a sex-specific difference ($p < 0.05$), with females displaying significantly lower methylation levels than males. Additionally, the latter showed significantly higher DNA methylation levels than the male offspring of parents challenged with *E. coli* ($p < 0.001$) (**Figure 6D** and **Supplementary Table 9**).

DISCUSSION

Theory predicts that immune responses will be sex-specific because the reproductive effort of females is higher than that of males. According to Bateman's principle, males improve their fitness by increasing their mating success whereas females increase fitness through longevity (Rolff, 2002). Current evidence suggests that these different investment strategies and life-history traits translate into the sex-specific expression of immunity-related genes in insects, which is in turn reflected by the sex-specific expression of regulatory microRNAs (Jacobs et al., 2016). Several studies have shown that parental investment into their offspring is achieved via TGIP which is also sex-dependent (Roth et al., 2010; Eggert et al., 2014). The higher specificity of maternal TGIP in insects can be explained by – but may not be limited to – the transfer of specific bacterial or fungal cells from the mother to the offspring (Freitak et al., 2014; Fisher and Hajek, 2015). However, it is unclear how male insects can transmit information about the pathogens they have encountered, and we have previously hypothesized that epigenetic mechanisms could explain this phenomenon (Vilcinskas, 2016; Vilcinskas, 2017). We selected the tobacco hornworm (*M. sexta*) because it is a widely used model of insect physiology and immunity and previous studies have demonstrated that TGIP occurs in this species (Trauer and Hilker, 2013; Trauer-Kizilelma and Hilker, 2015a,b; Rosengaus et al., 2017). Although speculating on the potential epigenetic dimension of their findings, these earlier studies did not address this topic by way of design. To the best of our knowledge the work of Castro-Vargas et al. (2017) is the only study which investigated the influence of an epigenetic regulator in TGIP. In this work the researchers found that RNA methylation is related to immune priming within but not across generations in *T. molitor* while DNA methylation was not detected (Castro-Vargas et al., 2017). As histone acetylation assays were previously established in our group for *G. mellonella* (Mukherjee et al., 2012), and DNA methylation was detected in closely related species, for example *B. mori* and *M. brassica* (Bewick et al., 2017), these two epigenetic mechnisms provided a promissing system to investigate the potential link between epigenetics and TGIP in our model organism *M. sexta*.

Given that pathogenic and non-pathogenic bacteria can influence TGIP in different ways, we fed hornworm larvae on diets supplemented with either the entomopathogenic species *S. entomophila* or the common gut inhabitant *E. coli* to mimic natural oral infections. The supplemented diets confirmed that *S. entomophila* and *E. coli* cause different developmental effects. Similar findings in *G. mellonella* have been attributed to the ability of pathogens, but not non-pathogenic species, to interfere with epigenetic mechanisms in the infected host (Mukherjee et al., 2015; Vilcinskas, 2017).

Next, we demonstrated that bacteria added to the diet of female larvae can translocate from the gut into the hemocoel and are ultimately deposited in the eggs, where they have the potential to elicit an immune response that may be sufficient to protect offspring from pathogens as they hatch (**Figure 3**). We also found that orally delivered *E. coli* and *S. entomophila* modulated the expression of selected immunity-related genes (encoding gloverin, two lysozyme isoforms, and a pro-phenoloxidase) in the gut of the infected F0 larvae, and also in their offspring.

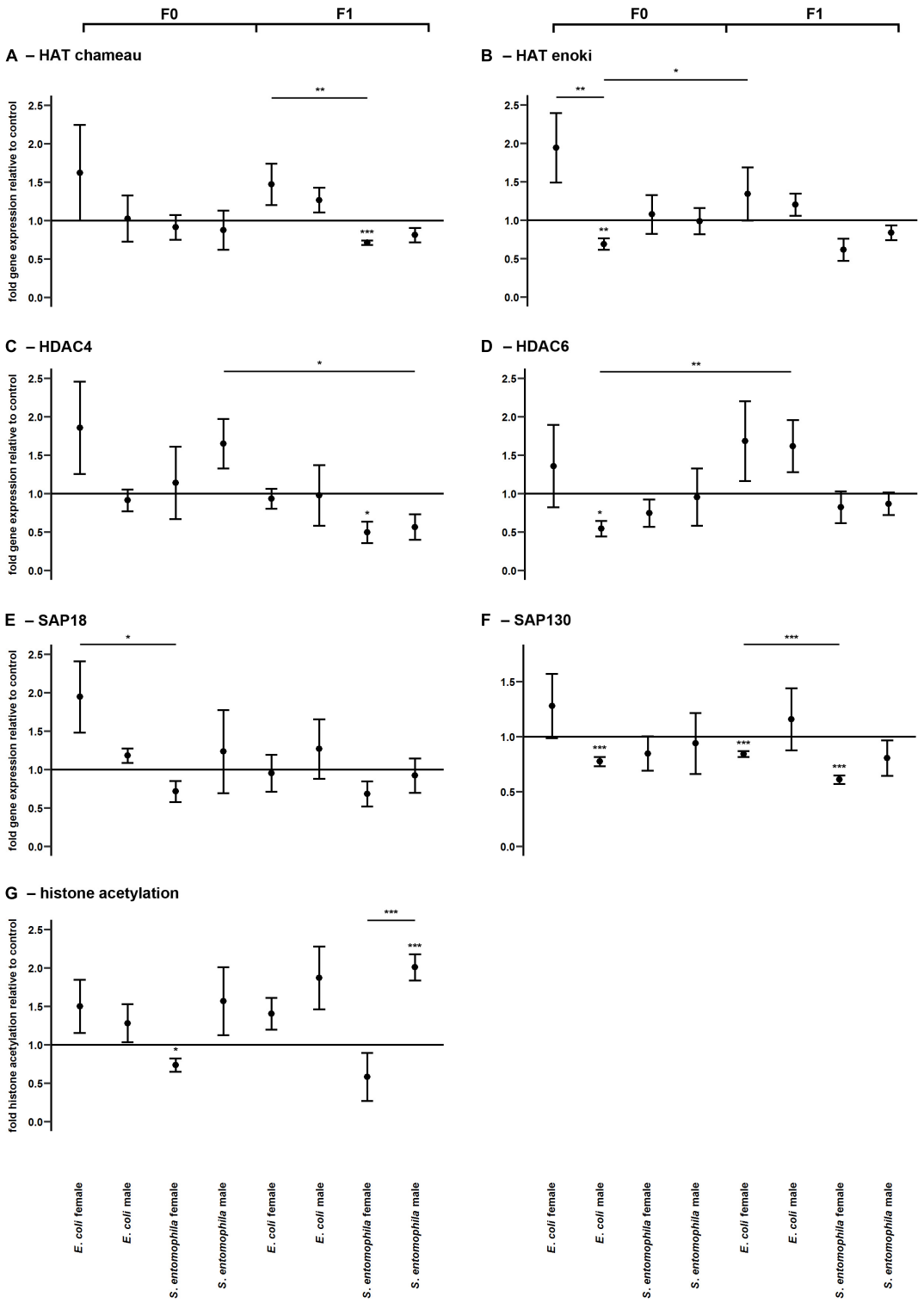

FIGURE 5 | Differential expression of genes encoding histone modifying enzymes and corresponding levels of histone acetylation. Relative expression levels of genes associated with histone modification in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** HAT chameau, **(B)** HAT enoki, **(C)** HDAC4, **(D)** HDAC6, **(E)** SAP18, and **(F)** SAP130. **(G)** Corresponding levels of relative histone H3 acetylation. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the RPL3 housekeeping gene. Results represent the mean values of three independent biological replicates for relative gene expression and four replicates for relative histone acetylation. Error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

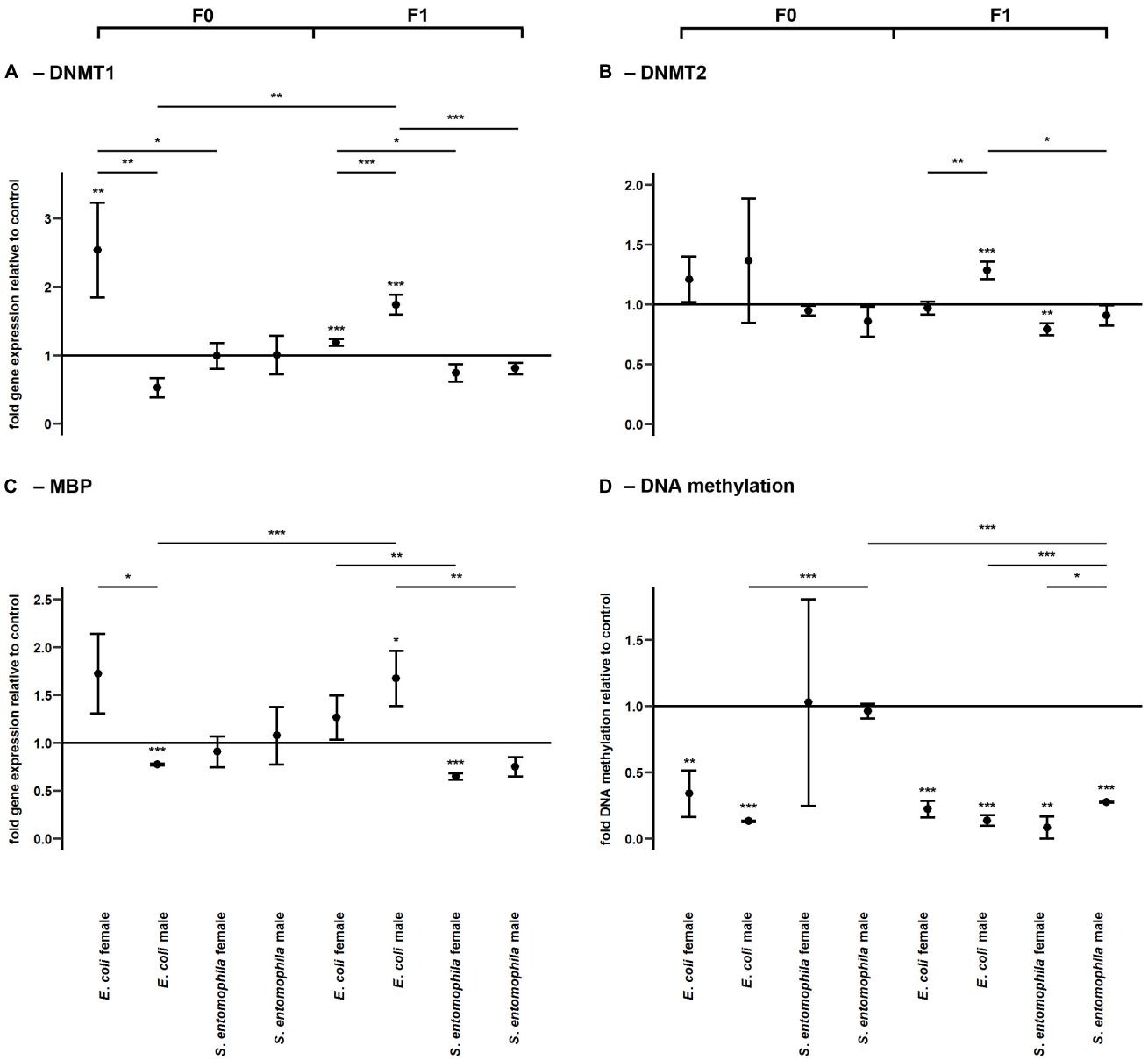

FIGURE 6 | Differential expression of genes encoding DNA modifying enzymes and corresponding levels of DNA methylation. Relative expression levels of genes associated with DNA methylation in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** DNMT1, **(B)** DNMT2, and **(C)** MBD. **(D)** Corresponding levels of relative global DNA methylation. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the *RPL3* housekeeping gene. Results represent the mean values of three independent biological replicates for relative gene expression and two replicates for the relative DNA methylation. Error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Interestingly, we observed significant sex-specific differences in the expression of gloverin in third-instar F1 larvae whose parents were exposed to *E. coli*, providing evidence for sex-specific TGIP in *M. sexta*. The near universal pattern was that male offspring of parents challenged with *E. coli* displayed the highest expression levels of immunity-related genes among all tested subgroups (**Figures 4A,C,D**). This agrees with an earlier report showing that *M. sexta* gloverin was induced to significantly higher levels in the hemocytes and midgut by Gram-negative *E. coli* than by other microorganisms (Xu et al., 2012). Male larvae therefore express antimicrobial peptides at higher levels than females, but the reason for this is unclear because the phenomenon does not seem to fit Bateman's principle. However, the activation of innate immunity constitutes fitness costs as well as benefits, so organisms are expected to optimize (but not necessarily maximize) their immune responses according to the circumstances. Optimality models predict that when pathogens are particularly detrimental to male mating

success but have a less severe effect on female fecundity or longevity, a superior male immune response will evolve (Stoehr and Kokko, 2006). For example, freshly eclosed *Pieris rapae* adult males display a stronger encapsulation response than females, but the female response becomes stronger as the individuals age (Stoehr, 2007). In our study, we analyzed gene expression only during the third larval stage. It is possible that the immune system dynamics are switched in favor of females during later larval stages, the pupal stage or at some point during the adult phase. However, for males to give preference to reproductive success over immunity, they would need to become sexually mature in the first place. If they succumb to pathogens during the larval stage, this trade-off becomes irrelevant. For example, in the case of *PPO2* (which is needed for the encapsulation response) we detected a near one-to-one transmission of the expression profile from parents challenged with *E. coli* to their male offspring (**Figure 4D**). Perhaps mothers specifically provide their male offspring with a form of immune competence that they would otherwise not exhibit due to a built-in trade-off. Moreover, in contrast to the results of an earlier investigation of transgenerational immune gene expression in *M. sexta* (Trauer-Kizilelma and Hilker, 2015b), we detected induced gloverin expression even though offspring remained unchallenged. This may reflect the different routes of infection and the different priming agents used in each study, and the different offspring stages assessed to investigate TGIP. The authors of this earlier study injected their parental generation with a peptidoglycan isolated from the Gram-positive bacterium *Micrococcus luteus* and sampled F1 eggs and ovaries of F1 females (Trauer-Kizilelma and Hilker, 2015b), whereas we used oral infection with Gram-negative *E. coli* and sampled male and female third-instar larvae. Similar findings to those reported herein were presented following the oral infection of *G. mellonella* with a mixture of Gram-negative *E. coli* and Gram-positive *M. luteus*, or with the entomopathogenic species *Pseudomonas entomophila* or *S. entomophila* (Freitak et al., 2014). The gloverin gene was upregulated in the eggs of challenged females and gloverin levels in their *E. coli*/+*M. luteus* challenge group were similar to the levels we observed in *M. sexta*.

Interestingly the bona fide pathogen we tested (*S. entomophila*) did not elicit any transgenerational immune responses, regardless of the offspring sex. If anything, we observed a slight downregulation of transcripts encoding immune effectors in this group (**Figure 4**). This may reflect the pathogen-induced circumvention of host efforts to protect their offspring against previously encountered microbes. If true, this would be consistent with the classical paradigm of host–parasite co-evolution and could represent a case of reciprocal epigenetic adaptations, as previously suggested (Vilcinskas, 2016). Accordingly, in the offspring generation, we found that the expression of HAT chameau was significantly downregulated in females whose parents had been exposed to *S. entomophila*, whereas the same gene was upregulated in females whose parents had been exposed to *E. coli*. We did not observe any differential regulation of HAT enoki in the *S. entomophila* treatment group but sex-specific regulation was evident in the *E. coli* treatment group (elevated in mothers but repressed in fathers) indicating that histone acetylation may underlie the sex-specific TGIP we observed (**Figures 5A,B**). Our interpretation relies on the general notion that the acetylation of histones H3 and H4 is highly correlated with gene expression, which seems to be conserved across higher eukaryotes (Zhang et al., 2015). At the same time, the expression of the HDAC SAP18 was significantly elevated in *E. coli*-fed mothers, which indicates ongoing differential gene regulation across the genome, consistent with adaptive processes that might contribute to TGIP. In line with this interpretation, HDAC6 and SAP130 were significantly downregulated in *E. coli*-fed fathers. Interestingly, the relative levels of histone H3 acetylation were lower only in the mothers challenged with *S. entomophila*, which again indicates pathogen-derived epigenetic interference that results in overall transcriptional repression (**Figure 5G**). This agrees with an earlier study showing that pathogenic bacteria can interfere with the regulation of HDACs and HATs in insects and can manipulate host immunity in *G. mellonella* (Mukherjee et al., 2012).

In the F1 offspring of parents challenged with *S. entomophila*, HDAC4 and SAP130 were significantly downregulated in female F1 larvae, which surprisingly did not correlate with the higher relative histone H3 acetylation levels. On the other hand, there was no differential regulation of histone acetylation modifiers in F1 male larvae, but the level of histone H3 acetylation was significantly higher. These results may indicate that the causal relationship between histone modifiers and marks is not as straightforward in lepidopteran species as previously assumed. Perhaps crosstalk with other histone marks such as methylation and ubiquitinylation have a dominant regulatory effect over the state of histone acetylation than the enzymes responsible for the actual addition and removal of acetyl groups. Alternatively, these results may represent prolonged sex-specific interference by the entomopathogen to disrupt the epigenetic machinery of the host well into the larval stage of the next generation, perhaps making males more susceptible to attack through the deregulation of immunity-related gene expression. Given that histone modifications occur locally due to the DNA sequence-dependent binding of transcription factors that recruit the HATs and HDACs (Zhang et al., 2015), the abundance of specific enzymes does not necessarily mean that global acetylation levels must change in the same manner.

In addition to histone acetylation, we also observed a sex-specific difference in the expression of genes related to DNA methylation when the parents were exposed to *E. coli*. DNMT1 and MBD were significantly upregulated in mothers but significantly downregulated in fathers (**Figures 6A,C**). Surprisingly, global DNA methylation levels were reduced in parents fed on diets containing *E. coli* but there was no significant difference between sexes. It is unclear why upregulation of the maintenance methyltransferase DNMT1 did not prevent the observed demethylation in *E. coli*-fed mothers. DNMT1 may perform additional functions other than maintaining the DNA methylation status across cell cycles

in *M. sexta*, or the oral challenge with *E. coli* may have triggered the major reprogramming of the methylome. It is conceivable that new genomic regions were methylated *de novo* while a larger, previously methylated portion of the genome became demethylated as a response to the infection, explaining the (partially sex-specific) gene expression we observed in their offspring. Ten-eleven translocation dioxygenases have been implicated in active DNA demethylation in vertebrates, but the demethylation apparatus in insects is unknown (Provataris et al., 2018).

Interestingly, in parents exposed to *S. entomophila*, there was no change in the expression of methylation-related enzymes or in the global DNA methylation status (**Figure 6D**). This further supports our hypothesis that the pathogen was able to interfere with or even subdue the epigenetic machinery of the host, as has recently been demonstrated in the diamondback moth (*Plutella xylostella*) during infections with the koinobiotic endoparasitic wasp *Cotesia plutellae* (Kumar and Kim, 2017). DNA methylation was reduced in parasitized larvae relative to non-parasitized controls, especially at late parasitic stages, along with reduced expression levels of DNMT1, DNMT2 and MBD. The mechanisms of epigenetic interference used by parasitic wasps and pathogenic bacteria are likely to differ substantially, given the phylogenetic distance between the invaders. Infection with *S. entomophila* did not alter DNA methylation in the parental generation but methylation levels were lower in the F1 offspring, particularly in females. Furthermore, and as observed in *P. xylostella*, the expression of DNMT1, DNMT2 and MBD in F1 larvae of both sexes was much lower when the parents had been challenged with *S. entomophila* compared to *E. coli* (**Figures 6A–C**). *S. entomophila* therefore appears to suppress TGIP by interfering with gene expression in the offspring. In contrast, in the female offspring of *E. coli*-fed parents there was no change or limited upregulation of the enzymes involved in DNA methylation whereas there was consistent upregulation in males, as observed for the expression of immunity-related genes. In both sexes, DNA methylation levels were lower than in the control treatment group. This configuration of enzymes versus methylation status again points toward an active and ongoing restructuring of epigenetically mediated gene regulation. Even though at first glance the reduced DNA methylation in both offspring groups seems to be analogous, this evidently does not translate into similar transcriptional profiles, as explained above. Interestingly, in the cotton bollworm *Helicoverpa armigera*, DNA methylation is tightly associated with stably expressed genes with basic housekeeping roles, such as transcription and translation (Jones et al., 2018). Even minute differences in such basic but diverse biological functions are likely to result in profoundly different transcriptional outcomes, depending on the methylation state of the corresponding genomic region.

CONCLUSION

We have shown that TGIP in *M. sexta* is associated with changes in both histone acetylation and DNA methylation. Effects in the offspring depended on the species of bacteria encountered by the parents, and were sex-specific for certain genes as well as for histone acetylation. We have also demonstrated that infection with non-pathogenic *E. coli* resulted in the differential expression of immunity-related genes and DNA methylation-modifying enzymes in the offspring generation, with the highest expression levels observed in males. The entomopathogen *S. entomophila* appears to influence most of the parameters we tested, consistent with counteracting the TGIP efforts of the host. The latter hypothesis warrants more research to determine the extent to which the observed effects reflect an epigenetic dimension of host–parasite coevolution. Our study shows that epigenetic mechanisms are promising tools to get further insight in the molecular mechanisms behind TGIP.

DATA AVAILABILITY

The datasets for this study can be found in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

AB and JG carried out the laboratory work related to the TGIP experiments. JG contributed to data analysis. RH and JG analyzed DNA methylation. KM analyzed histone acetylation. HV designed primers for qPCR and identification of epigenetic markers and immune genes. AV designed the study, provided funding, and supervised AB and JG. All authors drafted parts of the manuscript, gave approval for publication and agree to be accountable for the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00137/full#supplementary-material

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Acaricidal Mechanism of Scopoletin Against *Tetranychus cinnabarinus*

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Scopoletin is a promising acaricidal botanical natural compound against *Tetranychus cinnabarinus*, and its acaricidal mechanism maybe involve calcium overload according to our previous study. To seek potential candidate target genes of calcium overload induced by scopoletin in *T. cinnabarinus*, RNA-seq was utilized to detect changes in transcription levels. 24 and 48 h after treatment, 70 and 102 differentially expressed genes were obtained, respectively. Target genes included 3 signal transduction genes, 4 cell apoptosis genes, 4 energy metabolism genes, and 2 transcription factor genes. The role of 3 calcium signaling pathway-related genes, namely, G-protein-coupled neuropeptide receptor, Bcl-2 protein and guanylate kinase (designated *TcGPCR*, *TcBAG*, and *TcGUK*, respectively) in the calcium overload were investigated in this study. RT-qPCR detection showed that scopoletin treatment upregulated the expression level of *TcGPCR* and downregulated the expression level of *TcBAG* and *TcGUK*. The result of RNAi indicated that downregulation of *TcGPCR* decreased susceptibility to scopoletin, and downregulation of *TcBAG* and *TcGUK* enhanced susceptibility to scopoletin. Functional expression in Chinese hamster ovary cells showed that scopoletin induced a significant increase in intracellular free calcium $[Ca^{2+}]i$ levels by activating *TcGPCR.* These results demonstrated that the acaricidal mechanism of scopoletin was via disrupting intracellular Ca^{2+} homeostasis and calcium signaling pathway mediated by GPCR, BAG, and GUK.

Keywords: *Tetranychus cinnabarinus*, scopoletin, GPCR, BAG, GUK, Ca^{2+} homeostasis, calcium signaling pathway

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INTRODUCTION

The carmine spider mite, *Tetranychus cinnabarinus*, is one of the most polyphagous arthropod herbivores and feeds on more than 100 plant species, such as food and economic crops, ornamental plants, and weeds (Zhang et al., 2004; Çakmak et al., 2005; Sarwar, 2013). The carmine spider mite is parthenogenic and exhibits strong adaptability and fecundity. Moreover, this type of mite is one of the most difficult pests to control because it easily develops resistance to pesticides (Cruz et al., 2013).

Scopoletin (**Supplementary Figure S1**) is a kind of botanical natural phenolic coumarin (**Supplementary Figure S2**) and an important member of the group of phytoalexins isolated from many plants, such as *Erycibe obtusifolia* (Pan et al., 2011), *Aster tataricus* (Ng et al., 2003), *Foeniculum vulgare* (Kwon et al., 2002), *Artemisia annua* (Tzeng et al., 2007), *Sinomonium acutum* (Shaw et al., 2003), and *Melia azedarach* (Carpinella et al., 2005). Studies have shown that scopoletin

has a wide spectrum of biological activities, such as acaricidal (Zhou et al., 2017), anti-inflammatory (Ding et al., 2009; Jamuna et al., 2015), antitumoral (Cassady et al., 1979), antioxidative (Shaw et al., 2003), hepatoprotective (Cassady et al., 1979), insecticidal (Tripathi et al., 2011), antifungal (Prats et al., 2006), and alleopathic properties (Pérez and Nuñez, 1991). Especially, a previous study found that scopoletin exhibits excellent contact killing, as well as systemic, repellent, and oviposition inhibition activities against *T. cinnabarinus* (Zhou et al., 2017). Moreover, mites did not develop resistance against scopoletin after 18 generations possibly because of the multi-target mechanism of scopoletin against *T. cinnabarinus* (Zhang et al., 2011). Furthermore, after exposure to scopoletin, several typical neurotoxic symptoms, such as excitement and convulsions, were observed in mites, and the compound specifically inhibits the nervous system targets, AChE, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, and Ca^{2+}-ATPase, which indicates that scopoletin is a neurotoxin, in which Ca^{2+} plays a key role as an intracellular second messenger (Liang et al., 2011; Hou et al., 2015).

Intracellular free calcium [(Ca^{2+}) i] is one of the small signaling molecules regulating various biological functions in cells, including gene expression, protein synthesis, cell secretion, motility, metabolism, cell-cycle progression, and cell apoptosis (Nicotera and Orrenius, 1998). Under normal conditions, [Ca^{2+}]i concentration is maintained at 10–100 nM, and intracellular Ca^{2+} homeostasis maintains the normal function of cells (Bootman et al., 2001). However, sustained Ca^{2+} release from intracellular Ca^{2+} stores, Ca^{2+} influx through receptor- or voltage-dependent Ca^{2+} channels or blockage of re-uptake can perturb Ca^{2+} homeostasis, and the increased intracellular calcium concentration [(Ca^{2+})i] induces cell apoptosis (Orrenius et al., 2003). In human or mammalian cells, the increased [Ca^{2+}]i mediates the apoptosis of tumor cell induced by scopoletin in different cell types, such as T lymphoma cells (Manuele et al., 2006), PC3 cells (Liu et al., 2005), P-388 lymphocytic leukemia (Cassady et al., 1979), KB cells (Williams and Cassady, 1976), and Hepa 1c1c7 mouse hepatoma cells (Jang et al., 2003). Meanwhile, studies proved that the mode of action of scopoletin in insects was by inducing intracellular calcium overload. A significant increase in intracellular calcium level in *Spodoptera frugiperda* Sf9 cells was induced by scopoletin in a dose-dependent manner (**Figure 1**). Interestingly, the combination of Ca^{2+} and scopoletin can significantly improve its acaricidal activity (Hou et al., 2015). It is clear that the acaricidal mechanism of scopoletin is mainly by inducing calcium overload. However, other processes, such as MAPK signaling pathway, protein processing in endoplasmic reticulum, and fat digestion and absorption, may play a secondary role in the mode of action of scopoletin. Therefore, in this study, the molecular mechanism of calcium overload induced by scopoletin was investigated.

The main aim of the current study was to investigate the molecular mechanism of scopoletin inducing calcium overload in *T. cinnabarinus*, and attempted to provide evidence that the mode of action of the scopoletin was through the regulation of the expression of calcium signaling pathway-related genes, thus inducing calcium overload to kill mites. We conducted a comprehensive study that utilizes RNA-seq to detect changes in transcription levels. The role of candidate target genes, that is, calcium signaling pathway-related genes in the calcium overload were also investigated by RNA interference (RNAi) and a calcium reporter assay.

MATERIALS AND METHODS

Mite Rearing

The *T. cinnabarinus* colony was originally collected from cowpeas in Beibei, Chongqing, China and maintained for more than 16 years without exposure to any pesticides (Zhang et al., 2013). Specific permission was not required for the collection because it is a harmful agricultural insect and is distributed extensively. The mites were reared on potted in cowpea seedlings (*Vigna unguiculata*) in the insectary at 25 ± 1°C, 50% ± 5% RH, and 14 h:10 h (L:D) photoperiod.

Cell Lines and [Ca^{2+}]i Assay

Spodoptera frugiperda Sf9 cells were cultivated at 27°C and 5% CO_2 in 3 mL Grace's insect cell culture medium (Gibco, United States) containing 10% fetal bovine serum (FBS), 0.3% yeast extract, 0.3% lactalbumin hydrolysate, and 0.3% peptone. Chinese hamster ovary (CHO-WTA11) cells were cultured at 37°C and 5% CO_2 in the DMEM/F-12 medium (Invitrogen Life Technologies, Carlsbad, CA, United States) supplemented with 10% FBS, 250 ng/ml fungizone, 100 U/ml of penicillin, and 100 mg/ml of streptomycin.

In order to detect the effect of scopoletin (purity, 95%; Southwest University, Beibei, Chongqing, China) on intracellular free calcium [Ca^{2+}]i levels in insect cells, the Sf9 cells were treated with diluted scopoletin at concentrations of 0, 10, 50, and 90 μM for 24 h. The [Ca^{2+}]i level in the Sf9 cells was determined by Fura-2/AM fluorescence staining (Cao et al., 2016). Briefly, the harvested cells were incubated with Fura-2/AM (Beyotime, China) at final concentration of 5 μM at 30°C for 30 min. A fluorescence microscope (Carl Zeiss) was then used to observe the cells. The [Ca^{2+}]i level was represented by the mean fluorescence intensity (MFI) after the captured images were analyzed using Image-Pro Plus software (Media Cybernetics).

Bioassays and Scopoletin Treatment

The FAO-recommended slip-dip method was used to measure scopoletin toxicity against adult female *T. cinnabarinus* (Busvine, 1980). The details of the bioassay procedure were described by Zhang et al. (2013). In brief, 30 adult female individuals (3–5 days old) were placed on their backs on double-sided tape on glass. Then, the mites were dipped into each test solution for 5 s. Each dose (2, 1, 0.5, 0.25, 0.125, and 0.0625 g/ml) was performed in three replicates. Sterile distilled water with 0.1% (v/v) Tween-80 and 3% (v/v) acetone was designated as the control treatment. The mites were observed under an anatomical microscope after 48 h of rearing under controlled growth conditions as described above. Mites that exhibited immobility or irregularly trembling legs were considered dead. The lethal concentrations

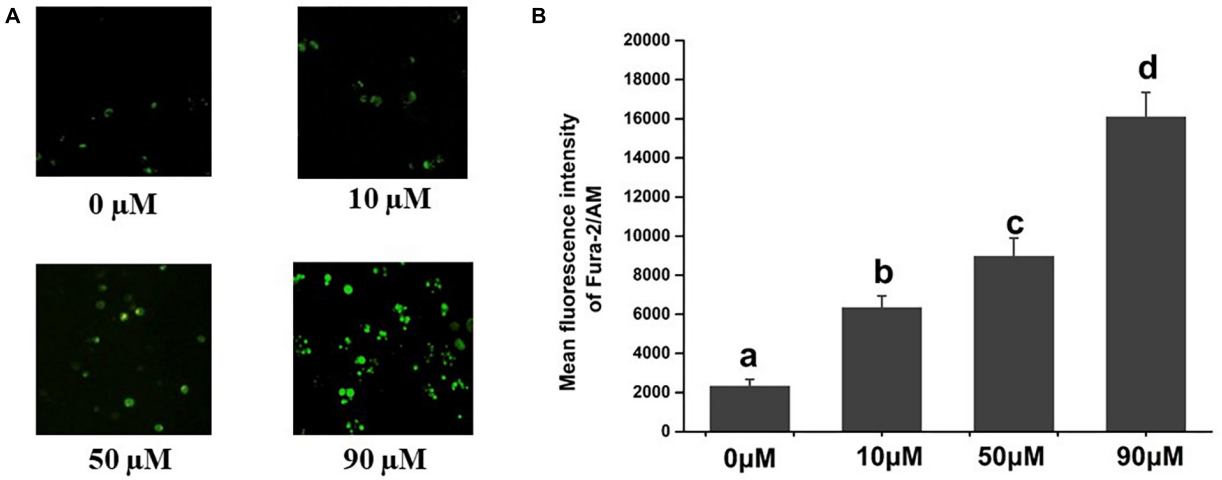

FIGURE 1 | Effects of scopoletin on intracellular free calcium [Ca^{2+}]i levels in insect Sf9 cells. 0, 10, 50, and 90 μM indicate Sf9 cells incubated with scopoletin at concentrations of 0, 10, 50, and 90 μM, respectively for 24 h. **(A)** The [Ca^{2+}]i level was detected by Fura-2/AM fluorescence staining. Positively stained calcium is shown as green zones in the captured images under a microscope. **(B)** The bar chart indicates the mean fluorescence intensity (MFI) of Fura-2/AM in Sf9 cells. Error bars represent the standard error of the calculated mean based on three biological replicates. Different letters on the error bars indicate significant difference according to Duncan's multiple tests (alpha = 0.05). i.e., No statistical difference between "a" and "a"; significant difference among "a," "b," "c," and "d."

for subsequent experiments were determined on the basis of log-probit analysis of concentration–mortality data.

For the analysis of the transcriptome changes in *T. cinnabarinus* treated with scopoletin or the solvent, scopoletin was dissolved in sterile distilled water containing 0.1% Tween 80 and 3% acetone to a final concentration of 0.938 mg/mL, the median lethal concentration (LC_{50}) of scopoletin against *T. cinnabarinus*. For the scopoletin exposure experiment, we adopted a slightly modified version of the leaf-disk dipping method described by Michel et al. (2010). More than 200 female adults (3–5 days old) were transferred to three freshly potted cowpea leaves in a small petri dish with water. Each detached cowpea leaf was dipped for 5 s in the test solution at the concentration indicated above. When the liquid dried around the mites, the insects were returned to the conditions as above. Then, sterile distilled water with 0.1% Tween-80 and 3% acetone was used as the solvent control. Three petri dishes from one independent experiment comprised a replicate and two biological replicates used for RNA purification and library preparation. After 24 and 48 h intervals, only the surviving female adult mites from the treated and control groups were collected and frozen at −80°C for RNA extraction.

RNA Extraction, Library Preparation, and Sequencing

The total RNA of each sample was extracted using the RNeasy® plus Micro Kit (Tiangen, Beijing, China) following the manufacturer's instructions. For checking the RNA quantity, the absorbance at 260 nm and absorbance ratio of $OD_{260/280}$ were measured using a Nanovue UV-Vis spectrophotometer (GE Healthcare, Fairfield, CT, United States). RNA integrity was further confirmed by 1% agarose gel electrophoresis.

The polyA mRNA was enriched from the total RNA using the Dynabeads mRNA Purification Kit (Invitrogen) and digested into short fragments (∼130 bp) with First-Strand Buffer (Invitrogen) at the appropriate temperature. The short fragments served as templates to synthesize first-strand cDNA with random hexamer primers, First-Strand Master Mix, and Super Script II reverse transcriptase (Invitrogen). Then, second-strand cDNA was synthesized using the Second-Strand Master Mix. After adenylation of the 3' ends of DNA fragments, the sequencing adaptors were ligated. AMPure XP beads were used to purify the short fragments; these cDNA were eluted in EB buffer, followed by polymerase chain reaction (PCR) amplification. An Agilent 2100 Bioanalyzer checked the quality of the library and the concentration of cDNA. The prepared libraries were sequenced on the Ion Proton platform (BGI, Shenzhen, China) using the sequencing strategy of single-end 150 bp.

Processing and Mapping of RNA-Seq Data

Primary sequencing data was first subjected to quality control. These data were produced by Ion Proton and were called raw reads. For filtering the raw reads, low-quality and adapter sequences were removed. The Q20, Q30, and GC contents of the filtered reads were calculated and checked. All obtained high-quality and clean reads were mapped against the reference genome of *Tetranychus urticae* with T-Map (Version 3.4.1, parametermapall-a2-n8-v-Y-u-o1stage1map4)[1]. Mismatches of three or less than three bases for each read (mean length = 150 bp) were allowed in the mapping. The unique and non-unique mapped reads were used for mapping scale calculation. The read per kilobase per million mapped reads (RPKM) of each

[1]https://www.ncbi.nlm.nih.gov/guide/genomes-maps/

gene was calculated using the following formula: RPKM = total exon reads/mapped reads in million × exon lengths in kb. The RPKM value was used as expression levels for differential expression analysis.

Differential Expression Analysis

To identify differentially expressed genes between different treatments, we used a rigorous algorithm as previously described (Audic and Claverie, 1997). The false discovery rate (FDR) was calculated to determine the threshold *p*-value in multiple tests. In this study, a threshold FDR ≤ 0.001 and an absolute value of $Log_2Ratio \geq 1$ were used to determine the significance of gene expression differences (Benjamini and Yekutieli, 2001). For depth analysis of differentially expressed genes, cluster analysis was performed using Cluster software and Java Treeview software. We then mapped, all differentially expressed genes to terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the gene orthology (GO) database for annotation.

Total RNA Extraction, cDNA Synthesis, and TcGPCR, TcBAG, and TcGUK Cloning

Total RNA was extracted from 300 adult (3–5 days old) *T. cinnabarinus* females. Extraction was performed as described in the above. Reverse transcription was performed with PrimeScript® First Strand cDNA Synthesis Kit (Takara, Dalian, China). Synthesized cDNA was stored at −20°C. To obtain the full-length *TcGPCR*, *TcBAG*, and *TcGUK*, we designed and synthesized specific primers (**Supplementary Table S1**) based on complete genomic sequences from the sister species *T. urticae*[2]. Specific PCR reactions were performed in a C1000™ Thermal Cycler (BIO-RAD, Hercules, CA, United States). PCR reactions were performed with a 25 μL reaction volume with 2.5 μL 10 × PCR buffer (Mg^{2+} free), 2.0 μL dNTPs (2.5 mM), 2.5 μL $MgCl_2$ (25 mM), 1 μL cDNA templates, 1 μL of each primer (10 mM), 0.2 μL rTaq™ polymerase (TaKaRa), and 14.8 μL double-distilled H_2O (ddH_2O). The PCR program was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 48°C to 60°C (based on the primer annealing temperature) for 30 s, 72°C extension for 1 min to 2 min (based on the predicted length of the amplified products), and a final extension for 10 min at 72°C. The amplified PCR fragments were gel-purified using the Gel Extraction Mini Kit (Tiangen, Beijing, China), ligated into pMD™ 19-T Vector (Takara, Dalian, China), and then transformed into *Escherichia coli* Trans5α-competent cells (Tiangen, Beijing, China). Recombinant plasmids were sequenced at the Beijing Genomics Institute (BGI, Beijing, China).

Gene Characterization and Phylogenetic Analysis

The nucleotide sequences of *TcGPCR*, *TcBAG*, and *TcGUK* nucleotide sequences were edited with DNAMAN 5.2.2. The deduced amino acid sequences of the GPCR, BAG, and GUK genes were aligned with ClustalW program (Hill et al., 2004; Bansal et al., 2011). The molecular weight and isoelectric point (pI) of the proteins were calculated by ExPASy Proteomics Server[3] (Bairoch, 1993). The signal peptide was predicted using SignalP 4.1[4] (Bendtsen et al., 2004), and the transmembrane region was analyzed using the TMHMM Server (v.2.0)[5] (Krogh et al., 2001). The *N*-glycosylation sites were predicted by the NetNGlyc 1.0 Server[6] (Gupta et al., 1997). The phylogenetic tree was constructed with MEGA 5.0 via the neighbor-joining method with 1000 bootstrap replicates (Tamura et al., 2011).

dsRNA Synthesis, dsRNA Feeding, and Knockdown *TcGPCR*, *TcBAG*, and *TcGUK* by RNAi

A set of T7 RNA polymerase promoter primers (**Supplementary Table S1**) were designed to amplify 160–600 bp lengths of the target genes to generate PCR products for *in vitro* transcription and dsRNA production (**Supplementary Table S1**). *TcGPCR*, *TcBAG*, *TcGUK* and the Green Fluorescent Protein (*GFP*) (ACY56286) gene were amplified by PCR. The PCR program was as described above. The recombinant plasmids were used as a template. The *GFP* gene was used as a negative control. The amplified segments were gel-purified and used in the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Lithuania, Europe). The dsRNAs were further purified with GeneJET RNA Purification Kit (Thermo Fisher Scientific, Lithuania, Europe). The size of the dsRNA products was determined by 1% agarose gel electrophoresis. The concentration of dsRNAs was determined with a spectrophotometer. dsRNAs were stored at −70°C. The systemic delivery of dsRNA via leaf-disk feeding was used to knock down *TcGPCR*, *TcBAG*, and *TcGUK* expressions, respectively. In brief, cowpea leaves were cut to a feeding arena (2.0 cm diameter) and dehydrated via incubation at 60°C for 3–5 min. Then, the leaves were treated with DEPC–water, dsRNA–*GFP*, or dsRNA–(*TcGPCR*, *TcBAG*, and *TcGUK*, respectively) (1000 ng/μL) for 3–4 h at room temperature. After complete absorption of the liquids, the leaves were placed on wet filter paper. Then, the leaf disks were placed on water-saturated sponges. Thirty female adults (3–5 days old and starved for 24 h) were placed on each dsRNA-permeated leaf disk. Then, the leaf disks were placed upside down on petri dishes (7 cm in diameter) to prevent mites from escaping. The dsRNA-treated leaf disks, which were infested by *T. cinnabarinus*, were placed under controlled growth conditions as described above. Finally, the mites were collected for subsequent experiments at 48 h post-feeding.

Quantitative Real-Time PCR (qPCR)

To verify the differential expressions of some genes generated by the abovementioned parameters by qPCR, we randomly selected 15 genes from significantly differentially expressed genes. To detect *TcGPCR*, *TcBAG*, and *TcGUK* expressions throughout the different life stages of the mites, approximately 2000 eggs, 1500

[2]http://bioinformatics.psb.ugent.be/orcae/overview/Teur

[3]http://cn.expasy.org/tools/pi_tool.html

[4]http://www.cbs.dtu.dk/services/SignalP/

[5]http://www.cbs.dtu.dk/services/TMHMM/

[6]http://www.cbs.dtu.dk/services/NetNGlyc/

larvae, 800 nymphs, and 200 adults were collected per sample with three replicates. To quantify *TcGPCR*, *TcBAG* and *TcGUK* expressions at 24 and 48 h, in response to different concentrations of scopoletin exposure, we collected 200 female adults per sample with three replicates. For examining the effect of scopoletin exposure on *TcGPCR*, *TcBAG*, and *TcGUK* expressions, female adults were treated with scopoletin, with 0.1% (v/v) Tween-80 and 3% (v/v) acetone as the surfactants. As in the slip-dip assay, the LC_{10}, LC_{30}, and LC_{50} of scopoletin corresponded to 0.099, 0.374, and 0.938 mg/mL, respectively. For the scopoletin exposure experiment, we adopted the leaf-disk dipping method described above and the detailed bioassay procedure that was described by Michel et al. (2010). Each experiment was replicated for a minimum of three times and used independent biological samples. For examining the effectiveness of RNAi, approximately 200 female adult mites were collected per sample at 48 h post-dsRNA feeding. Three replicated samples were prepared. The specific primers used for qPCR were designed by Primer 3.0[7] (**Supplementary Table S1**; Misener and Krawetz, 2000). *RPS18* (FJ608659) was used as the stable reference gene for all qPCR assays (**Supplementary Table S1**; Sun et al., 2010). qPCR was conducted with a Mx3000P thermal cycler (Agilent Technologies, Inc., Wilmington, NC, United States) with 20 μL reaction mixtures that contained 1 μL cDNA template (200 ng/μL), 10 μL iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA, United States), 1 μL of each gene-specific primer (0.2 mM), and 7 μL ddH_2O. The optimized qPCR protocol used for amplification was 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, 60°C for 30 s, and elongation at 72°C for 30 s. Melt curve analyses (from 60 to 95°C) were included to ensure the consistency of the amplified products. The quantification of expression level was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Susceptibility Test of *T. cinnabarinus* to Scopoletin After RNAi of *TcGPCR*, *TcBAG*, and *TcGUK*

Lethal doses of scopoletin (LC_{50} of the scopoletin) were applied in the bioassays. We adopted the slip-dip method described above and the detailed bioassay procedure that was described by Ding et al. (2013). The LC_{50} values of scopoletin were used as diagnostic doses to compare the changes in susceptibility to acaricide in *T. cinnabarinus* at 48 h post-feeding of dsRNA-(*TcGPCR, TcBAG*, and *TcGUK,* respectively).

Heterologous Expression and Functional Assay

To construct the plasmid for transient expression, the ORF of the *TcGPCR* was inserted into the expression vector pcDNA3.1(+) with the restriction enzyme BamHI and XbaI (TaKaRa). The sequences of the inserts were confirmed by sequencing (BGI) prior to heterologous expression. High-quality plasmid DNA prepared using the EndoFree Maxi Plasmid Kit (Tiangen) was employed for transient transfection. CHO-WTA11 cells supplemented with aequorin and Gα16 were used for heterologous expression. The cells were collected 30 h later and pre-incubated with the coelenterazine (Invitrogen) for the functional assay according to the published protocols (Aikins et al., 2008; Jiang et al., 2014). Luminescence caused by intracellular calcium mobilization was measured using a TriStar^2 LB 942 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany). Ten-fold serial dilutions of the scopoletion were used for the treatment of the cells. Based on luminescence values, a concentration-response curve of the receptor to the scopoletin was developed using logistic fitting in Origin 8.6 (OriginLab[8]). The experiments were conducted in three biological replicates.

Statistical Analysis

The MFI of Fura-2/AM in Sf9 cells, the differences in the expression levels of *TcGPCR*, *TcBAG*, and *TcGUK* during four developmental stages, RNAi knockdown efficiencies, and mortality rates were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple tests in SPSS (v.16.0, SPSS Inc., Chicago, IL, United States) at a alpha = 0.05.

RESULTS

Analysis of Acaricidal Toxicity

Table 1 presents the median lethal concentration values (LC_{50}) calculated for scopoletin against adult female *T. cinnabarinus*. The estimated LC_{50} values of scopoletin was 0.938 mg/mL. The LC_{50} of scopoletin indicated its potential as an acaricidal compound against *T. cinnabarinus*.

Effects of Scopoletin on Intracellular Free Calcium $[Ca^{2+}]i$ Levels in Sf9 Cells

The images of Fura-2/AM staining of the Sf9 cells are shown in **Figure 1**. As a calcium indicator, the intensities of Fura-2/AM staining were used to determine the $[Ca^{2+}]i$ concentration. Treatment with scopoletin significantly elevated the $[Ca^{2+}]i$ levels in insect Sf9 cells in a concentration-dependent manner (**Figure 1**).

RNA-Seq Data Analysis

For investigating the transcriptional changes in *T. cinnabarinus* after scopoletin treatment, the purified mRNA from scopoletin- or solvent-treated mites were sequenced on the

[7]http://frodo.wi.mit.edu/

[8]http://www.originlab.com

TABLE 1 | Toxicity of scopoletin against adult females of *T. cinnabarinus* after 48 h exposure time.

Acaricide	N	LC_{50} (mg.mL^{-1})[a] 95%CI[b]	Slope (±SE)	χ^{2c}	*P*
scopoletin	540	0.938 (0.576~2.292)	1.314 ± 0.15	6.321	0.097

[a]LC_{50}, median lethal concentration. [b]CI, 95% confidence interval. [c]Chi-square testing linearity, $P < 0.05$.

Proton platform. After the removal of duplicate sequences, adaptor sequences, and low-quality reads, 52,496,305 clean sequence reads were generated from scopoletin-treated mites, and 52,286,859 clean sequence reads were generated from solvent-treated mites (**Supplementary Table S2**). All sequencing data were submitted to the GEO web site[9] with the accession number GSE92959. The whole genome sequence of *T. cinnabarinus* is still unavailable; therefore, the genomic information of the sister species *T. urticae* was used as the reference genome for map reading. More than 80% of these reads could be successfully mapped to the reference genome, indicating the overall good quality of RNA-seq, as well as the close genetic relationship between *T. cinnabarinus* and *T. urticae*. When the total read numbers approached 5 million per sample, sequencing saturation analysis showed that the number of detected genes tends to be saturated, and the amount of sequencing data can be determined to meet the requirements (**Supplementary Figure S2**). Each of our libraries produced up to 10 million reads, indicating that the depth of sequencing was sufficient to cover most of the transcripts of this organism.

Differential Gene Expression Between Scopoletin- and Solvent-Treated Mites

A total of 18,414 protein coding gene models in the *T. urticae* genome database (Grbiæ et al., 2011). More than 14,000 genes were detected for expression in each sample by mapping all clean reads to the reference genome (**Supplementary Table S2**). According to RPKM values, 70 and 102 genes were identified as significantly differentially expressed genes between scopoletin- and solvent-treated mites for 24 and 48 h, respectively (**Supplementary Table S3**, **S4**). Most genes were time-specific except for 18 genes that were shared by two time points, as found by comparing the differentially expressed genes at two time points (**Figure 2A** and **Supplementary Table S4**). Among the significantly differentially expressed genes, 41 genes were upregulated and 29 genes were downregulated by scopoletin treatment for 24 h, and 61 genes were upregulated and 41 genes were downregulated by scopoletin treatment for 48 h (**Figure 2B**). The $\log_2$ fold change was from -12.8 to 13.4. At 24 and 48 h post-treatment, the number of upregulated significantly differentially expressed genes was consistently higher than that of downregulated genes. Moreover, the number of significantly differentially expressed genes in the treatment at 48 h was markedly higher than that at 24 h by scopoletin. This difference suggested that certain genes are regulated by scopoletin with the change in scopoletin treatment time and that the significantly differentially expressed genes may play a key role in the acaricidal mechanism of scopoletin against *T. cinnabarinus*.

[9]http://www.ncbi.nlm.nih.gov/geo/

GO Enrichment and KEGG Pathway Analysis of Differentially Expressed Genes

To understand the molecular function of genes involved in the response of *T. cinnabarinus* to scopoletin treatment, we used GO database assignments to classify the functions of the predicted genes by mapping all the differentially expressed genes to terms into the GO database and comparing them with the whole reference genome background (**Figure 3**). Based on three GO classes, namely, biological processes, cellular components, and molecular functions, the differentially expressed genes from 24 h post-treatment were categorized into 31 GO subgroups (**Figure 3A**) and differentially expressed genes from 48 h post-treatment were categorized into 29 GO subgroups (**Figure 3B**). In the biological processes category, the "cellular processes" category was prevalent, followed by "metabolic process" throughout the GO classification. At 24 h post-treatment, one enriched term (mitotic nuclear division) presented a proportion of 50%. At 48 h post-treatment, three enriched terms, including ion binding (33.3%), regulation of signal transduction (25%), and nervous system development (25%), were observed. In the cellular components category, the most highly represented subgroups were "cell" and "cell part." In the molecular functions classification, the major subgroups were "catalytic activity" and "binding." Interestingly, the major categorized subgroups were relatively similar for 24 and 48 h

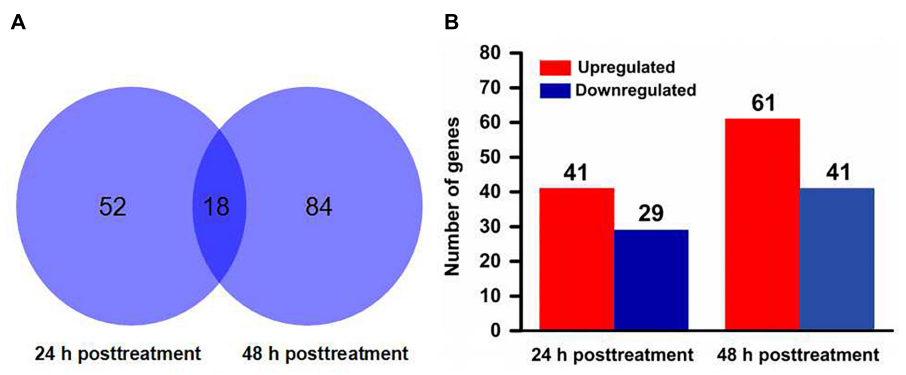

FIGURE 2 | Distribution of differentially expressed genes in *Tetranychus cinnabarinus* in response to scopoletin. **(A)** Venn diagram showing the total number of differentially expressed genes at 24 and 48 h posttreatment, and the number of overlapped genes between two time points. **(B)** The number of up-regulated and down-regulated genes at 24 and 48 h after scopoletin treatment.

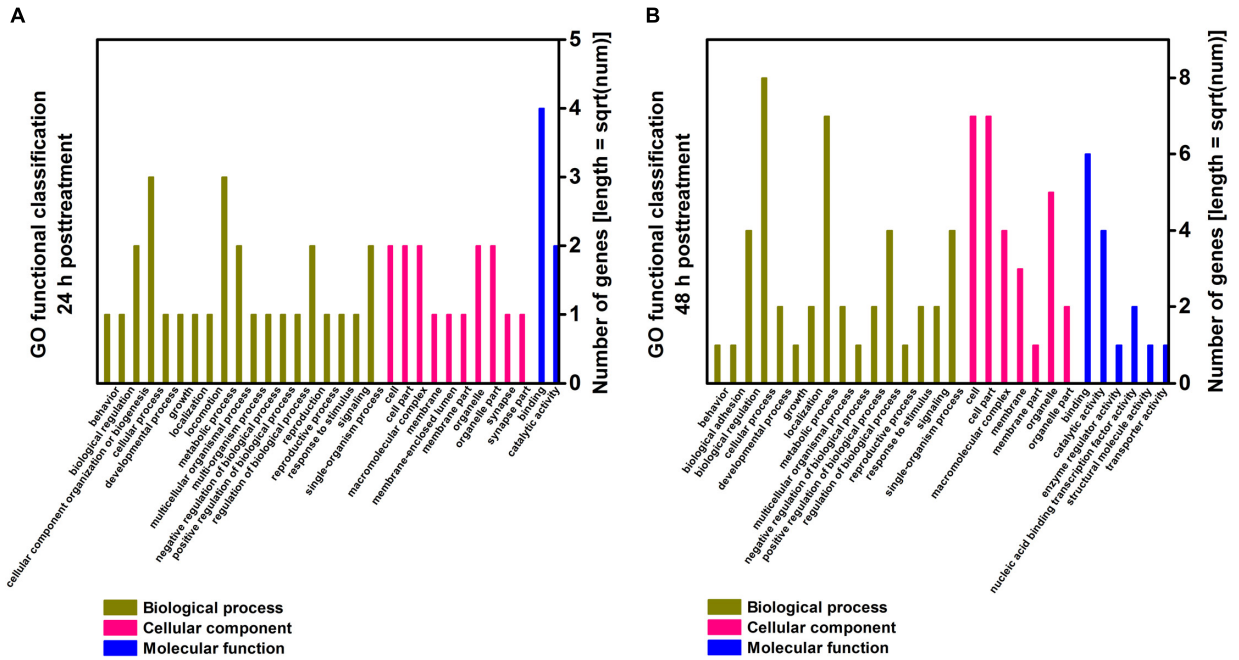

FIGURE 3 | Gene ontology (GO) enrichment analysis of differentially expressed genes in *T. cinnabarinus* after scopoletin or solvent treatment. **(A)** Functional categories of differentially expressed genes at 24 h posttreatment. **(B)** Functional categories of differentially expressed genes at 48 h posttreatment. Three main categories, biological process, cellular component and molecular function, are summarized.

post-treatment, thereby indicating a similar response pattern of mites toward scopoletin treatment at different time points.

To annotate the differentially expressed genes, we aligned the genes into the KEGG database for functional prediction and classification (**Figure 4**). Among the top 20 pathways at 24 and 48 h post-treatment, "protein processing in endoplasmic reticulum" represented the major biochemical pathway (**Figure 4**). Pathways, such as phosphatidylinositol signaling system, spliceosome, and gastric acid secretion, were significantly enriched at 24 h post-treatment (**Figure 4A**), whereas calcium signaling pathway, MAPK signaling pathway, and fat digestion and absorption were well represented at 48 h post-treatment (**Figure 4B**).

Identification of Candidate Genes Involved in Mite Detoxification and Acaricidal Mechanism

On the basis of the previous analysis on differentially expressed genes, we manually selected candidate genes associated with mite detoxification and acaricidal mechanism, such as cell proliferation, substance transportation, cell apoptosis, detoxification, and metabolism (**Tables 2**, **3**). The gene products of these candidates could be classified into several categories, such as signal transduction protein, apoptosis protein, energy metabolism protein, and channel protein, according to their biological functions. Genes involved in signal transduction were guanylate kinase, G-protein coupled neuropeptide receptor, and glycerol-3-phosphate dehydrogenase. We identified senescence-associated protein, Bcl-2 protein, RAB5-interacting protein as cell apoptosis-related products. Moreover, C4-dicarboxylate-binding protein was detected as an energy metabolism-related protein. Among the selected candidate genes, genes associated with signal transduction and cell apoptosis were dominant. Interestingly, several candidates, such as the C4-dicarboxylate-binding protein, were upregulated at 24 h post-treatment and downregulated at 48 h post-treatment. However, except for its acaricidal activity, scopoletin is widely used as a medicine for human beings. Surprisingly, genes with similar functional annotations to the targets of scopoletin were found in our study. For example, Bcl-2 protein, GUK, and RAB5-interacting protein were differentially expressed in mites treated with scopoletin.

Validation of RNA-Seq Data by RT-qPCR

To confirm the RNA-seq results, we selected 5 upregulated genes and 10 downregulated genes from the differentially expressed genes involved in mite detoxification and acaricide mechanism either at 24 or 48 h post-treatment. These genes were used for quantitative reverse transcription PCR (RT-qPCR) analysis. The results of RT-qPCR showed that all tested genes presented a similar differential expression trend compared with the RNA-seq data (**Figure 5**). For example, the ADP-ribosylation factor tetur17g02410 and the exostosin-1 tetur01g05810 were upregulated by 5.6 and 10.8 $\log_2$ fold changes, respectively, in the RNA-seq and by 2.9 and 3.2 $\log_2$ fold changes, respectively, in RT-qPCR. Moreover, the results of RT-qPCR showed that the $\log_2$ fold change of the tested genes did not perfectly match that in the RNA-seq possibly because of calculation and sequencing bias.

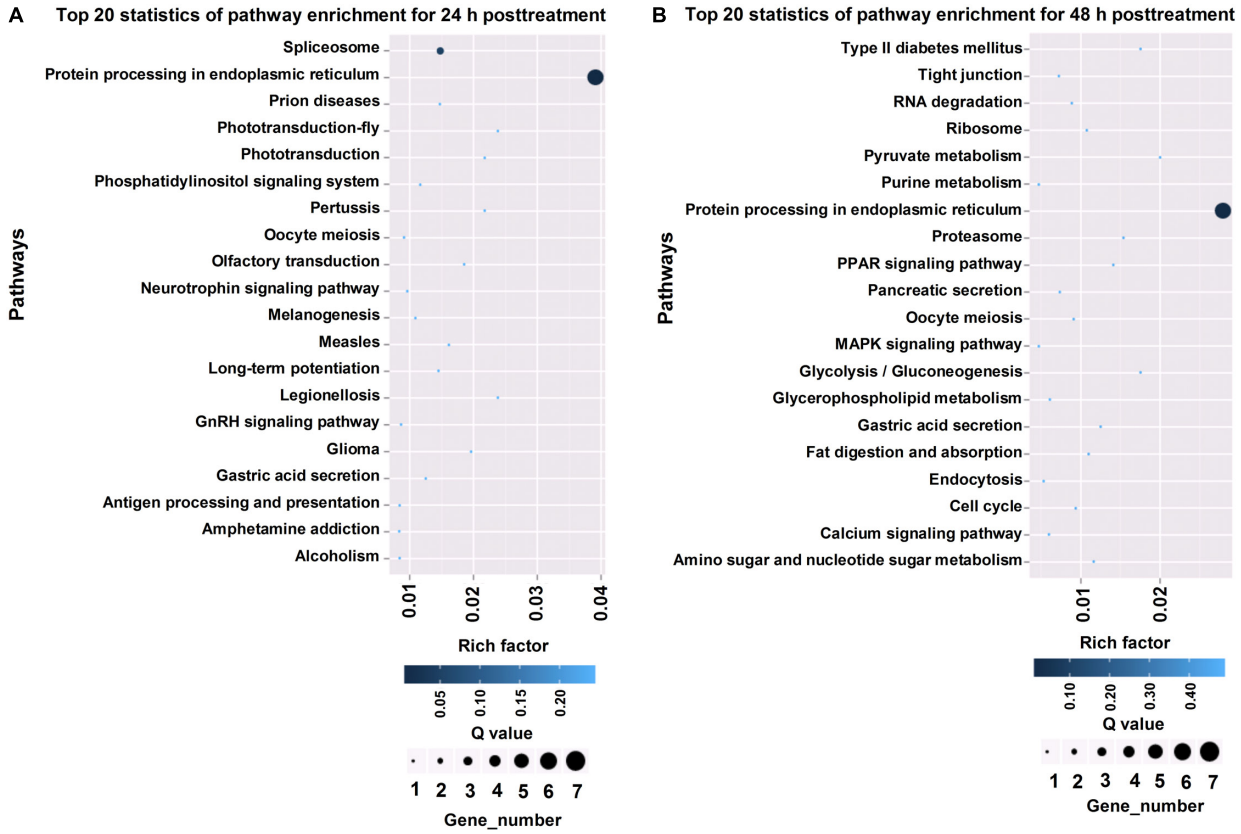

FIGURE 4 | KEGG pathway analysis of differentially expressed genes in *T. cinnabarinus* in response to scpoletin. **(A)** Top 20 enriched KEGG pathways of differentially expressed genes at 24 h posttreatment. **(B)** Top 20 enriched KEGG pathways of differentially expressed genes at 48 h posttreatment. Rich factor is defined by the ratio of the number of differentially expressed genes enriched in the pathway and the number of all genes enriched in the same pathway.

TABLE 2 | Selected genes involved in mite detoxification and acaricide metabolism at 24 h posttreatment.

Gene ID	Description	RPKM		Log_2 fold change
		CK	Scopoletin	
tetur11g05740	Lipase	11.6255	1.4205	−3.03
tetur32g01740	Bcl-2 protein	8.4983	1.6847	−2.33
tetur03g04050	Thioredoxin-like protein 4A	15.6866	5.7149	−1.46
tetur06g00140	Senescence-associated protein	242.9565	107.2315	−1.18
tetur07g05920	Guanylate kinase	46.8228	22.8164	−1.04
tetur11g01680	Heat shock protein 70	128.5005	63.8484	−1.01
tetur21g00510	RAB5-interacting protein	16.3942	52.5307	1.68
tetur02g02050	C4-dicarboxylate-binding protein	1.7990	7.9400	2.14
tetur01g05810	Exostosin-1	0.0100	18.0346	10.82

However, the RT-qPCR results almost validated the upregulation and downregulation directions obtained from RNA-seq results.

cDNA Cloning and Characterization of *TcGPCR, TcBAG*, and *TcGUK*

The deduced amino acid sequences and full-length cDNAs of *TcGPCR, TcGUK*, and *TcBAG*, which contained open reading frames (ORFs), were deposited in GenBank under the following accession numbers: KY660538 (*TcGPCR*), KY660539 (*TcBAG*), and KY660540 (*TcGUK*). **Supplementary Table S5** presents the lengths of the deduced amino acid sequences, predicted protein molecular weights, and theoretical isoelectric points. *TcGPCR* was predicted to possess seven transmembrane (TM) helices, as shown in **Figure 6**. These regions were reported as rhodopsin-like GPCR (GPCRA) common structure frameworks (Monaci et al., 1990). The GPCRA represents a widespread protein family that includes hormones, neurotransmitters,

TABLE 3 | Selected genes involved in mite detoxification and acaricide metabolism at 48 h posttreatment.

Gene ID	Description	RPKM		Log_2 fold change
		CK	Scopoletin	
tetur07g05920	Guanylate kinase	71.2255	0.0100	−12.80
tetur02g02050	C4-dicarboxylate-binding protein	6.6234	0.0100	−9.37
tetur201g00020	Prohibitin 2	8.6853	0.5558	−3.97
tetur08g07000	AMP-dependent synthetase and ligase	9.9772	0.8585	−3.54
tetur02g05380	Scaffold protein	9.1194	0.8489	−3.43
tetur02g02600	26S protease regulatory subunit 6A	13.2758	1.6563	−3.00
tetur05g04350	Glycosyltransferase subunit 4	32.6193	4.6704	−2.80
tetur06g00140	Senescence-associated protein	266.4575	38.5752	−2.79
tetur651g00010	Ribosomal protein S12	21.0008	4.4955	−2.22
tetur39g00730	Vitellogenin1	17.4034	4.1871	−2.06
tetur23g01300	Glycerol-3-phosphate dehydrogenase	9.9892	2.5329	−1.98
tetur02g12070	Tanscription factor SOX-2	17.6862	6.0849	−1.54
tetur15g01820	DM DNA binding domain	29.7538	12.7198	−1.23
tetur11g01430	Similar to Negative elongation factor E CG5994-PA	45.0860	94.5915	1.07
tetur01g05420	BmGATA-beta:Transcription factor BCFI	3.9215	15.9409	2.02
tetur12g04490	DEAD-box ATP dependent DNA helicase	2.5244	11.3534	2.17
tetur08g06300	ADP-ribosylation factor-like protein 6	6.2265	30.8088	2.31
tetur02g03830	G-protein coupled neuropeptide receptor	3.0684	18.2442	2.57
tetur21g01620	Similar to GA21569-PA	0.1296	6.2176	5.58
tetur17g02410	ADP-ribosylation factor	0.0914	4.5619	5.64
tetur01g05810	Exostosin-1	0.0100	22.5082	11.14
tetur04g07080	Alpha-D-phosphohexomutase	0.0100	109.2025	13.41

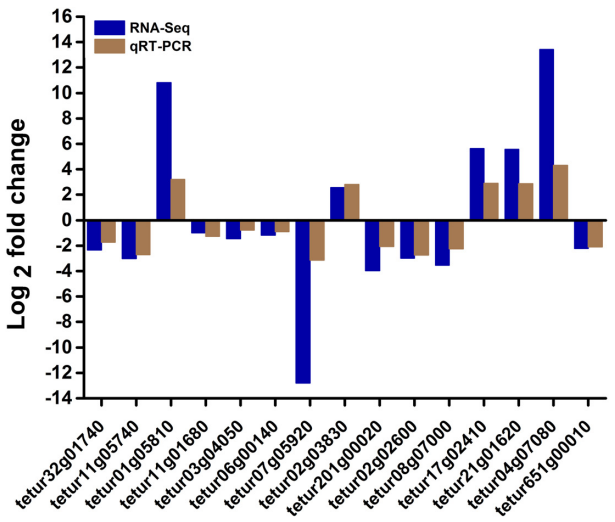

FIGURE 5 | RT-qPCR validation of some differentially expressed genes in RNA-seq. The relative expression levels of fifteen differentially expressed genes in scopoletin- or solvent-treated *T. cinnabarinus* were determined by RT-qPCR. *RPS18* was used as the reference gene to normalize the gene expression using the $\Delta\Delta$Cq method. The y-axis indicates the log_2 fold change of each gene in RT-qPCR and RNA-seq.

and light receptors involved in signal transmission (Casey and Gilman, 1988). *TcBAG* was predicted to include a BAG domain that has anti-apoptotic activity and increases the anti-cell death function of Bcl-2 induced by various stimuli (**Figure 6**; Doong et al., 2002). *TcGUK* is predicted to possess a guanylate kinase-like domain (GK) whose function is to mediate the interaction of protein molecules, which is related to cell adhesion and orientation of mitotic spindle (**Figure 6**; Momand et al., 2000; Von et al., 2003; Yang et al., 2004).

Phylogenetic Analysis of *TcGPCR*, *TcBAG*, and *TcGUK*

Phylogenetic analysis was performed by MEGA 5.0 with the maximum-likelihood method on the basis of the deduced amino acid sequences of *TcGPCR*, *TcBAG*, and *TcGUK*, as well as other known GPCR, BAG, and GUK proteins, including orthologs from arachnids and insects. All GPCR, BAG, and GUK sequences, which possess complete ORFs, were obtained from the *T. urticae* genome and the National Center for Biotechnology Information (Bethesda, MD)[10] (**Supplementary Table S6**). The result showed that *TcGPCR*, *TcBAG*, and *TcGUK* share the highest sequence similarity with the GPCR, BAG, and GUK of *T. urticae* (*TuGPCR*, *TuBAG*, and *TuGUK*), respectively (**Figure 7**), suggesting evolutionary relatedness and possibly similar physiological functions that exist between *TcGPCR* and *TuGPCR*, between *TcBAG* and *TuBAG*, and between *TcGUK* and *TuGUK*.

[10]http://www.ncbi.nlm.nih.gov/

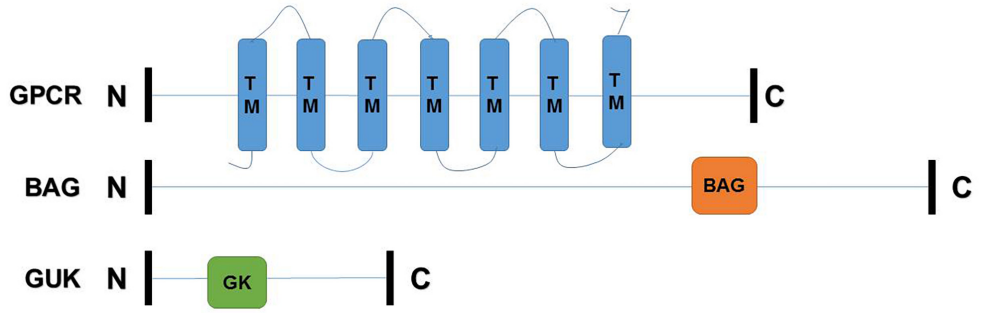

FIGURE 6 | Schematic drawing of *TcGPCR, TcBAG, TcGUK*. C, C-terminal region; N, N-terminal region; TM, transmembrane helices; BAG, BAG domain; GK, guanylate kinase-like domain.

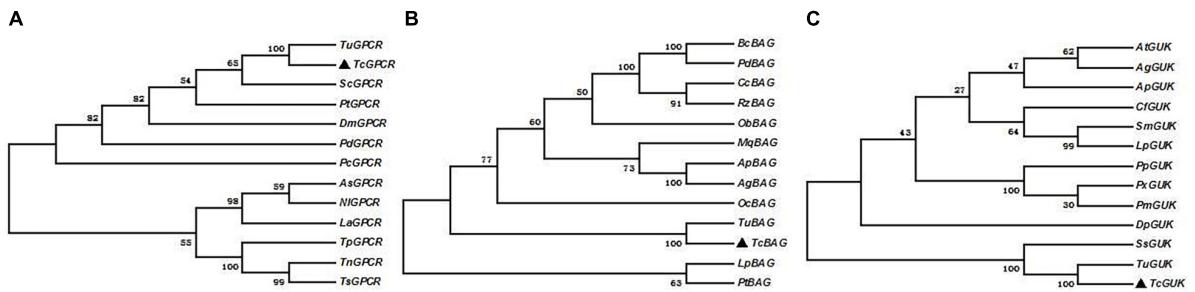

FIGURE 7 | Phylogenic analysis of *TcGPCRs, TcBAGs* and *TcGUKs*, respectively. Maximum likelihood tree constructed by MEGA 5.0. Phylogeny testing was conducted via the bootstrap method with 1000 replications. **A**, **B**, and **C** were *TcGPCRs, TcBAGs,* and *TcGUKs*, respectively.

Expression Patterns of *TcGPCR, TcBAG*, and *TcGUK* in Different Developmental Stages and Scopoletin Treatment

The expression levels of *TcGPCR*, *TcBAG*, and *TcGUK* genes during different developmental stages (egg, larva, nymph, and female adult) and upon acaricide treatment were evaluated via qPCR. The results showed that the calcium channel-related genes (*TcGPCR*, *TcBAG*, and *TcGUK*) were expressed throughout all life stages, which suggested that *TcGPCR*, *TcBAG*, and *TcGUK* are involved in biological processes throughout developmental and growth stages. Specifically, the calcium channel-related genes (*TcGPCR*, *TcBAG*, and *TcGUK*) were significantly highly expressed during the larval and nymphal stages compared with other developmental stages (**Figure 8**). The mRNA expression levels of *TcGPCR*, *TcBAG*, and *TcGUK* in larva and nymph were approximately 139-, 147-, and 5-fold higher than those in eggs and adults, respectively (**Figure 8**).

The results of the scopoletin treatment experiment showed that, compared with the control, the BAG (at 24 h post-treatment) and GUK (at 24 and 48 h post-treatment) genes were

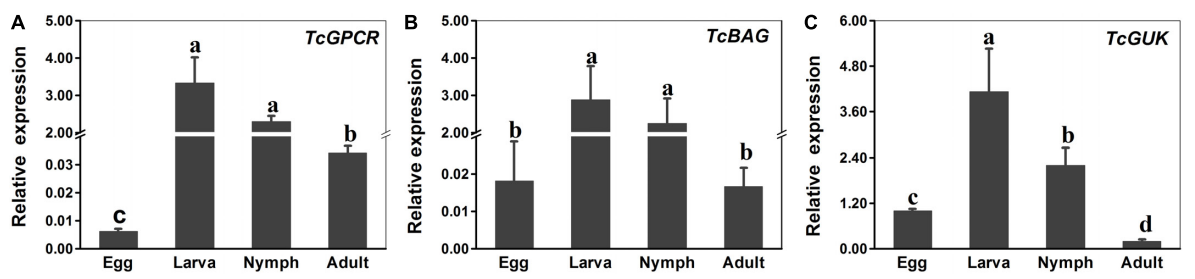

FIGURE 8 | RT-qPCR evaluation of the developmentally specific expression patterns of GPCR, BAG and GUK genes in *T. cinnabarinus*, respectively. **(A)** RT-qPCR analysis of *TcGPCR* expression in different life stages. **(B)** RT-qPCR analysis of *TcBAG* expression in different life stages. **(C)** RT-qPCR analysis of *TcGUK* expression in different life stages. The following life stages were analyzed: egg, larvae, nymph, and adult. Error bars represent the standard error of the calculated mean based on three biological replicates. Different letters on the error bars indicate significant difference according to Duncan's multiple tests (alpha = 0.05). i.e., No statistical difference between "a" and "a"; significant difference among "a," "b," "c," and "d." *RPS18* was used as the reference gene.

downregulated, and the GPCR gene (at 48 h post-treatment) was upregulated (**Figure 9**). Statistical analysis suggested that, compared with the control (CK), at LC_{50}, LC_{30}, and LC_{10} doses of scopoletin, the relative expression levels of *TcBAG* were 3.4-, 2.5-, and 2.0-fold lower at 24 h post treatment; the relative expression levels of *TcGUK* were 1.8-, 1.3-, and 2.4-fold lower at 24 h post-treatment and were 8.9-, 6.4-, and 5.1-fold lower at 48 h post-treatment; and the relative expression levels of *TcGPCR* were 7.1-, 1.7-, and 1.1-fold higher at 48 h post-treatment, respectively. However, the relative expression levels of *TcGPCR* after 24 h of scopoletin treatment and *TcBAG* after 48 h of scopoletin treatment were not significantly different compared with the control at three different concentrations.

RNAi via dsRNA Knockdown

For investigating the transcript knockdown efficiency of the calcium channel-related genes (*TcGPCR*, *TcBAG*, and *TcGUK*) expression, relative mRNA expression levels were measured via qPCR at 48 h post-dsRNA feeding. The results showed that the transcript levels of *TcGPCR*, *TcBAG*, and *TcGUK* significantly decreased to 38.63, 43.12, and 40.13% after feeding of dsRNA-*TcGPCR*, dsRNA-*TcBAG*, and dsRNA-*TcGUK* compared with feeding of DEPC–water or dsRNA-*GFP*, respectively (**Figure 10**). No significant transcript efficiency difference exists between the two controls (water and dsGFP) (**Figure 10**). These results revealed that the *TcGPCR*, *TcBAG*, and *TcGUK* transcripts were successfully knocked down by RNAi in *T. cinnabarinus*.

Susceptibility Test of *T. cinnabarinus* to Scopoletin After RNAi of *TcGPCR*, *TcBAG*, and *TcGUK*

The susceptibilities to scopoletin at 48 h after feeding (dsRNA-*TcGPCR*, dsRNA-*TcBAG*, and dsRNA-*TcGUK*) feeding were detected by slip-dip method. When the *TcGPCR*, *TcBAG* and *TcGUK* in the LC_{50} assays of scopoletin were knocked down by RNAi in *T. cinnabarinus,* mortality significantly decreased to 16.40% and significantly increased to 16.98, and 25.23% in mites fed with dsRNA-*TcGPCR*, dsRNA-*TcBAG*, and dsRNA-*TcGUK* compared with mites treated with DEPC–water, respectively (**Figure 11**). No significant mortality difference existed between DEPC–water and dsRNA-*GFP* (**Figure 11**). These results demonstrated that the RNAi of *TcGPCR* reduces the susceptibility of *T. cinnabarinus* to scopoletin and the RNAi of *TcBAG* and *TcGUK* enhences the susceptibility of *T. cinnabarinus* to scopoletin.

Functional Assay

To confirm that scopoletin induced an increase in intracellular free calcium $[Ca^{2+}]i$ levels by activating *TcGPCR*, we performed a cell-based assay with intracellular calcium mobilization in CHO cells. The results showed that a significant increase in intracellular calcium level in CHO cells expressing *TcGPCR* was induced by scopoletin in a concentration-dependent manner with a very low 50% effective concentration (EC_{50}) value of 0.28 μM (**Figure 12**).

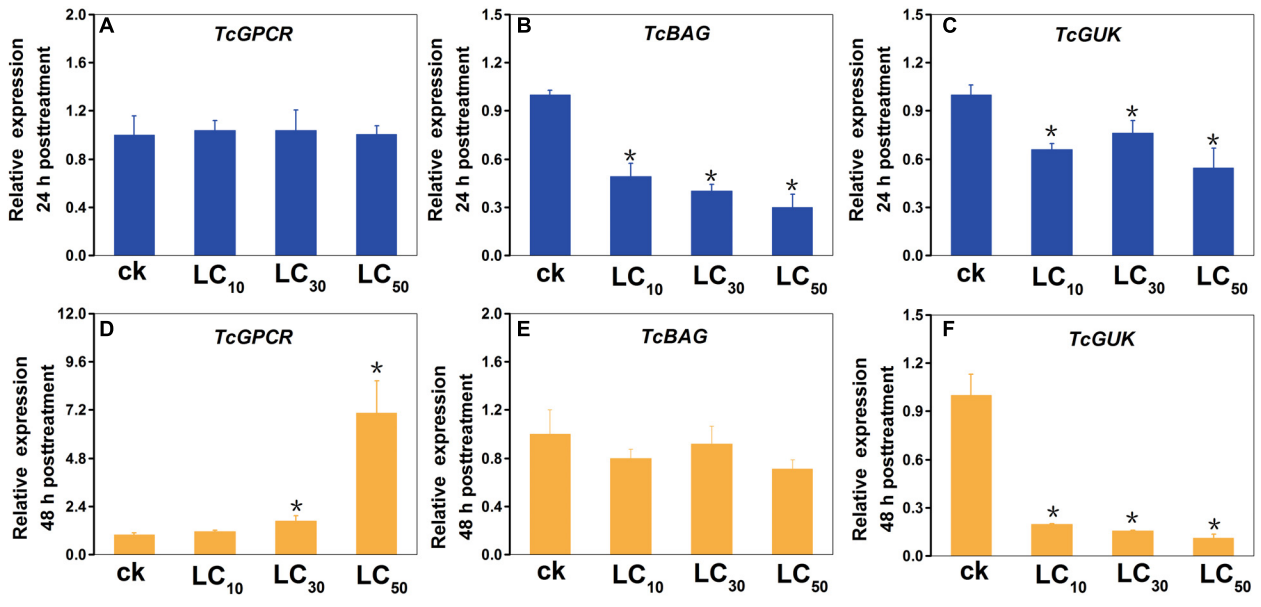

FIGURE 9 | Expression profiles of *TcGPCR, TcBAG,* and *TcGUK* transcripts after scopoletin treatment for 24 h and 48 h at three different concentrations, respectively. Relative expression of the *TcGPCR* **(A,D)**, *TcBAG* **(B,E)** and *TcGUK* **(C,F)** of *T. cinnabarinus* exposed to 0.099, 0.374, and 0.938 mg/L scopoletin (LC_{10}, LC_{30}, and LC_{50}) in 0.1% (v/v) Tween-80 and 3% (v/v) acetone at the adult stage for 24 and 48 h using a slip-dip bioassay were analyzed using RT-qPCR, respectively. Error bars represent the standard error of the calculated mean based on three biological replicates. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). An asterisk (*) on the error bar indicates a significant difference between the treatment and group (CK) according to *t*-tests, $*p < 0.05$. *RPS18* was used as the reference gene.

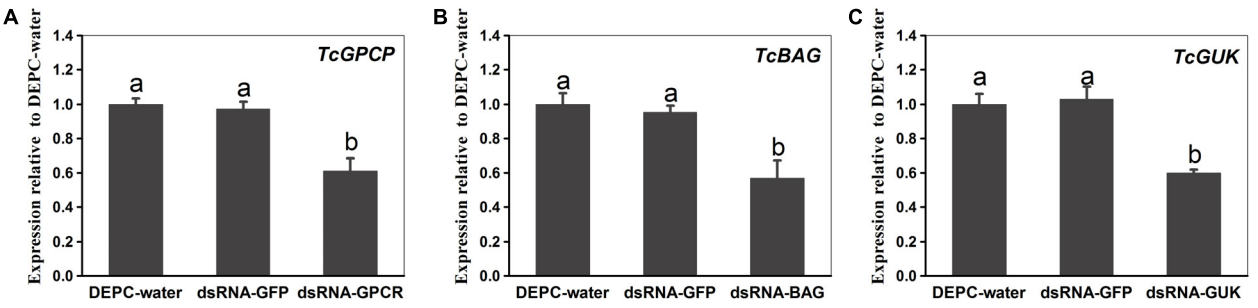

FIGURE 10 | Quantitative PCR expression analysis of *TcGPCR, TcBAG,* and *TcGUK* after RNAi at 48 h post-dsRNA feeding, relative to expression levels after DEPC-water treatment, respectively. **(A)** Expression levels of *TcGPCR*. **(B)** Expression levels of *TcBAG*. **(C)** Expression levels of *TcGUK*. Different letters on the error bars indicate significant difference according to Duncan's multiple tests (alpha = 0.05). i.e., No statistical difference between "a" and "a"; significant difference between "a" and "b."

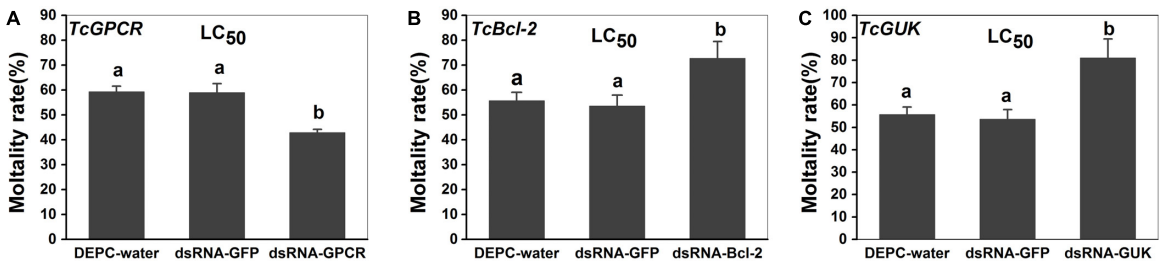

FIGURE 11 | Knockdown of *TcGPCR* expression reduced susceptibility to scopoletin, and knockdown of *TcBAG* and *TcGUK* expression increased susceptibility to scopoletin in mites, respectively. **(A)** Mortality of *TcGPCR*-silenced *T. cinnabarinus* to scopoletin at LC_{50}. **(B)** Mortality of *TcBAG*-silenced *T. cinnabarinus* to scopoletin at LC_{50}. **(C)** Mortality of *TcGUK*-silenced *T. cinnabarinus* to scopoletin at LC_{50}. Error bars represent the standard error of the calculated mean based on three biological replicates. Different letters on the error bars indicate significant difference according to Duncan's multiple tests (alpha = 0.05). i.e., No statistical difference between "a" and "a"; significant difference between "a" and "b."

DISCUSSION

In this study, the effect of scopoletin on intracellular free calcium $[Ca^{2+}]i$ levels in insect Sf9 cells was investigated. We found that a significant increase in intracellular calcium level in insect Sf9 cells was induced by scopoletin in a concentration-dependent manner, indicating that the mode of action of scopoletin in insects was by inducing intracellular calcium overload. Meanwhile, the combination of Ca^{2+} and scopoletin can significantly improve its acaricidal activity (Hou et al., 2015), suggesting that the acaricidal mechanism of scopoletin involves calcium overload. As an important phenolic phytoalexin in plants, scopoletin presents numerous pharmacological activities, such as antitumor activity. Scopoletin also exerts its effect on inducing apoptosis of tumor cells by increasing intracellular calcium concentration (Manuele et al., 2006; Happi et al., 2012).

Thus, to search for candidate target genes of scopoletin inducing calcium overload in *T. cinnabarinus*, we applied transcriptomics on *T. cinnabarinus* treated with scopoletin or the solvent. A total of 52,496,305 and 52,286,859 reads were obtained from *T. cinnabarinus* treated with scopoletin or solvent at 24 and 48 h post-treatment, respectively. The obtained reads were mapped to the genome of *T. urticae* Koch, a sister species of *T. cinnabarinus* (Ueckermann et al., 2013). More than 80% of the reads were successfully mapped to the reference genome, thereby providing good mapping results for downstream analysis. We identified 70 and 102 differentially expressed genes upon scopoletin treatment at 24 and 48 h

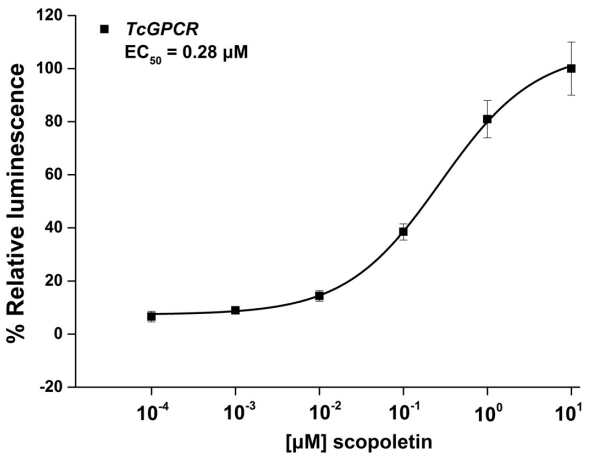

FIGURE 12 | Relative activity of Chinese hamster ovary cells transfected with *T. cinnabarinus* G-protein-coupled neuropeptide receptor (GPCR) and activated with scopoletin. Concentration-response curves for luminescence induced by scopoletin. Results are means ± SD (n = 3).

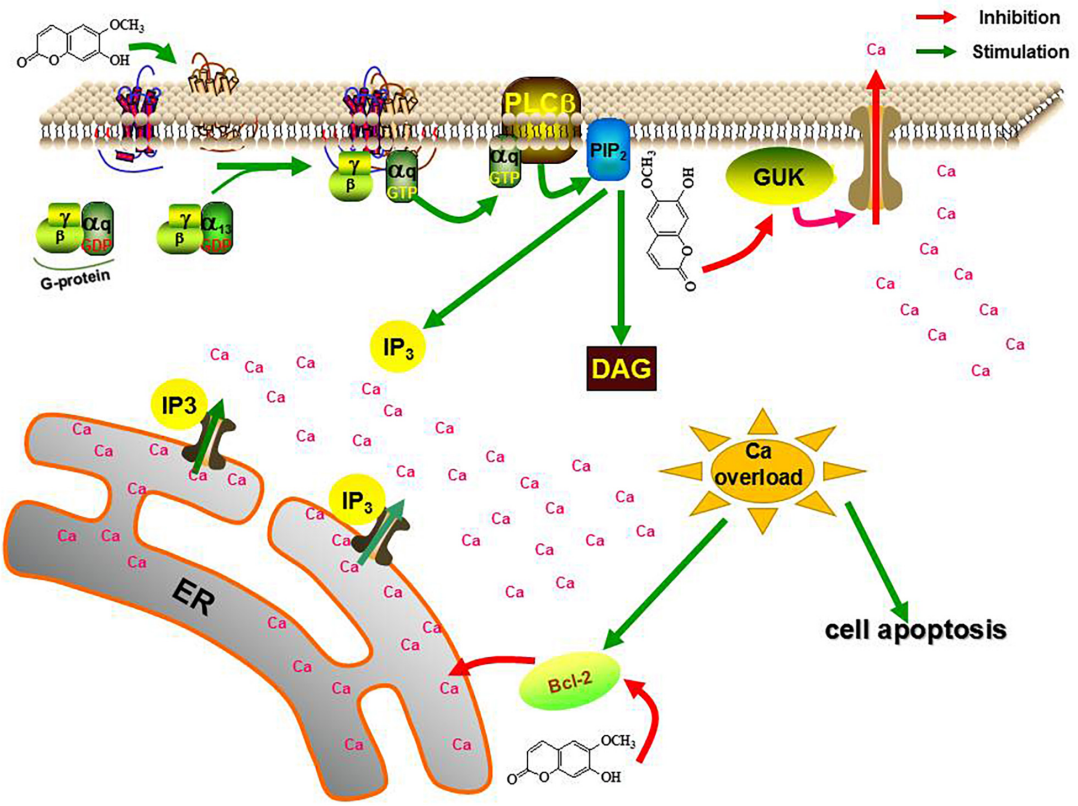

FIGURE 13 | The role of *TcGPCR*, *TcBAG* and *TcGUK* on the acaricidal mechanism of scopoletin. PLCβ, phospholipase Cβ; PIP_2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; IP_3, inositol trisphosphate; ER, endoplasmic reticulum.

post-treatment, respectively. The number of upregulated genes was markedly higher than that of downregulated genes at 24 and 48 h post-treatment, thereby suggesting that more genes were activated by scopoletin at the two time points. However, the fold changes of certain differentially expressed genes, such as guanylate kinase, senescence-associated protein, and exostosin-1, were different between the two time points. The differential expression is closely related to a previous report that the poisoning symptoms of *T. cinnabarinus* change after scopoletin treatment over time (Liang et al., 2011). These symptoms indicated that different defense or lethal responses may be involved in *T. cinnabarinus* treated with scopoletin at different time points.

In this study, GO enrichment analysis of differentially expressed genes showed that "cellular process" was the dominant group at both time points. KEGG pathways showed that "protein processing in endoplasmic reticulum" represented the major biochemical pathway at 24 and 48 h post-treatment, whereas calcium signaling pathway, MAPK signaling pathway, and fat digestion and absorption were well represented at 48 h post-treatment. Furthermore, we manually selected candidate genes associated with mite detoxification and acaricidal mechanism, such as signal transduction genes (e.g., GUK, GPCR, and glycerol-3-phosphate dehydrogenase) (Sato et al., 2016), cell apoptosis genes (e.g., Bcl-2 protein, RAB5-interacting protein, HSP70, and prohibitin 2) (Hoffenberg et al., 2000; Kasashima et al., 2006), and energy metabolism genes (e.g., lipase, vitellogenin1, C4-dicarboxylate-binding protein, and AMP-dependent synthetase and ligase) (Dinh et al., 2002; Kawakami et al., 2009; Shaw et al., 2010; Tanaka et al., 2017), according to their biological functions. Among the selected candidate genes, genes associated with signal transduction and cell apoptosis were dominant. In addition, the differential expression of 2 transcription factor genes (e.g., transcription factor BCFI and SOX-2) was induced by scopoletin, indicating that the transcription factors were involved in the regulation of gene expression in the acaricidal mechanism of scopoletin.

Among the differentially expressed signal transduction genes identified in our study, GPCR was upregulated in *T. cinnabarinus* upon scopoletin treatment. Moreover, specific expression detection showed that scopoletin treatment upregulates the expression level of *TcGPCR*. GPCR composes one of the largest families of cell-surface proteins which involve in signal transmission and play crucial roles in diverse processes, such as development, metabolism, ecdysis, and reproduction in insects (Monaci et al., 1990; Van et al., 2010). GPCR can activate calcium channels present in the membrane of the endoplasmic reticulum, which induces the release of calcium into the cytoplasm (Caers et al., 2014). Thus, in the present study, to confirm that scopoletin induced an increase in intracellular free calcium $[Ca^{2+}]i$ levels

by activating *TcGPCR*, we performed a cell-based assay with intracellular calcium mobilization in CHO cells. Indeed, the pharmacological data demonstrated that a significant increase in intracellular calcium level in CHO cells expressing *TcGPCR* was induced by scopoletin in a dose-dependent manner. Moreover, in this study, the susceptibility to scopoletin decreases when *TcGPCR* in the LC_{50} assays is suppressed via RNAi, indicating that the downregulation of GPCR reduces susceptibility to scopoletin. Taken all together, these results suggested that the calcium overload in the scopoletin-treated mites was mediated by the overexpression of GPCR.

In addition, the downregulation of GUK by scopoletin was observed in this study. Moreover, specific expression detection showed that scopoletin treatment downregulates the expression of *TcGUK*. GUK belongs to the superfamily of the membrane-associated guanylate kinase (MAGUK), which forms a complex with Ca^{2+} efflux pump of the plasma membrane Ca^{2+}-ATPase (PMCA) to regulate calcium homeostasis (Aravindan et al., 2012). PMCA is responsible for the expulsion of Ca^{2+} from the cytosol of all eukaryotic cells (Zoccola et al., 2004). Aravindan et al. (2012) reported that GUK removes excess Ca^{2+} from cells by positively regulating the activity of PMCA. Elevated Ca^{2+} may result from increased influx or decreased efflux. Meanwhile, in this study, the susceptibility to scopoletin raises when *TcGUK* in the LC_{50} assays are suppressed via RNAi, indicating that the downregulation of GUK increases susceptibility to scopoletin. Thus, in our study, these results suggested that the downregulation of GUK may result in the closure of the efflux channel of calcium in the cell membrane, thereby inhibiting the outflow of intracellular Ca^{2+}, which disrupts calcium homeostasis and promotes the overload of intracellular calcium. However, the regulation of intracellular calcium signaling is extremely complex. Therefore, the mechanism by which GUK downregulation mediates calcium overload in the scopoletin-treated mites needs further elucidation.

We identified several genes that were inhibited by scopoletin, including the apoptosis regulatory protein, Bcl-2 protein (BAG). Moreover, specific expression detection showed that scopoletin treatment downregulates the expression of *TcBAG*. Bcl-2 protein is a pro-survival protein that inhibits apoptosis induced by calcium signaling (Shibasaki et al., 1997). For example, the anti-apoptotic action of Bcl-2 reportedly involves enhancing the storage of calcium by upregulating the expression levels of calcium pump genes (Shibasaki et al., 1997; Zhu et al., 1999). Other reports indicate that Bcl-2 increases membrane permeability, thereby reducing the concentration of Ca^{2+} in the endoplasmic reticulum, resulting in a decrease in the amount of released Ca^{2+} during signal transduction and inhibiting apoptosis (Pinton et al., 2000; Schlossmann et al., 2000). Additionally, in this study, the susceptibility to scopoletin raises when *TcBAG* in the LC_{50} assays are suppressed via RNAi, indicating that the downregulation of Bcl-2 increases susceptibility to scopoletin. In this case, the Bcl-2 protein gene was inhibited by scopoletin, suggesting that the anti-apoptotic function induced by calcium signaling was disturbed in scopoletin-treated mites. Thus, the overloading of calcium induces cell apoptosis, and downregulation of Bcl-2 protein may promote apoptosis.

In our study, calcium signaling pathway-related genes (GPCR, BAG, and GUK) played crucial roles in the acaricidal mechanism of scopoletin against *T. cinnabarinus*. In consequence, the identification and characterization of calcium signaling pathway-related genes from mites will help in determining the involvement of GPCR, BAG, and GUK in the responses of mites to specific acaricides. Moreover, the present study will help us understand the biological functions of GPCR, BAG, and GUK. In this study, we cloned and characterized the full-length cDNA of GPCR, BAG, and GUK gene in *T. cinnabarinus* (designated as *TcGPCR*, *TcBAG*, and *TcGUK*, respectively). The structure analysis of *TcGPCR* demonstrates that this gene possesses seven transmembrane (7TM) helix domains that indicate the common structural framework of transmembrane signal transduction (Monaci et al., 1990). The structure analysis of *TcBAG* indicates that this gene possesses a BAG domain with anti-apoptotic activity and increases the anti-cell death function of Bcl-2 induced by calcium signaling (Shibasaki et al., 1997; Doong et al., 2002). Doong et al. (2002) reported that the gene was dependent on its interaction with heat shock protein 70 (HSP70) to exhibit anti-apoptotic activity. However, in this study, HSP70 was downregulated in BAG-suppressed mites, indicating that scopoletin promotes apoptosis. Moreover, *TcGUK* is predicted to possess a guanylate kinase-like domain (GK) whose function is to mediate the interaction of protein molecules, which is related to cell adhesion and orientation of mitotic spindle (Momand et al., 2000; Von et al., 2003; Yang et al., 2004). In addition, the expression levels of the calcium signaling pathway-related genes (*TcGPCR*, *TcBAG*, and *TcGUK*) were detected in all four tested developmental stages of *T. cinnabarinus*, indicating that the GPCR, BAG, and GUK genes are important during the whole life cycle of mites. However, the expression levels of the calcium signaling pathway-related genes (*TcGPCR*, *TcBAG*, and *TcGUK*) during the larval and nymphal stages were significantly higher than those in the other developmental stages of *T. cinnabarinus*, indicating that the three calcium signaling pathway-related genes coordinated and interacted during the regulation of mite development.

CONCLUSION

In the present study, we found that the acaricidal mechanism of scopoletin involves calcium overload. Therefore, to reveal the molecular mechanism and search for candidate target genes of calcium overload induced by scopoletin in mites, we utilize RNA-seq to detect changes in transcription levels. We identified 70 and 102 differentially expressed genes upon scopoletin treatment at 24 and 48 h post-treatment, respectively. GO enrichment analysis of differentially expressed genes showed that "cellular process" was the dominant group at both time points. KEGG pathways showed that "protein processing in endoplasmic reticulum" represented the major biochemical pathway at 24 and 48 h post-treatment, whereas calcium signaling pathway, MAPK signaling pathway, and fat digestion

and absorption were well represented at 48 h post-treatment. The target genes associated with the acaricidal mechanism of scopoletin included 3 signal transduction genes (GUK, GPCR, and glycerol-3-phosphate dehydrogenase), 4 cell apoptosis genes (Bcl-2 protein, RAB5-interacting protein, HSP70, and prohibitin 2), 4 energy metabolism genes (lipase, vitellogenin1, C4-dicarboxylate-binding protein, and AMP-dependent synthetase and ligase), and 2 transcription factor genes (transcription factor BCFI and SOX-2).

Mechanically, the calcium overload in the scopoletin-treated mites was mediated by calcium signaling pathway-related genes. Thus, the differential expression of three calcium signaling pathway-related genes, namely, GPCR, BAG, and GUK, may medicate calcium overload induced by scopoletin in RNA-seq. Specific expression detection shows that scopoletin treatment upregulates the expression levels of *TcGPCR* and downregulates the expression levels of *TcBAG* and *TcGUK*. Moreover, the RNAi of GPCR gene expression decreased the susceptibility of *T. cinnabarinus* to scopoletin, and the RNAi of BAG and GUK gene expressions enhanced the susceptibility of *T. cinnabarinus* to scopoletin. What is more, functional expression data strongly suggest that scopoletin induced a significant increase in intracellular free calcium $[Ca^{2+}]i$ levels by activating *TcGPCR* in CHO cells. Our results showed that the acaricidal mechanism of scopoletin against *T. cinnabarinus* was by disrupting intracellular Ca^{2+} homeostasis and calcium signaling pathway mediated by GPCR, BAG, and GUK (**Figure 13**). Our findings enhance the understanding of the acaricidal mechanism of scopoletin in *T. cinnabarinus* and clarify designing strategies to control pest mites.

AUTHOR CONTRIBUTIONS

HZ, Y-qZ, and WD conceived and designed the experiments and wrote and revised the manuscript. HZ, TL, X-jL, F-yG, and TG performed the experiments and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00164/full#supplementary-material

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REVIEW
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Immune Defenses of a Beneficial Pest: The Mealworm Beetle, *Tenebrio molitor*

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The mealworm beetle, *Tenebrio molitor*, is currently considered as a pest when infesting stored grains or grain products. However, mealworms are now being promoted as a beneficial insect because their high nutrient content makes them a viable food source and because they are capable of degrading polystyrene and plastic waste. These attributes make *T. molitor* attractive for mass rearing, which may promote disease transmission within the insect colonies. Disease resistance is of paramount importance for both the control and the culture of mealworms, and several biotic and abiotic environmental factors affect the success of their anti-parasitic defenses, both positively and negatively. After providing a detailed description of *T. molitor*'s anti-parasitic defenses, we review the main biotic and abiotic environmental factors that alter their presentation, and we discuss their implications for the purpose of controlling the development and health of this insect.

Keywords: *Tenebrio molitor*, innate immunity, pest control, insect farming, ecoimmunology

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INTRODUCTION

Insects conflict with human activities due to their ability to successfully colonize and adapt to the majority of terrestrial habitats. They are considered as pests when they damage crops and/or parasitize livestock, which decrease food production, or when they pose a health hazard to humans or other domesticated animals. By contrast, insects are deemed beneficial when they perform valuable services such as pollination and pest control, or when they are bred for being used in human activities. Such a distinction is rather subjective and only arises in light of desired outcomes from a human perspective. In this context, the mealworm beetle, *Tenebrio molitor*, could be seen as either a pest or a beneficial insect. On the one hand, *T. molitor* has generally been considered as a pest because they consume or degrade the quality of stored grains and grain products. On the other hand, mealworms are beneficial, as their larvae are often used as pet food. They also offer a promising alternative protein-rich animal feed and are recommended as a source of human nutrition. Furthermore, *T. molitor* larvae may be further useful due to their ability to efficiently degrade polystyrene and plastic waste (Brandon et al., 2018). For these reasons, *T. molitor* is currently being considered for production at an industrial scale.

T. molitor is the host of a wide range of pathogens and parasites such as entomopathogenic microbes, protozoa, and tapeworms, which reduce the mealworm survival or reproductive success. While some of these parasites might be used as biological insecticides to control unwanted

population development, they might also be a source of concern in mass rearing facilities. Like other insects, *T. molitor* possesses an arsenal of behavioral, physical, and physiological mechanisms that aim to prevent exogenic invasions or to lower the consequences of a successful infection. The efficacy of these lines of defense may independently or synergistically vary according to biotic and abiotic environmental factors including temperature, food, population density, and individual past interaction with pathogens and parasites. Characterization of such phenotypic plasticity may provide valuable insights for the purpose of improving control or protection of the insect populations. Here we review the prominent *T. molitor* anti-parasitic defense systems and the main environmental factors affecting their presentation. The impact of the environmental factors is considered from the phenotypic to the population level. The processes affecting mealworm anti-parasitic defenses are discussed in the context of controlling the development and health of the insect populations.

ANTI-PARASITIC DEFENSES IN THE MEALWORM BEETLE

Behavioral Immunity

Behavioral immunity refers to altered behaviors used by a host to avoid infection, reduce parasite growth, and/or alleviate disease symptoms. Such anti-parasitic behaviors are increasingly recognized in insects, including *T. molitor*. Behavioral immunity involves anti-parasitic behaviors categorized into three main infectious outcomes (de Roode and Lefèvre, 2012). First, behavioral immunity may provide qualitative resistance by avoiding contact with parasites and pathogens. Behavioral immunity comprises spatial and temporal avoidance of potentially infected places, individuals or food, implementation of hygienic behaviors such as grooming, and adapted social contacts, such as mate choice based on a partner's immunocompetence. This range of behavior was reported when *T. molitor* were exposed to the tapeworm *Hymenolepis diminuta*. *H. diminuta* is a rodent parasite that uses the mealworm beetle as an intermediate host. Beetles become infected by consuming eggs of the parasite when feeding on infectious rodent feces. Infection of the beetle is maximized by an increased attractiveness of infected rodents' feces compared to non-infected feces (Pappas et al., 1995). Infected male beetles, which pay a higher reproductive cost than do infected females (Hurd and Arme, 1986; Hurd and Parry, 1991; Worden et al., 2000), have developed an avoidance behavior for feces that harbor *H. diminuta*, thus decreasing their probability of coming into contact with the tapeworm (Shea, 2010). In addition, females developed qualitative resistance through mate choice, as they are able to evaluate male immunocompetence *via* pheromone signaling and then choose a more immunologically fit mate (Rantala et al., 2002). By choosing a male more refractory to pathogens, females reduce the probability of being infected by their mate and may transmit an enhanced level of immunocompetence to their offspring (Hamilton and Zuk, 1982).

Second, host behaviors may provide quantitative resistance by preventing parasite or pathogen replication. These behaviors involve therapeutic medication, behavioral fever, and grooming, which are particularly beneficial in dense insect populations, where diseases can efficiently spread. No infection outbreak was reported from mealworm mass rearing, but alternatives to antibiotic use could be beneficial to avoid the rise of resistant pathogens. Particularly, adopting therapeutic behavioral medication would be of great interest for the prevention and control of diseases in large populations of beetles. Therapeutic medication can be defined "as a series of behaviors through which infected hosts exploit additional species or compounds to reduce or clear infections, whether mediated through defensive or nutritional properties" (de Roode and Lefèvre, 2012). So far, therapeutic medication has not been reported in the mealworm beetle. Hence, further investigations are needed on this aspect of the mealworm behavioral immunity.

Third, the host may tolerate infections by limiting the negative effects on their reproductive success. This limitation is mainly achieved through increasing their reproductive effort but often at the expense of their longevity. For instance, mealworm beetles tolerate a high number of cysticercoids of the parasite *H. diminuta* at the expense of their own fitness. Nevertheless, in response to parasite infection, males produce improved spermatophores that contain superior nuptial gifts that will be passed to their mating females. This increases female fecundity, and therefore, a higher number of eggs are fertilized by the male (Hurd and Ardin, 2003). Specifically, as beetle longevity is compromised by the parasite, infected males may gain a reproductive benefit, before dying from the infection, by increasing the total protein content of the spermatophores they transfer to females during mating (Carver et al., 1999; Hurd and Ardin, 2003). Males that are experiencing a non-infectious immune-challenge, e.g., a piece of nylon filament inserted into their hemocoel, present a similar increased reproductive effort. Indeed, the immune challenge may induce in males, the perception of a lower survival probability consequent to their simulated infection status, causing the insect to make a last attempt to achieve a maximized level of reproductive success. Consequently, females find those artificially challenged males more sexually attractive, probably due to an increased production of sexual pheromone consequent to the challenge (Sadd et al., 2006; Kivleniece et al., 2010; Krams et al., 2011). The underlying signaling may transit through juvenile hormone (JH), a hormone secreted by the *corpora allata* that is involved in the control of morphogenesis and reproduction in insects, as *T. molitor* males injected with JH are preferred by females (Rantala et al., 2003). So far, comparable adjustment of the reproductive effort upon infection has never been reported in females.

Cuticle Immunity

The insect integument forms a robust barrier that successfully prevents most parasites and pathogens from colonizing the hemocoel (Moret and Moreau, 2012). It usually constitutes the first barrier between an insect and endogenous invaders. The integument includes an outer layer, called the cuticle, which is produced by a monolayer of epidermal cells. This layer of cells, or epidermis, is separated from the underlying tissues by a thin matrix called the basal lamina. The basal lamina provides a defensive boundary

on the insect's surface due to the thickness of the cuticle and the degrees of sclerotization, or cross-linking, and melanization within cuticular layers. Melanization in the cuticle strengthens its property to act as a physical barrier against the penetration of parasites (Stleger et al., 1988; Hajek and Stleger, 1994). In addition, melanin is toxic to microorganisms and has potent antimicrobial activity (Soderhall and Ajaxon, 1982). Cuticular melanization naturally occurs during the process of molting in insect larvae and nymphs, and right after adult emergence from pupation (Vigneron et al., 2014). However, this process is also induced in response to a mechanical scratch to avoid loss of hemolymph (Benoit et al., 2017) or to microbial invasion (Golkar et al., 1993). In the mealworm beetle, the degree of cuticular melanization is a strong indicator of resistance to the entomopathogenic fungus, *Metarhizium anisopliae*. Indeed, darker beetles are more resistant than lighter ones (Barnes and Siva-Jothy, 2000). This could be explained by the thicker and less porous cuticle displayed by darker insects compared to lighter ones (Evison et al., 2017). A breach in the cuticle also triggers the production of antimicrobial peptides by the epidermal cells, such as cecropins, which are transported in the vicinity of a microbial challenge to abraded cuticle (Brey et al., 1993).

Insect growth and development involve a series of molts during which the old cuticle is partially digested, while a new cuticle is formed and the remnant is discarded. In addition to allowing insect growth, molting may serve as a defense mechanism by reducing the negative effects of a wound or a parasite invasion. For instance, molting quickly in response to a parasite exposure prevents parasites from remaining attached to the cuticle; subsequently, reducing the probability of a successful infection (Duneau and Ebert, 2012; Kim and Roberts, 2012). The benefit of such molting could be exploited by the host inducing precocious molts in response to parasite early attachment (Duneau and Ebert, 2012; Moret and Moreau, 2012). In *T. molitor*, whether wounding or parasite attachment can induce larvae to perform more molts is not known. However, larvae grow through a variable number of molts from 8 to 20. The variability in this number can be partially explained by the availability of resources, the quality of the diet, or the density of the population (Connat et al., 1991; Morales-Ramos et al., 2010). This suggests that the mealworm beetle can adjust its development in response to its environment. Hence, it would be highly relevant to investigate the capacity of the mealworm to molt subsequently to the pressure caused by a wound or a pathogen attempting to invade the insect.

The Hemocoelic Immune System

Once a parasite or a pathogen has breached the integumental defenses, the insect has to produce a rapid and effective response that localizes and neutralizes the growth and development of the microbe. Like in other insects, hemocoelic defenses of *T. molitor* rely on innate immune effector systems. These involve recognition by pattern recognition receptors (PRRs) that detect a range of conserved non-self microbe-associated molecular patterns (MAMPs), such as bacterial lipopolysaccharides (LPS) and peptidoglycans (PGN), fungal and bacterial β-1,3 glucans, and other sugar moieties. *T. molitor* possesses most known insect PRRs (Johnston et al., 2014). Specifically, these include peptidoglycan recognition proteins (PGRPs) that bind bacterial PGN, Gram-negative binding proteins (GNBPs) that bind LPS, and together with glucan-binding proteins (GBPs), recognizing β-glucans (**Figure 1**; Zhang et al., 2003; Park et al., 2007; Kim et al., 2008; Lee et al., 2009; Johnston et al., 2014; Yang et al., 2017). Upon recognition, these PRRs trigger the action of various signaling pathways, including the prophenoloxidase cascade regulating melanization processes (Park et al., 2007), the Toll and immune deficiency (IMD) pathways leading to the synthesis of AMPs (Kim et al., 2008; Roh et al., 2009; Yu et al., 2010; Johnston et al., 2014), and hemocyte-driven phagocytosis (Zhu et al., 2013; Kim et al., 2017). Insect innate immune response relies mainly on those pathways, which, *via* their synergic actions, form efficient cellular and humoral responses.

Cellular defenses primarily involve the action of immune cells called hemocytes, which drive phagocytosis, nodulation, and encapsulation of endogenous organisms. Insects possess several types of circulating hemocytes that are morphologically and functionally distinct, and the prevalence of which is variable in the hemolymph. The mealworm beetle presents four main types of hemocytes: granulocytes, plasmatocytes, oenocytoids, and prohemocytes (**Figure 2**; Chung and Moon, 2004; Urbanski et al., 2018). Granulocytes account for 50–60% of the observed hemocytes. They are oval cells of about 10 μm in size containing visible dense granules in their cytoplasm and are involved in phagocytosis. Plasmatocytes are the second most abundant hemocytes, representing 23–28% of the total hemocytes. Plasmatocytes are large elongated cells that are likely involved in encapsulation. Oenocytoids are the rarest type as they account for 1–2% of circulating hemocytes. They are large oval cells with a centrally located nucleus and presumably produce enzymes of the melanization cascades. Finally, prohemocytes represent 10–15% of the circulating hemocytes. They are small oval cells less than 10 μm in size with a very large nucleus, probably functioning as precursors of hemocytes.

Hemocyte phagocytosis is achieved upon the recognition of microbes, either directly or after their opsonization by thioester proteins (TEPs), using Scavenger and Nimrod receptors or using the highly variable, alternatively spliced Dscam (Cherry and Silverman, 2006). Particularly, *T. molitor* Scavenger Receptor class C (SR-C) plays a crucial role in the ability of the insect to phagocytose fungi and bacteria (Kim et al., 2017). When an endogenous object is too big to be phagocytized, the cellular immune response also relies on melanization and encapsulation processes. Melanization corresponds to the production of melanin around foreign objects including bacteria, protozoan parasites, nematodes, or parasitoid eggs (Zhu et al., 2013). Upon wounding or recognition of a foreign object by GNBPs and PGRPs, prophenoloxidase (proPO), a zymogen present in some hemocytes and in the plasma, is cleaved through a cascade of serine proteases to liberate the active phenoloxidase (PO). This enzyme catalyzes the production of melanin. In arthropods, levels of melanin and circulating proPO enzymes are used to evaluate immune functions and status. For instance, larvae of the African armyworm, *Spodoptera exempta*, reared at high densities exhibit higher proPO levels in the hemolymph and higher resistance to

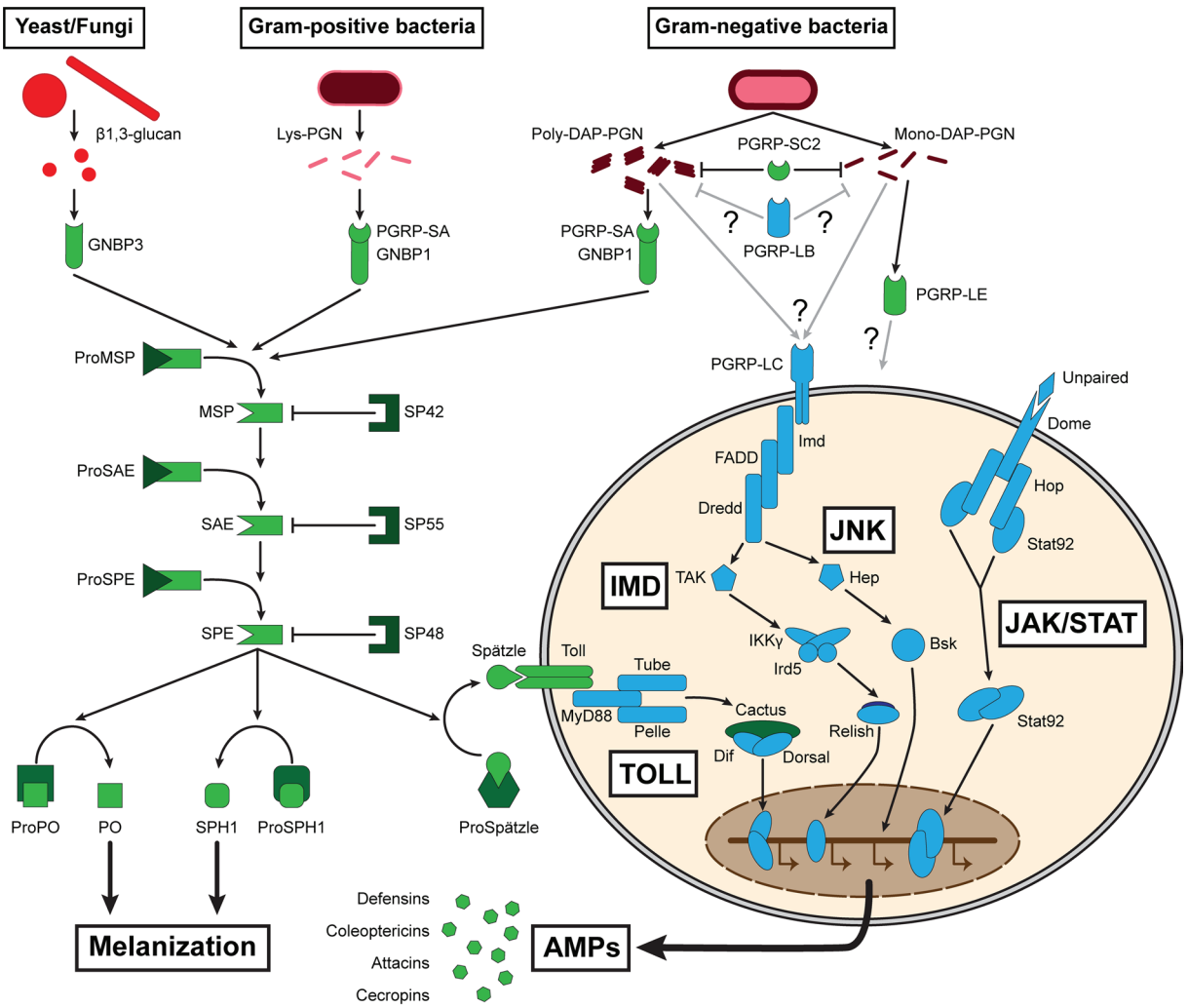

FIGURE 1 | Summary of the major pathways constituting *T. molitor* humoral immune response. Proteins that have been functionally investigated are represented in green, while proteins evidenced at the genomic and/or transcriptomic levels are represented in blue. Lys: lysine; DAP: diaminopimelic acid; PGN: peptidoglycan; PGRP: peptidoglycan recognition protein; GNBP: Gram-negative binding protein; MSP: modular serine protease; SAE: Spätzle-processing enzyme-activating enzyme; SPE: Spätzle-processing enzyme; SP: serine protease; PO: phenoloxidase; MyD88: myeloid differentiation primary response 88; Dif: dorsal-related immunity factor; Imd: immune deficiency; FADD: Fas-associated death domain ortholog; Dredd: death-related ced-3/Nedd2-like protein; TAK: TGF-β activated kinase; IKKγ: inhibitor of nuclear factor-κB kinase subunit gamma, also known as Kenny; Ird5: immune response deficient 5; Hep: hemipterous; Bsk: basket; Dome: domeless; Hop: hopscotch, also known as Jak: Janus kinase; Stat: signal transducer and activator of transcription; AMP: antimicrobial peptide; JNK: c-Jun N-terminal kinase.

nucleopolyhedroviruses than those reared solitarily (Reeson et al., 1998; Wilson et al., 2001). In addition, parasitized bumblebees (*Bombus terrestris*) exhibit twice as much PO activity in their hemolymph than do non-parasitized nest mates (Brown et al., 2003). *T. molitor* melanization processes play an important role in the ecology of the insect, as cuticle darkness polymorphisms correlate with PO activity, with a darker cuticle meaning a higher PO activity (Armitage and Siva-Jothy, 2005). The plasticity of those traits could indicate a higher cost for the insect to maintain a more efficient immune system (Barnes and Siva-Jothy, 2000). This could be due to the fact that the enzymatic cascade leading to melanization is accompanied by the production of cytotoxic intermediates, such as phenols, quinones, and reactive oxygen species (Nappi and Vass, 1993; Nappi and Ottaviani, 2000; Sugumaran et al., 2000; Nappi and Christensen, 2005), which help to kill invading organisms, but at the cost of deleterious effects for the host (Sadd and Siva-Jothy, 2006). However, fecundity and lifespan of darker insects reared in laboratory conditions were not impacted (Krams et al., 2016). Unidentified trade-offs structuring insect life history traits may prevent the fixation of the darker cuticle phenotype in the wild.

Those intermediates are also part of a more systemic immune response as they are liberated in the insect hemolymph along with the inducible synthesis of AMPs produced by the fat body. This response constitutes the humoral immune defense, which is triggered upon microbe recognition *via* the Toll and IMD signal transduction cascades, complemented by c-Jun N-terminal kinase (JNK), and Janus kinase/Signal Transducer and Activator

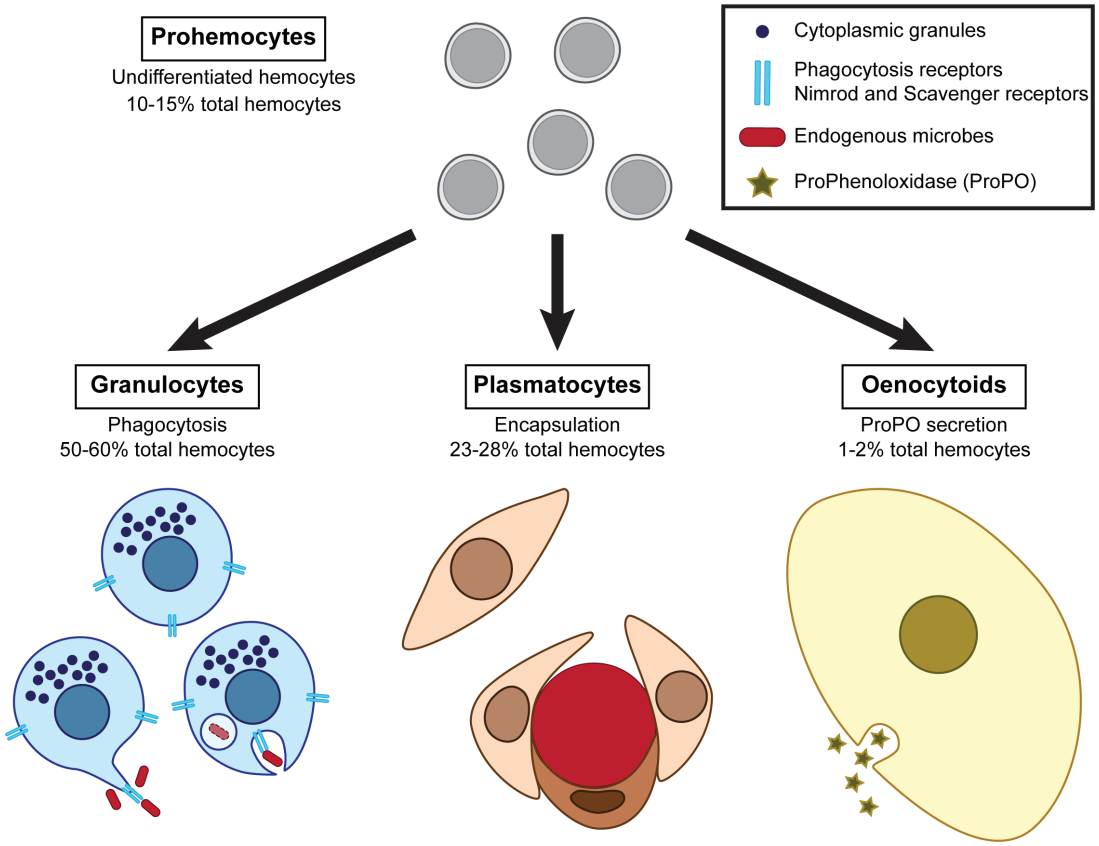

FIGURE 2 | Diversity of the hemocytes composing *T. molitor* cellular immune response and their associated functions.

of Transcription (JAK/STAT) pathways (**Figure 1**; Buchon et al., 2014). These pathways activate the NF-κB transcription factors Relish, Dorsal, and Dif, which induce expression of antimicrobial peptides (Kounatidis and Ligoxygakis, 2012). These pathways are conserved in many insects including *T. molitor* (Johnston et al., 2014). Contrary to the transient and immediate induction of cellular immune effectors, *T. molitor* antimicrobial production is induced within 48 h following MAMP detection and last at least 14 days (Haine et al., 2008; Johnston et al., 2014). The mealworm beetle presents several genes potentially coding for antimicrobial peptides, including the Tenecins 1, 2, 3, and 4, for which the proteins have been isolated and assessed for their immune functions (Moon et al., 1994; Lee et al., 1996; Kim et al., 1998; Roh et al., 2009; Park et al., 2010b; Chae et al., 2012). Tenecins 1, 2, and 4 are inducible defensin, coleoptericin, and attacin, respectively, which are regulated by the Toll and IMD pathways, and mainly display antibacterial activities (Kim et al., 1998; Kim et al., 2001; Roh et al., 2009; Park et al., 2010b; Chae et al., 2012). Tenecin 3 is a constitutively expressed Thaumatin, the role of which is suggested to prevent fungal infections (Chae et al., 2012; Maistrou et al., 2018). Interestingly, AMPs are induced in *T. molitor* eggs in response to a septic injury (Jacobs et al., 2017). Moreover, the levels of immune gene expression in the eggs reach comparable levels to the expression in adults. This suggests that eggs contain immunocompetent cells in addition to maternal effects to defend themselves against potential invaders. Investigation of egg immunity in *Tribolium castaneum* has shown that most of the immune response originates from the serosa, an extra layer of cells that envelops the yolk and the developing embryo (Jacobs et al., 2014). It is not known if *T. molitor* serosa is capable of such immune response, but this is likely due to their close relation to *T. castaneum* and the similar environment they exploit.

SOURCE OF VARIATION IN ANTI-PARASITIC DEFENSES

Density

The risk for an individual to be infected with pathogens and parasites increases when it lives in a population with higher density. Hence, it would be beneficial for individuals developing in such conditions to invest more in their defense mechanisms than individuals experiencing a low-density environment. This hypothesis was introduced as "density-dependent prophylaxis" (DDP) and predicts that individuals developing in high-density conditions will exhibit a more efficient immune response against parasites and pathogens (Wilson and Reeson, 1998). This concept was first defined investigating the noctuid moth *Spodoptera exempta*, which presents a higher resistance to baculovirus when developing in high-density conditions (Wilson and Reeson, 1998). These density-dependent effects are associated with elevated antibacterial activity and higher numbers of circulating hemocytes

in the hemolymph (Reeson et al., 1998; Wilson and Reeson, 1998). DDP is observed in several insects, including both holometabolous insects (Cotter et al., 2004a,b) such as *T. molitor* (Barnes and Siva-Jothy, 2000) and hemimetabolous insects (Wilson et al., 2002), suggesting that it is widely conserved among distant species and even in other invertebrates (Mills, 2012).

Interestingly, DDP usually correlates with melanism polyphenism, for which insects living in high-density population are darker (**Figure 3**; Barnes and Siva-Jothy, 2000; Wilson et al., 2001; Wilson et al., 2002; Cotter et al., 2004a). This degree of melanization is the main factor correlating with higher resistance to microbial invaders (Barnes and Siva-Jothy, 2000; Cotter et al., 2004a). Hence, it can be argued that the DDP-driven improved resistance to pathogens comes from the higher number of darker individuals generated in high-density conditions rather than being a shared trend among insect reared in high-density conditions, independently of their color (Barnes and Siva-Jothy, 2000; Cotter et al., 2004a). This is especially supported in *T. molitor* as darker individuals, compared to paler insects, present a thicker and less porous exocuticle (Silva et al., 2016) and an increased PO activity (Evison et al., 2017) and do not suffer the same deleterious effects following a mock hemolymph infection (Krams et al., 2016) independently of the population density. Nevertheless, the plasticity of the melanization phenotypes in response to population density added to the absence of predominance of darker individuals among *T. molitor* populations suggests underlying trade-offs preventing the fixation of the darker phenotype.

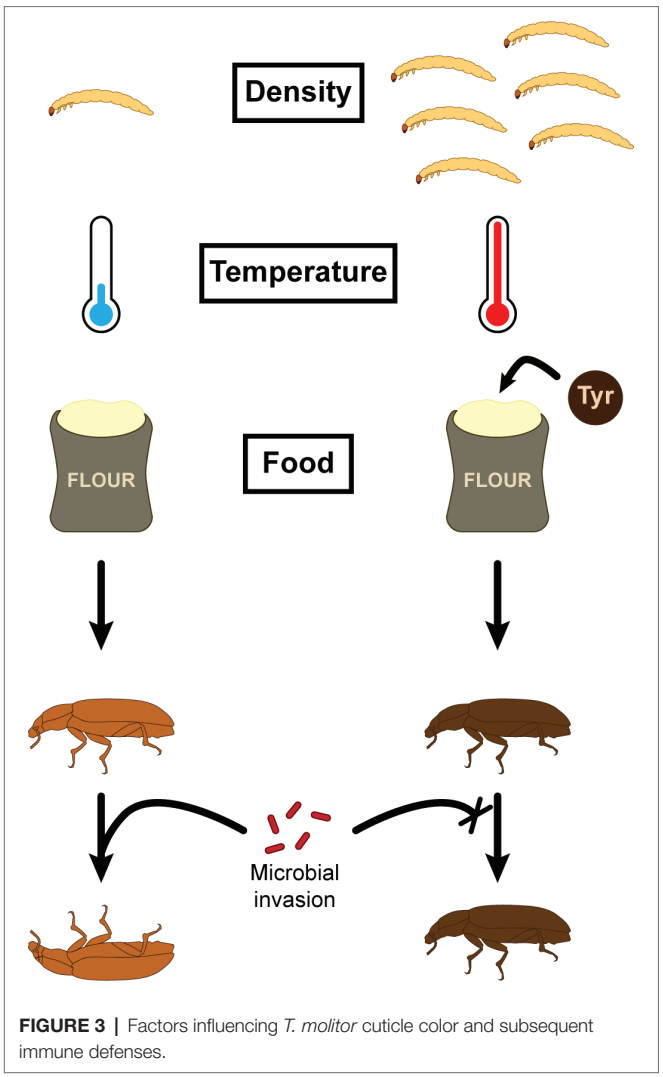

FIGURE 3 | Factors influencing *T. molitor* cuticle color and subsequent immune defenses.

While population density influences insect melanization, the underlying molecular mechanisms governing DDP are still unclear. Nevertheless, DDP demonstrates that interaction between individuals can structure their development, including the immune defenses. Horizontally transferred immunity from insects exposed to an infection to naïve insects is another aspect of individual interaction shaping the efficiency of immune defenses. Social and behavioral immunity have been mainly described in eusocial insects (Traniello et al., 2002; Ugelvig and Cremer, 2007) and may be relevant in highly interacted insect populations (Elliot and Hart, 2010). A recent study investigated whether *T. molitor* displays social immunity through exposing *Staphylococcus aureus*-infected insects to naïve individuals (Gallagher et al., 2018). The authors did not report any evidence leading to the conclusion that social immunization exists in the mealworm beetle. However, they observed an improved tolerance to bacterial infection in naïve insects that were interacting with insects injected with heat-killed bacteria (Gallagher et al., 2018). This suggests that *T. molitor* can sense and respond to immune-related signals originating from pathogen-exposed individual.

Density- and infection-sensing can also modulate resistance mechanisms across generations, as shown by the water flea, *Daphnia magna* (Michel and Hall, 2016), and the cotton leafworm, *Spodoptera littoralis* (Wilson and Graham, 2015). In those models, crowding the parental generation conditioned the immune response of the offspring associated to increased levels of parasite resistance. No such result has been yet demonstrated in *T. molitor*. This insect can protect the egg through the transfer of maternal immune effectors and the induction of egg immune genes in response to a parental microbial challenge (Dhinaut et al., 2018b). Hence, because the mealworm displays DDP, it would be highly relevant to investigate whether high-density conditions could lead to improve egg immunity.

Temperature

With the rising concern for climate change, a growing number of studies have focused on how temperature fluctuation impacts insect biology. One recent aspect of this field of investigations relates to the influence of temperature on insect immunity. Temperature stress triggers the production of heat-shock protein (HSP) that helps organisms to sustain said stress. Interestingly, those proteins are also expressed in *T. castaneum* following a microbial infection (Altincicek et al., 2008). Another example comes from *Musca domestica* that is expressing Hsp70 in response to a bacterial stimulation, and for which the knockdown *via* RNA interference (RNAi) leads to a decrease resistance to infection (Tang et al., 2012). Altogether, these results show that temperature-related genes are intimately intricate with immune pathways, suggesting a potential influence of temperature on immune defenses.

Several recent studies have investigated the effects of temperature on life history traits of insects and conclusions vary depending on the model studied (Prokkola et al., 2013; Kaunisto et al., 2015; Silva and Elliot, 2016; Yin et al., 2016; Laughton et al., 2017). For example, when reared in a higher set of temperatures, the Indian meal moth, *Plodia punctella*, presents more hemocytes (Laughton et al., 2017). Conversely, the velvet bean caterpillar, *Anticarsia gemmatalis*, presents fewer hemocytes when developing at a higher temperature (Silva and Elliot, 2016). As organisms evolve and build trade-offs between their life history traits influenced by their environment, it is expected that mechanisms underlying resistance or tolerance to stress correlate differently to the impacted life history traits. It is especially relevant for insects, which, because they are ectothermic, must resist the variations of their environmental temperature. In *T. molitor*, investigations have shown that encapsulation and cuticle darkness negatively and positively correlate with temperature, respectively (**Figure 3**; Prokkola et al., 2013). Moreover, while developing in warmer temperature conditions, the mealworm beetle experiences a shortened larval development and presents longer elytra. It shows that the insect biology in its whole is impacted by the temperature, which imposes trade-offs between different life history traits of an individual.

While temperature stress impacts insect immune defenses, little is known about how and whether it also affects the immune system of offspring. This inquiry has been explored in *T. castaneum*, where the offspring resistance to a bacterial challenge was found to be improved when both parents received a cold shock, while no effects were observed when the parents received a heat shock (Eggert et al., 2015). In addition, a cold shock experienced by the mother or both parents induced higher PO activity in the offspring, while a heat shock of either parent, or both, reduced the PO activity of their offspring. In conclusion, temperature is an important parameter that can significantly influence the biology of individuals, including their immune system. Hence, it would be beneficial to further investigate how temperature relates to *T. molitor* in the aim to better promote or regulate its population.

Food

Food quality and quantity are critical to immune defenses against parasites and pathogens. While leveraging food amount and quality for restricting or controlling pest populations is difficult, we may use nutrients that directly or indirectly improve the immune system of insects that we would like to maintain or mass rear.

As immune functions require metabolic resources, food restriction can impair immune activity. For instance, adult *T. molitor* PO activity can be reduced by half during short-term food privation, but it returns rapidly to initial levels when given access to food again (Siva-Jothy and Thompson, 2002). Furthermore, following an immune challenge, *T. molitor* larvae can eat five times more food per day than usual to compensate for the caloric expense of the immune response (Catalan et al., 2011). Hence, unsurprisingly, food supply is important to keep insects healthy. While the amount of food available matters, its nutritional composition is also important, especially with regard to its protein to carbohydrate ratio (Ponton et al., 2011). For instance, infected caterpillars select food containing higher protein to carbohydrate ratio, which improves their resistance to viral or bacterial infection (Lee et al., 2006; Povey et al., 2009). The same observation has been made for *T. molitor,* whose healthy larvae usually prefer diets with lower protein to carbohydrate ratio but shift toward food with higher protein contents after immune challenge with bacteria (Catalan et al., 2011). As a consequence of this diet shift, hemocyte circulation and antibacterial activity are enhanced in the hemolymph, which presumably maximizes resistance against bacterial infection. However, PO activity is not affected by this shift in diet-choice, suggesting that either PO activity is likely less sensitive to protein intake, or it is limited by a trade-off consequent to the upregulation of antibacterial activity (Moret and Schmid-Hempel, 2001; Cotter et al., 2004b; Moret and Schmid-Hempel, 2009). In fact, diet effects on PO activity appear controversial in insects, as it has been found to be slightly enhanced by diets rich in either proteins (Lee et al., 2006; Povey et al., 2009) or carbohydrates (Koella and Sorensen, 2002; Cotter et al., 2011). Nevertheless, PO activity appears less variable with respect to diet than other immune traits (Catalan et al., 2011; Cotter et al., 2011; Evison et al., 2017). On the one hand, excess levels of PO activity could be dangerous, as uncontrolled activation of PO in the hemocoel would result in the production of toxic quinones and reactive oxygen species, which could harm self-tissues and organs (Nappi and Vass, 1993; Sadd and Siva-Jothy, 2006). Therefore, preventing excessive diet-mediated upregulation of PO activity might be required. On the other hand, PO is also involved in a large set of physiological functions independent of immunity (Hiruma and Riddiford, 1988). Thus, preventing its excessive downregulation may help to maintain homeostasis of those other physiological functions.

Interestingly, immune-challenged *T. molitor* larvae exhibit significant weight loss when fed either protein- or carbohydrate-rich diets, while their weight remains stable when they were given both protein- and carbohydrate-rich diets (Catalan et al., 2011). This suggests that no single blend of ingested nutrients can optimize all the physiological needs, and that the composition of the diets ingested by the insects represents a trade-off between optimizing different traits (Cotter et al., 2011). Hence, a relevant way to maximize growth and immunity of mealworm beetles would be to supply the insect with nutrient adjusted according to their physiological needs.

While diet composition appears to exert a moderate effect on hemocoelic PO activity, it may nevertheless influence cuticle melanization and sclerotization, which subsequently impacts cuticular color (Reeson et al., 1998; Wilson et al., 2001; Cotter et al., 2004a) and resistance to important entomopathogens (Wilson and Reeson, 1998; Barnes and Siva-Jothy, 2000; Wilson et al., 2001; Dubovskiy et al., 2013). PO enzymes mediate melanization and sclerotization of the cuticle (Andersen, 2010), and central to these latter processes is the production of 3, 4-dihydroxyphenylalanine (DOPA) from the hydroxylation of the semi-essential amino acid tyrosine (Vavricka et al., 2010). *T. molitor* exhibits plastic variation in cuticle color, and darker individuals are often more resistant to fungal diseases (Barnes and Siva-Jothy, 2000). Darker individuals exhibit alteration in the physical and chemical properties of the cuticle such as thickening and a higher degree of melanization (Silva et al.,

2016; Evison et al., 2017). The experimental supplementation of *T. molitor* larvae with the amino acid Tyrosine led to the development of adults with a darker cuticle (**Figure 3**; Evison et al., 2017). Interestingly, the cuticle was also thicker, but only in females, suggesting that males and females are allocating their tyrosine resources differently. In addition, while tyrosine supplementation resulted in improved cuticle defenses, it did not affect hemocoelic melanin-mediated defenses (Evison et al., 2017), suggesting that the regulation of both lines of defense could be uncoupled despite their similar precursor.

Insect immune activities are associated with the production and release of cytotoxic compounds such as reactive oxygen and nitrogen species (ROS and RNS, respectively) (Nappi and Vass, 1993). While these toxic substances help to kill invading organisms, they also cause self-damage in *T. molitor* (Sadd and Siva-Jothy, 2006), which results in a significant lifespan reduction (Pursall and Rolff, 2011; Khan et al., 2017). While insects rely on endogenous antioxidants to scavenge these free radicals, this process might be supported by dietary sources of antioxidants (Chew and Park, 2004). For instance, carotenoids have the ability to scavenge free radicals produced by immune activities (El-Agamey et al., 2004) and the potential to interact with endogenous antioxidant enzymes (Lee et al., 2011; Babin et al., 2015). Importantly, in addition to their potent antioxidant property, carotenoids stimulate the immune system of both vertebrates (Blount et al., 2003; Park et al., 2010a) and invertebrates (Flores et al., 2007; Babin et al., 2010; Babin et al., 2015). Contrary to these general observations, life-time dietary supplementation of *T. molitor* with astaxanthin, a carotenoid with strong antioxidant activity (Chew and Park, 2004), strongly depressed the insect immune system and decreased its resistance to bacterial infection (Dhinaut et al., 2017). Investigations pointed that this could result from the interaction between the pigment and nitric oxide (NO), which stimulates both cellular and humoral immunity of insects (Imamura et al., 2002; Kraaijeveld et al., 2011; Eleftherianos et al., 2014; Sanzhaeva et al., 2016). Indeed, astaxanthin may either inhibit the activity of the nitric oxide synthase, the enzyme responsible of NO production from L-Arginine (Hussein et al., 2006), or interfere with NO cellular signaling by scavenging a fraction of circulating NO, and consequently, downregulating base levels of immune activities. In addition, astaxanthin may also have regulatory effects on the host's metabolism, which would collaterally impair immune activities (Hussein et al., 2007; Yang et al., 2011). Astaxanthin interacts with nuclear receptors of the peroxisome proliferator-activated receptor superfamily, which regulates lipid and glucose metabolism in vertebrate (Jia et al., 2011). Such an alteration of the host metabolism may reduce the allocation of energetic resources to the immune system. If these receptors are conserved among taxa, similar regulatory effects may also occur in insects. These results suggest that supplying *T. molitor* with carotenoids, especially with astaxanthin, is rather detrimental. Hence, the use of this carotenoid might not be adequate when rearing this insect. By contrast, its immune-depressive effect could be used to improve the success of microbial insecticides, where the insect is detrimental (**Figure 4**).

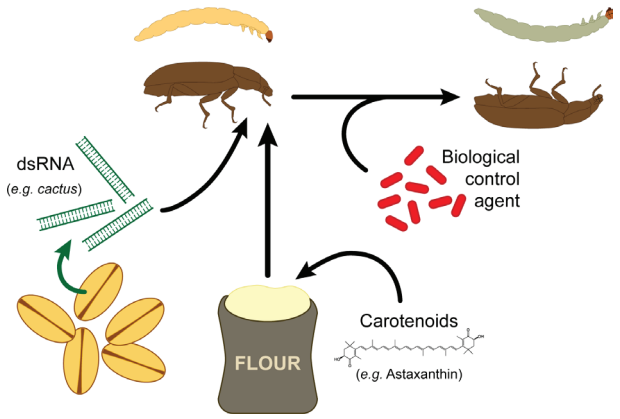

FIGURE 4 | Potential methods to weaken *T. molitor* immune system with the aim of enhancing population control using biological agents.

Previous Experience of Pathogens: Immune Priming

Like other invertebrates, *T. molitor* lacks immune effectors that are responsible for the acquired immune response of vertebrates. However, the invertebrate immune system is capable of functional modulation similar to the acquired immune response of vertebrates (Moret and Siva-Jothy, 2003; Moret, 2006). This form of innate immune memory in an invertebrate is termed "immune priming," which is broadly defined as increased protection to a pathogen following previous exposure (Little and Kraaijeveld, 2004). Immune priming may exhibit a variable degree of specificity, from cross-reactive (non-specific) (Moret and Siva-Jothy, 2003) to highly specific (more effective against the pathogen encountered during the primary challenge), especially when cellular processes are involved for the latter (Pham et al., 2007; Roth and Kurtz, 2009). Priming response can also be obtained from a challenge with an inert immune elicitor such as a nylon implant, which can provide immune protection against a subsequent fungal infection (Krams et al., 2013). This suggests that immune priming originates, at least partially, from activation of immune defenses rather than solely from the presence of MAMPs.

Functionally, individual immune priming may rely on three types of responses (Coustau et al., 2016). First, it may involve a sustained response, corresponding to the long-lasting upregulation of the same immune effectors after the initial immune challenge. Second, a recalled response results in a faster and stronger response after a secondary infection in a way that is reminiscent of the vertebrate acquired immune response. Third, priming may induce an immune shift, involving different immune effector systems during the primary and the secondary immune responses. Current evidence suggests that individual immune priming in *T. molitor* is achieved through a sustained antibacterial activity, which can be active for at least 20 days after a primary immune challenge by injection of a suspension of killed Gram-positive bacteria (Makarova et al., 2016; Dhinaut et al., 2018a). Hemocyte concentration also increased two-fold upon the secondary challenge with

the bacteria (Dhinaut et al., 2018a), which might be consistent with a hemocyte-mediated recall response. However, while this enhanced hemocyte concentration could be involved in the priming response, this relationship is speculative because of the persistent antibacterial activity in the hemolymph resulting from the primary challenge (Dhinaut et al., 2018a). No such long-lasting antibacterial activity and change in hemocyte concentration was found in primed insects with Gram-negative bacteria, and consistently, the priming with Gram-positive bacteria provided the most effective protection against microbial reinfection (Dhinaut et al., 2018a). Therefore, individual priming responses induced by Gram-positive bacteria are stronger and more protective than those induced by Gram-negative bacteria in *T. molitor*, possibly because Gram-positive bacteria have played an important evolutionary role in shaping the immune system of this insect.

An individual may not only gain immune protection from its own immunological experience, but it can also benefit from that of its parents through "trans-generational immune priming" (TGIP). TGIP allows immune-challenged parents to produce more resistant offspring (**Figure 5**; Moret, 2006). In *T. molitor*, TGIP effects were revealed through enhanced immune activity in primed eggs (Moreau et al., 2012; Zanchi et al., 2012; Dubuffet et al., 2015; Dhinaut et al., 2018b), larvae (Moret, 2006), and adult offspring (Zanchi et al., 2011; Dhinaut et al., 2018b). Furthermore, enhanced immunity in the offspring may result either from the immune challenge of fathers or mothers, although paternal and maternal TGIP are associated with the enhancement of different immune effectors in the offspring (Zanchi et al., 2011). Similar to individual immune priming, TGIP of offspring does not appear to be pathogen-specific (Dhinaut et al., 2018b). For example, the offspring of mothers primed with the Gram-negative bacteria, *Serratia entomophila*, and those primed with the Gram-positive bacteria, *Bacillus thuringiensis,* had a similar enhanced survival to bacterial infection (Dhinaut et al., 2018b). However, while the maternal challenge with *S. entomophila* had no apparent effect on base levels of cellular or humoral immune defenses of the offspring, the maternal challenge with *B. thuringiensis* slightly enhanced PO activity of the offspring (Dhinaut et al., 2018a). In addition, the offspring of mothers immunized with purified LPS from bacterial cell wall displayed increased base levels of hemocyte concentration (Zanchi et al., 2011). Hence, offspring immunity is affected differently through TGIP depending on the nature of the maternal challenge.

Although TGIP occurs in several invertebrate species, investigations on its molecular mechanisms have just begun. In *T. castaneum*, bacterial components cross the midgut epithelium and are stored in eggs (Knorr et al., 2015), probably *via* a vitellogenin-mediated transfer. Vitellogenin, the major egg storage resource for embryo nutrition, also displays multiple immune functions by acting as a multivalent pattern recognition receptor with opsonin and antibacterial activity (Singh et al., 2013). Vitellogenin can recognize bacteria by specifically binding to MAMPs, such as PGN and LPS, and mediate the translocation of bacterial proteins to the eggs (Salmela et al., 2015). This transfer was associated with an increased expression of immune genes in the eggs (Knorr et al., 2015; Salmela et al., 2015). Interestingly, *T. molitor* females provide enhanced antibacterial activity to eggs that are produced from the second to the eighth day after the maternal challenge only (Zanchi et al., 2012). Offspring resulting from eggs laid after this restricted period of time are not protected by enhanced antibacterial activity, but they still exhibit a higher concentration of hemocytes in their hemolymph at the adult stage (Zanchi et al., 2011). These results suggest that mechanisms regulating protection of eggs and adult offspring are probably different. Hence, TGIP likely involves independent mechanisms that are acting simultaneously or sequentially over the development of the insect.

How and where the signal of a primary infection is "memorized" appears of paramount importance in the understanding of within and trans-generational immune priming. While hemocytes may play a role in immune memory, evidence is still scarce. For instance, during the antiviral immune response of *Drosophila*, infected cells generate double-stranded RNA (dsRNA) to inhibit viral molecule expression *via* RNAi (Tassetto et al., 2017). Part of the produced dsRNA is taken up by hemocytes, which then produce virus-derived complementary DNAs (vDNA) used as templates for *de novo* synthesis of small interference RNAs (siRNAs) targeting viral sequences (Tassetto et al., 2017). These siRNAs, secreted in exosome-like vesicles of immune cells, may represent the source of information storage. Furthermore, as TGIP requires the transfer of the information from the primary challenge to the offspring, the integration of viral elements into the genome through recombination with specific classes of retrotransposons and their organization into large loci of endogenous viral elements (EVEs) may represent a reservoir of immune memory in *Aedes aegypti* (Whitfield et al., 2017). Similar processes are unknown in *T. molitor*, which was also not reported to exhibit immune priming against virus infection.

Epigenetic reprogramming was also proposed as an important process to support within and trans-generational immune priming (Ottaviani, 2015). Epigenetic reprogramming of immune cells could be achieved through remodeling of DNA methylation patterns, changes in histone marks, modifications of chromatin structure, or changes in miRNA or lncRNA expression patterns. While indications of transcriptomic changes involving enzymes that control DNA methylation and histone acetylation in *Galleria mellonella* have been documented (Heitmueller et al., 2017), experimental evidence failed to link epigenetic differences to immune priming, both within and across generations (Eggert et al., 2014; Norouzitallab et al., 2015). A recent study has investigated the occurrence of methylation from total DNA and RNA extraction in *T. molitor* subjected to either individual or trans-generational immune priming by the fungus *Metarhizium anisopliae* or the bacteria *Micrococcus lysodeikticus* (Castro-Vargas et al., 2017). No global changes in DNA methylation resulting from either within or across generation immune priming were detected. However, whether DNA methylation was affecting smaller relevant portions of the genomic DNA, for instance, targeting the regulatory regions of a restricted number of genes was not investigated. In addition, a low proportion of RNA methylation results from individual immune

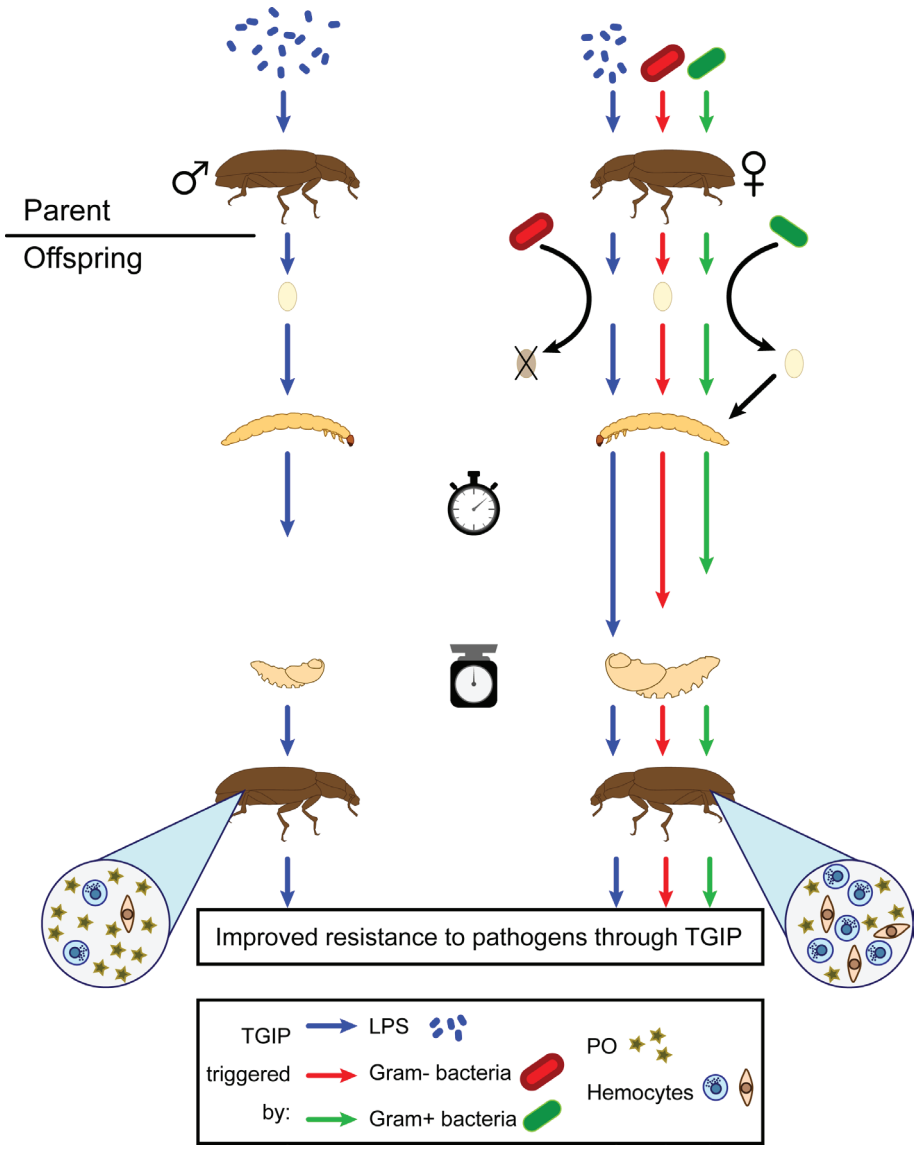

FIGURE 5 | Subsequent effects of TGIP on *T. molitor* offspring traits. TGIP enhances offspring resistance to pathogens if either the father (left) or the mother (right) has been primed. However, the sex of the parent triggering the TGIP produces different consequences on the offspring traits. Offspring originated from a mother primed with either LPS (blue), Gram-negative bacteria (red), or Gram-positive bacteria (green) are also affected differently by the TGIP. Larval developmental time (stopwatch) is represented by the length of the arrows between larvae and pupae (arbitrary scale). Weight of the pupae (scale) is represented by the size of the scheme at this stage (arbitrary scale). Density of hemocytes and hemolymph phenoloxidase (PO) activity is represented by the relative abundance of their corresponding symbols in the circles originated from the offspring beetles.

priming, indicating that RNA methylation could be involved in the process. However, no such change results from TGIP. Further study is needed to identify the types of RNA involved in methylation and their implication in the individual priming process.

The involvement of microRNA, lncRNA, and changes in chromatin structure has not been investigated in TGIP, despite their involvement in invertebrate immunity and host-pathogen interactions (Asgari, 2013). In *G. mellonella,* experimental selection for resistance to *Bacillus thuringiensis* resulted in trans-generational modification of acetylation of specific histones, DNA methylation, and transcription of genes encoding the enzymatic writers and erasers of these epigenetic mechanisms (Mukherjee et al., 2017). Hence, considering the prominent role of epigenetics in many trans-generational adaptation processes in animals and its implication in the modulation of several immune response pathways, its involvement in immune priming, especially in TGIP, might be a promising avenue to explore in greater depth.

Immune priming, either within or across generation, is beneficial by enhancing individual immune protection against repeated infections. However, this process likely exerts

energy-related costs that would constrain the expression of other important functions. These costs might be bearable upon high risks of repeated infection but could be heavy when re-infection is unlikely (Tate, 2017). The cost of individual immune priming includes the cost of the initial immune response, upon a primary contact with the pathogen, and the additional cost of keeping the immune system upregulated for an extended period of time (*i.e.* memory), which are almost impossible to discriminate. The cost of TGIP is likely shared by both parents and offspring. On the one hand, parents, especially mothers, may support part of the cost of TGIP by producing and transferring immune substances to their eggs in addition to paying the usual immune activation costs resulting from the priming infection (Moret and Schmid-Hempel, 2000). For instance, bacterially immune-challenged females of *T. molitor* transiently endow a variable proportion of their eggs with antibacterial activity, which negatively correlates with female fecundity (Zanchi et al., 2012). Furthermore, the level of antibacterial activity found in eggs correlates negatively to that of their mother's hemolymph, suggesting that mothers trade-off their own immunity against that of their eggs (Moreau et al., 2012). On the other hand, enhanced immunity in the offspring may compromise other important functions. TGIP enhances immunity in the offspring of *T. castaneum* and *T. molitor* at the expense of a prolonged larval development time (Roth et al., 2010; Zanchi et al., 2011; Dhinaut et al., 2018a). A prolonged larval development time increases the probability of mortality (Bell, 1980), especially in tenebrionid beetles, which exhibits cannibalism on juveniles (Ichikawa and Kurauchi, 2009). However, such a cost in *T. molitor* depends on the bacterial pathogen to which mothers were previously exposed, as larval development time of maternally primed offspring with Gram-positive bacteria was much shorter than maternally primed offspring with Gram-negative bacteria (Dhinaut et al., 2018a). As pathogens may vary in the selective pressure they impose on hosts, *T. molitor* may have evolved an optimal immune priming against the most pervasive and threatening range of pathogens it encounters. In other insects, primed offspring exhibit reduced fecundity at the adult stage (Trauer and Hilker, 2013) and reduced resistance to a different parasite type to which the mother was exposed (Sadd and Schmid-Hempel, 2009). Further investigations are needed to reveal whether *T. molitor* primed offspring are paying comparable costs to TGIP. These negative effects associated to TGIP may result from the offspring trading-off their immunity against other functions. Alternatively, they may be the consequence of a reduced parental investment per offspring resulting from the cost of the parental immune challenge. In this case, reduced parental investment into their progeny should be observed early in the offspring's life. However, recent evidence showed that immune-challenged *T. molitor* females produced eggs with a stronger hatching success and that the resulting young larvae show enhanced survival to starvation within the first month post hatching (Dhinaut et al., 2018b), although they are known to exhibit a prolonged developmental time later on (Zanchi et al., 2011; Dhinaut et al., 2018a). This suggests that TGIP cost is likely to arise from offspring trade-offs and not from a reduced parental investment.

Microbiota

The importance of characterizing the mealworm's microbial community is proportional to the increased interest in this insect as a food source. Indeed, understanding the microbiota of insects that are used for consumption is an essential for identifying potential spoilage bacteria and food pathogens. The bacterial community from living, processed, and laboratory-reared mealworms reared for consumption indicated that their microbiota was dominated by Tenericutes, Firmicutes, and Proteobacteria (Jung et al., 2014; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2018). The gut-associated microbiota is an important mediator of host development and growth. For instance, microbiota-free mosquito larvae (axenic individuals) developmentally arrest due to the lack of bacteria-mediated hypoxia (Coon et al., 2014; Coon et al., 2017). Another striking example is the contribution of the gut-associated bacteria *Lactobacillus plantarum* to *Drosophila* growth (Storelli et al., 2011). The bacterium promotes protein assimilation from *Drosophila*'s diet, optimizing diet-derived amino acid levels in the hemolymph. This activates the target of rapamycin (TOR) signaling pathway, which triggers the insulin-like and ecdysone pathways that promote growth rate and reduce growth duration, respectively (Storelli et al., 2011). While no such intimate interaction has yet been described between *T. molitor* and its microbiota, axenic *T. molitor* experiences a change in digestive enzyme expression, supporting the hypothesis that associated microbes are involved in the insect's physiological homeostasis. Especially, the microbiota may help the mealworm to defend against the detrimental effects of food-derived toxic compounds such plant-derived glucoside salicin (Genta et al., 2006). In addition, microbe-free *T. molitor* does not produce pentadecene (Genta et al., 2006), a volatile that functions as a defensive secretion against predators in *T. castaneum* (Arnaud et al., 2002).

As previously mentioned, another growing interest for *T. molitor* concerns its ability to digest polystyrene foam. While polystyrene foam decreases *T. molitor* fecundity (Nukmal et al., 2018), the insect can fully develop using the plastic as its primary source of food. This makes the insect a relevant alternative to recycle polystyrene. Interestingly, when the mealworm's microbiota is disrupted following an antibiotic treatment, the insect loses its ability to digest polystyrene, indicating that its associated microbes play a crucial role in the digestion process (Yang et al., 2015). Especially, the bacterium *Exiguobacterium* sp. (Firmicutes) was isolated from the midgut of mealworms and was demonstrated to degrade the polystyrene *in vitro* (Yang et al., 2015). This shows that specific members of the microbial community confer the mealworm its ability to digest polystyrene. Hence, targeting the microbial community of *T. molitor* could help to boost its polystyrene digestion efficiency. This could be achieved *via* isolation of bacteria originating from the mealworm microbiota that would be genetically engineered to produce polystyrene-degrading enzymes.

In addition to supporting insect growth, indigenous microbes can mediate the development and function of their host immune system. In tsetse flies, microbe-free adults exhibit a severely compromised immune system that is characterized by a significantly depleted population of hemocytes (Weiss et al., 2011). Interestingly, larval *Drosophila's* indigenous microbiota

regulates orthologous hematopoietic pathways in their host (Benoit et al., 2017). These examples demonstrate the intricate impact that the microbiota plays on host immune development. Such an association between *T. molitor* and its microbiota has yet to be investigated. Nevertheless, the enhanced immune response conferred by oral priming and TGIP demonstrates that immune mechanisms are adjustable according to *T. molitor* interaction with microbes. Also, it was suggested that microbiota influences oral priming in the red flour beetle *T. castaneum* (Futo et al., 2016). Hence, exploring whether *T. molitor*'s microbiota could influence TGIP would be a major milestone to understand the molecular mechanisms underlying this process.

The microbiota of *T. molitor* includes microbial taxa that may be pathogenic for human and animals, such as *Enterobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae* (Jung et al., 2014; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2018). Methods used to process mealworm larvae for food, such as pulverization, could allow microbes to be released from the insect gut, which, if not eliminated, may be hazardous to livestock and human consumers (Klunder et al., 2012; Megido et al., 2016; Stoops et al., 2016; Garofalo et al., 2017; Grau et al., 2017). Thus, the presence of pathogens in mealworms would serve as an impediment to the use of this insect as a source of human and animal food. How the immune system could cope with controlling pathogens or modifying the microbiota structure is still an open question. However, the activation of the immune system through the production of antimicrobial peptides before processing the larvae could help to prevent an unwanted microbial community growing within insect-processed products. Indeed, insect antimicrobial peptides are active against a large range of microbes (Bulet et al., 1999). Immune priming could be used to trigger the long-lasting production of antimicrobial peptides in the hemolymph of the insect (Dhinaut et al., 2018a) prior to processing mealworm larvae. In consequence, primed insects would incorporate antimicrobial peptides that prevent unwanted microbes from contaminating the mealworm-derived food and feed.

PROMOTING AND CONTROLLING *T. MOLITOR* POPULATIONS

T. molitor is increasing being recognized as an alternative source of protein-rich food with a low ecological footprint (Grau et al., 2017). Hence, insect farms have started to mass produced mealworms despite the risk of an infection outbreak. Each group of insect pathogens presents unique biological characteristics for which a clear understanding of the interaction with the host is required to implement efficient control (Eilenberg et al., 2015). Nevertheless, controlling disease involves improving the insect's first line of defense, with the aim being to reduce the probability of infection. In *T. molitor*, one promising approach involves enhancing the integrity of the cuticle so as to render adult insects more resistant (**Figure 3**). Indeed, darker beetles present a thicker cuticle that is less likely to be circumvented by external pathogens (Silva et al., 2016; Evison et al., 2017), and they present enhanced immune parameters, such as the PO activity and hemocyte concentration (Armitage and Siva-Jothy, 2005). As *T. molitor* population density positively correlates with cuticle darkness, we can expect that mass rearing conditions are already optimized for this parameter. Temperature and food can also influence the quality of the cuticle, as higher temperature and better access to the aromatic amino acid tyrosine increase the darkness of the cuticle (Prokkola et al., 2013; Evison et al., 2017). While supplementation of tyrosine could be easily achieved, increasing the rearing temperature would cause some deleterious effects on the insect immune system, which makes it a versatile parameter to account for.

Immune priming could be an asset for the mass production of healthy insects while keeping in mind the deleterious effects on other traits of the offspring. However, all the studies investigating immune priming in *T. molitor* used systemic injection of immune elicitors to obtain primed insects. This may prove difficult in mass-reared insects due to their large number. If *T. molitor* could be orally primed like *T. castaneum* (Knorr et al., 2015), the provision of inactivated bacterial materials in the food should be valuable to prevent disease outbreaks in the insect cultures (Grau et al., 2017). Alternatively, managing individually the parental line to enhance offspring immune parameters would be an easier task than trying to apply a method to each mass-produced insect. TGIP or a cold shock experienced by the parents could potentially improve the immunity of the offspring (Eggert et al., 2015). Hence, applying such stress only to insects destined for reproduction could enhance the overall immunity of the colony (**Figure 5**).

Immune priming may also have strong implications for the control of populations of pest insects, such as *T. molitor*, using microbial bio-insecticides. Indeed, when failing to kill the insects, biocontrol agents may subsequently enhance the insect resistance or tolerance, rendering their use less efficient over time. The control of unwanted populations might be even further complicated when, like in *T. molitor*, the priming response provides cross-specific protection that would impair the efficiency of control strategies using different microbial pathogens. Furthermore, insects may not necessarily need to suffer from the infection by the pathogen to become primed, as the consumption of dead bacteria in the food could be sufficient to prime them, as shown for *T. castaneum* (Knorr et al., 2015). Such an infection-free priming process may keep the insects vigorous enough to maintain prolific reproduction while becoming more immunocompetent.

Optimizing the production of insects such as *T. molitor* would allow other uses, such as producing pharmaceuticals or using them for de-pollution purposes. Interest in using insect AMPs as an alternative to antibiotics in livestock production has been growing over the past decade (Li et al., 2014; Wang et al., 2016). Given the broad spectrum activity exhibited by insect AMPs, their production in heterologous systems can reveal difficult. However, *T. molitor's* large mass, in conjunction with the ability to mass produce the insect, makes it useful for generating AMPs *via* immune stimulation during the rearing process. Because the purification of AMPs could prove difficult, the use of a whole insect extract could be a viable alternative.

T. molitor is especially relevant for its ability to digest polystyrene foam. It would be highly beneficial to optimize production of the insect, in combination with improving its digestive capacity via microbiota manipulation, as an alternative to conventional polystyrene recycling schemes. Fundamentally, *T. molitor* and its associated microbes are highly suited candidates for investigating microbe-driven impacts on insect development, with a special focus on the immune system maturation. Understanding the molecular dialog between the insect and its microbiota opens up the possibility for exploiting this interaction as a target for pest control strategies to undermine the insect defenses, or, on the contrary, for enhancing the insect's defenses to optimize its application as a beneficial resource.

Recently, the insect immune system has been studied in the context of developing new tools for insect pest control. A proposed approach consists on using RNA interference (RNAi) to target genes that are crucial for the survival of insects (Baum et al., 2007). RNAi uses homologous double-stranded RNA (dsRNA) to downregulate specific mRNAs, which, for the purpose of pest control, would target genes leading to a severe decrease in fitness. This method was demonstrated successful on insects that feed on plants expressing hairpin dsRNA constructs, or on crops sprayed with dsRNA (Baum et al., 2007; Mao et al., 2007; Whyard et al., 2009; Huvenne and Smagghe, 2010). Recently, this method was applied to downregulate the expression of genes involved in *T. castaneum* Toll signaling pathway, especially focusing the pathway regulator *cactus* and its interacting genes (Bingsohn et al., 2017). RNAi-driven knockdown of *cactus* leads to the death of the insect, validating the relevance of targeting this gene as a novel control method. However, as *cactus*, and more globally the Toll pathway, is required for dorsoventral patterning in *Drosophila melanogaster* (Belvin and Anderson, 1996), the observed effect on *T. castaneum* may be due to a disruption in developmental processes rather than immune-related functions. Using such methods also brings the issue of collateral damage due to an unspecific effect of the RNAi, which could target conserved genes across different species that reside in the target insect's environment. As developmental genes are usually highly conserved across taxa, targeting genes specifically involved in the immune system of an organism under infection could prevent such an effect. Knockdown of immune genes could be combined with the use of *T. molitor* parasites such as the Apicomplexan gregarines (Harry, 1967; Rodriguez et al., 2007) or the ectoparasitoid *Scleroderma guani* (Zhu et al., 2013) to successfully control the pest (**Figure 4**).

AUTHOR CONTRIBUTIONS

AV and YM conceived the ideas. AV, CJ, TR, and YM performed the literature search and contributed to the writing of the manuscript.

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MINI REVIEW
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Functional Multiplicity of an Insect Cytokine Family Assists Defense Against Environmental Stress

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The widespread distribution of insects over many ecological niches owes much to evolution of multiple mechanisms to defend against environmental stress, especially because their ectothermic nature and small body size render them particularly susceptible to extremes in temperature and water availability. In this review, we will summarize the latest information describing a single, multifunctional cytokine family that is deployed by six orders of insect species to combat a diverse variety of environmental stresses. The originating member of this peptide family was identified in *Mythimna* (formerly called *Pseudaletia*) *separata* armyworm; the cytokine was named growth-blocking peptide (GBP), reflecting its actions in combating parasitic invasion. The peptide's name has been retained, though the list of its regulatory activities has greatly expanded. All members of this family are small peptides, 19–25 amino acid residues, whose major source is fat body. They are now known to regulate embryonic morphogenesis, larval growth rates, feeding activities, immune responses, nutrition, and aging. In this review, we will describe recent developments in our understanding of the mechanisms of action of the GBP family, but we will also highlight remaining gaps in our knowledge.

Keywords: cytokine, growth-blocking peptide (GBP), stress-responsive peptide (SRP), Mthl10, hormesis

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INTRODUCTION

Growth-blocking peptide (GBP) was initially found as a peptidergic factor which blocks JH esterase activation in the hemolymph of early last instar larvae of host *Mythimna* (formerly called *Pseudaletia*) *separata* armyworm upon infection by the parasitic wasp *Cotesia kariyai* (Hayakawa, 1990). GBP-induced suppression of hemolymph JH esterase is a protective measure that delays larval growth and development (Hayakawa, 1991). Although the mechanism by which *M. separata* (*Ms*) GBP suppresses hemolymph JH esterase activation is still unknown, this initial observation led us to focus on its hormone-like function (Hayakawa, 1992). Further characterization of *Ms*GBP signaling elucidated that it elevates dopamine concentrations in the hemolymph through enhanced expression of tyrosine hydroxylase and DOPA decarboxylase in the integument and the brain (Noguchi et al., 1995, 2003). This up-regulation of gene expression was subsequently attributed to *Ms*GBP-induced activation of phospholipase C (PLC), release of inositol triphosphate (IP3), and the elevation of cytoplasmic Ca^{2+} concentrations (Ninomiya and Hayakawa, 2007; Ninomiya et al., 2008). Although the relationship between

dopamine elevation and JH esterase repression has not been yet clarified, both events have negative impact on the growth rates of insect larvae (Noguchi et al., 1995).

In the years since the discovery of *Ms*GBP, over 10 GBP orthologous peptides have been found in several lepidopteran species (Hayakawa, 1995, 2006). They all consist of 23–25 amino acids, and they share more than 70% sequence identity, yet they show diverse functions: paralysis induction, plasmatocyte spreading, and cardioacceleration. However, to date, the only known receptor for a GBP is that identified in *Drosophila*-Mthl10 (see below). *Ms*GBP itself was demonstrated to have this multifunctionality (Strand et al., 2000). Subsequent studies established further functions of the GBP family such as cell growth activator, early morphogenetic mediator, and humoral immune mediator (Ohnishi et al., 2001; Tsuzuki et al., 2005, 2012). Nevertheless, as is common practice, this cytokine family is still named after its originating function as a growth-blocking peptide (Hayakawa, 2006).

NONLEPIDOPTERAN GBP

Many GBP orthologous peptides had been reported in Lepidoptera, but it was not until 2012 that the first nonlepidopteran GBP was discovered (Tsuzuki et al., 2012). To identify their primary structures, hemolymph peptides that induce cell growth and plasmatocyte spreading activities were purified from Tenebrionid and bluebottle fly larvae (Matsumoto et al., 2012; Tsuzuki et al., 2012). The functional orthologs identified by these studies comprised 19–24 amino acids, and subsequent homology searches expanded the presence of GBP-like peptides to five orders. Comparisons of these peptides enabled us to extract the consensus motif C-x(2)-G-x(4,6)-G-x(1,2)-C-[KR] (Matsumoto et al., 2012). More recently, this motif has been found in *Locusta migratoria* and *Schistocerca gregaria* GBPs (Duressa et al., 2015). Here, we describe the phylogenetic relationship derived from precursor protein sequences of all known members of the GBP family and GBP orthologs which were identified by homology searches (**Figure 1**). It is interesting that the GBP motif shares a significant similarity with the portion of the mammalian epidermal growth factor (EGF) motif (**Figure 2**) that forms the C-terminal region, in which Arg41 and Leu47 have been reported to be crucial for binding to the EGF receptor (Ogiso et al., 2002). NMR analysis demonstrated that the GBP motif core structure (residues 7–22) is predicted to show an EGF-like fold stabilized by a disulfide bond and a short ß-hairpin turn (Aizawa et al., 1999). This characteristic tertiary structure has been reported to be common in lepidopteran GBP orthologs which had been previously referred as to "ENF-peptide" that was named after the consensus N-terminal amino acid sequence (Volkman et al., 1999; Yu et al., 1999).

Although several other insect cytokines, such as Spätzle (DeLotto and DeLotto, 1998; Weber et al., 2003), Unpaired (Zeidler et al., 1999; Karsten et al., 2002; Yang et al., 2015), and Eiger (Moreno et al., 2002), have been reported, most of them were identified by searching for *Drosophila* orthologs of human cytokines. Therefore, GBP is unique in that following its original discovery in the armyworm, and it has since been identified in many other insect species, but no human ortholog has been found yet (Vanha-Aho et al., 2016). It is therefore particularly intriguing that *Drosophila melanogaster* (*Dm*) GBP exhibits some sequence similarity with human defensin BD2, a member of the immunomodulatory ß-defensin family, that can also regulate cell proliferation; BD2 is small, cationic peptides produced by specific proteolytic processing just like *Dm*GBP (Shafee et al., 2017). Furthermore, both the GBP and defensin families recruit the inositol phosphate (IP)/Ca^{2+} signaling cascade to serve their biological actions in common (Niyonsaba et al., 2007; Ninomiya et al., 2008; Zhou et al., 2012; Tsuzuki et al., 2014).

DROSOPHILA GBP

Following on from the identification of *Dm*GBP, three major developments have been made concerning its functions and signaling mechanisms as follows. First, *Dm*GBP was demonstrated to elevate anti-microbial peptide (AMP) expression independently of the canonical receptors that at that time were known to be associated with the inflammatory pathways mediated by Toll- and IMD-dependent pathways (Tsuzuki et al., 2012). Instead, the adaptor protein IMD is recruited to an activated *Dm*GBP receptor which thereby activates JNK. This signaling pathway stimulates expression of a unique set of AMP genes, mainly *Mechnikowin* and *Diptericin.* The *Dm*GBP-dependent *AMP* expression occurs not only in larvae infected with pathogens but also in larvae exposed to noninfectious stress such as high/low temperatures or mechanical perturbation; thus, GBP has more general roles in maintaining insect homeostasis.

Second, it was demonstrated that *Dm*GBP activates an IP/Ca^{2+} signaling cascade that dictates the timing and the intensity of the separate cellular and humoral components of the innate immune response which, moreover, are reciprocally regulated (Tsuzuki et al., 2014). *Dm*GBP protects against pathogens by activating cellular defense program (phagocytosis and encapsulation), while inhibiting humoral pathways (production and release of AMPs), through an IP/Ca^{2+} signaling-mediated activation of a receptor-regulated kinase cascade (the PVR/ERK pathway).

Third, by screening a dsRNA library that targets genes encoding membrane proteins, the *Dm*GBP receptor has been determined to be the G-protein-coupled receptor *Methuselah-like 10* (*Mthl10*) (Sung et al., 2017). Knockdown of *Mthl10* by RNAi resulted in increased mortality upon bacterial infection and impaired adaptation to an environmental stress such as cold temperature.

It was recently reported that *Dm*GBP regulates the release of insulin-like peptides (ILPs) from the brain depending on nutrient levels in the hemolymph through target of rapamycin (TOR) in *Drosophila* (Koyama and Mirth, 2016). Thus, it was investigated if the GBP elicited those effects by acting through Mthl10. *Mthl10* was found to be expressed in ILP-producing cells of the brain and *Mthl10* knockdown decreased ILP expression (Sung et al., 2017). *Mthl10* knockdown was also demonstrated to be associated with

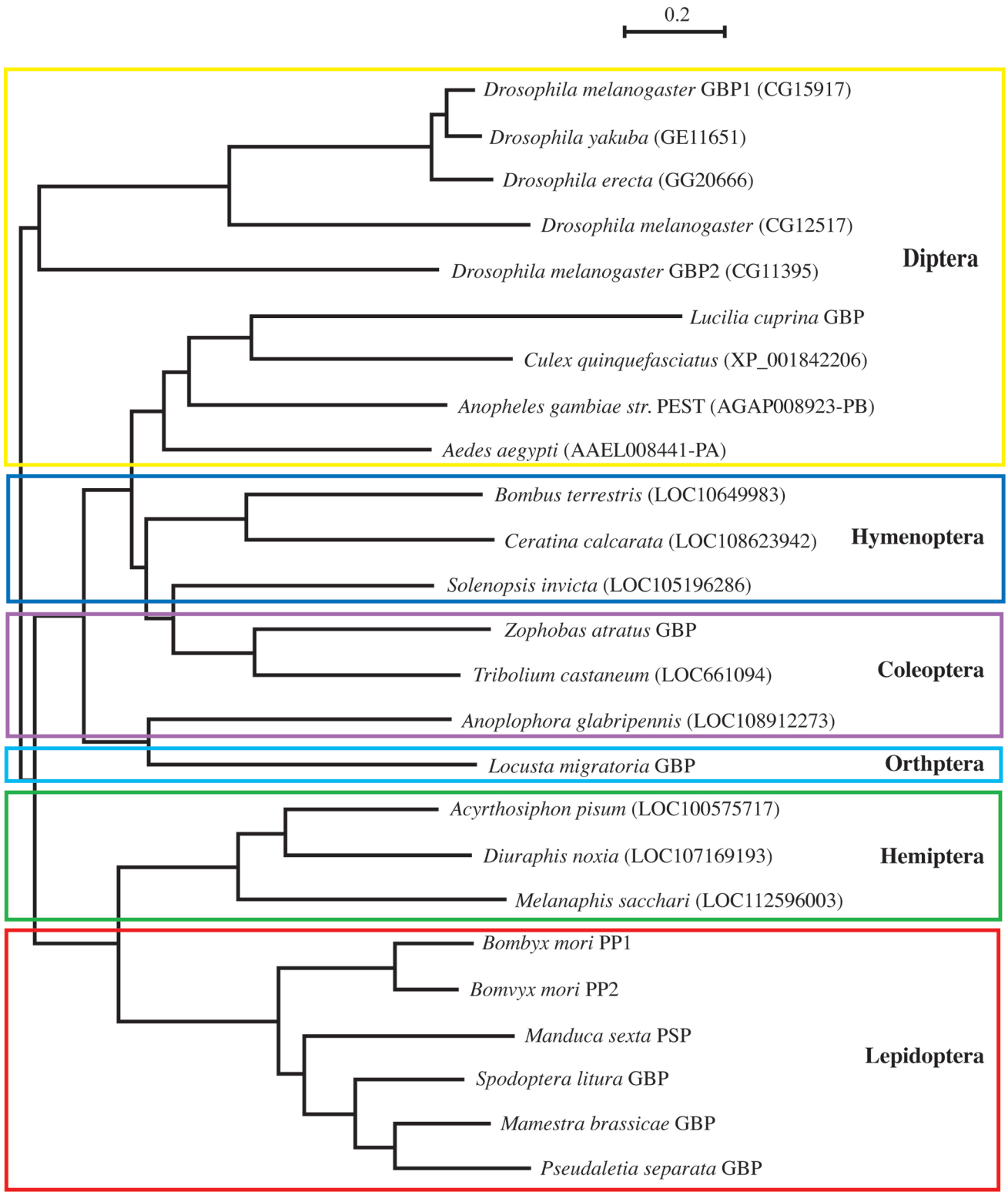

FIGURE 1 | A phylogenetic tree derived from precursor polypeptide sequences of GBP and GBP-like gene family spanning six orders of insects by using the neighbor-joining method with protein-Poisson distances (Saitou and Nei, 1987). The following sequences were identified on database of the NCBI/Blast: *Diuraphis noxia* LOC107169193 (XP_015374346.1), *Melanaphis sacchari* LOC112596003 (XP_025197225.1), *Solenopsis invicta* LOC105196286 (XP_011160410), *Ceratina calcarata* LOC108623942 (XP_017878339), and *Anoplophora glabripennis* LOC108912273 (XP_018572983). *Locusta migratoria* GBP was reported by Durressa et al. (Duressa et al., 2015). Other peptide sequences are in the prior report (Matsumoto et al., 2012). PP: paralytic peptide and PSP: plasmatocyte spreading peptide. Scale bar means a number of amino acid substitution per site.

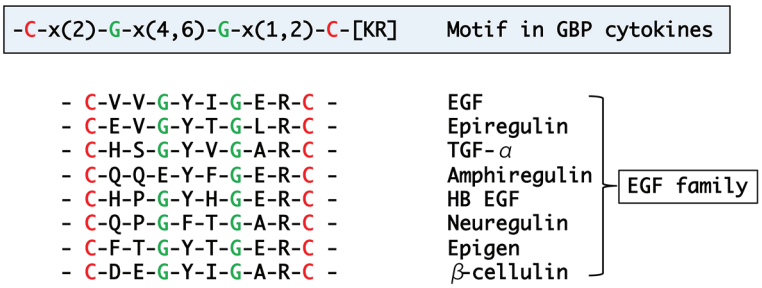

FIGURE 2 | A motif found in the active peptide regions of GBP/GBP-like genes and alignment of mammalian EGF peptide family.

increased longevity of flies, while *DmGBP* overexpression shortened lifespans. Furthermore, the GBP-induced shorter-lived phenotype was not observed in a strain with simultaneous knockdown of *Mthl10*. These observations provided solid evidence that Mthl10-mediated integration of various immunological and metabolic properties of *Dm*GBP is essential to maintain health and homeostasis that are critical for normal lifespan in insects.

GBP SIGNALING AND ITS REGULATION

In mammals, the cytokine TNF triggers the production of proinflammatory cytokines such as IL-1ß and IL-6 (Cunha et al., 1991; Lorenzetti et al., 2002). Furthermore, IL-6 expression has been demonstrated to be induced by IL-1ß in epithelial cells (Moon et al., 2000; Khan et al., 2014). Another insect cytokine, stress-responsive peptide (SRP), was recently identified; its expression is enhanced by *Ms*GBP in the armyworm (Yamaguchi et al., 2012; Matsumura et al., 2018). Physiological functions of SRP are similar to those of *Ms*GBP. For example, both *Ms*GBP and SRP showed larval growth retardation when they are injected into early last instar larvae. Although *Ms*GBP elicits a slightly stronger growth inhibitory effect than SRP, co-injection of both peptides has a greater effect than that due to *Ms*GBP alone (Yamaguchi et al., 2012; Matsumura et al., 2018). The negative impact on larval growth seems to be mainly due to the *Ms*GBP and/or SRP-induced decrease in larval feeding activities: co-injection of both cytokines caused a slightly more severe reduction in appetite than injection of each individual factor alone. Similar effects by cytokines have been reported in mouse IL-1ß and IL-6: both cytokines synergistically enhanced STAT3/NF-κB-dependent gene expression in the mouse liver during the acute inflammation phase (Goldstein et al., 2017). It might be worth investigating the functional parallelism between *Ms*GBP—SRP and IL-1ß—IL-6 to clarify evolutional feature of cytokine functions. Furthermore, it was demonstrated that *Ms*GBP does not elevate *SRP* expression when injected with SRP into the armyworm larvae (Matsumura et al., 2018), indicating that *Ms*GBP cannot activate *SRP* expression as long as SRP is present in the hemolymph above a threshold concentration. This might be analogous to the fact that an excessive immune response, through strong stress, stimulates a negative feedback mechanism in mammals, which protects the organism from an overproduction of proinflammatory cytokines (Elenkov and Chrousos, 2002).

Another mode of GBP signaling regulation is the control of its hemolymph concentrations by GBP-binding protein (GBP-BP) that functions as a scavenger of *Ms*GBP in the armyworm (Matsumoto et al., 2003). As mentioned above, *Dm*GBP initially tends to prioritize neutralization of an invading pathogen by activating cellular defense reactions (spreading, phagocytosis, and encapsulation). *Ms*GBP regulates not only immune active plasmatocytes and granulocytes in Lepidoptera (Lavine and Strand, 2002), but also another hemocyte class, the oenocytoids. The latter cells possess densely packed GBP-BP molecules, which are released by *Ms*GBP-induced cell lysis that occurs after the cellular immune responses of plasmatocytes (Matsumoto et al., 2003). Therefore, *Ms*GBP has temporally dependent actions, first to stimulate the immune cells and afterwards to silence its own action by releasing GBP-BP through specific hemolysis of oenocytoids. Although an equivalent GBP-BP has not been identified in *Drosophila,* orthologous genes and proteins have been identified in several Lepidoptera such as *Manduca sexta* (Chevignon et al., 2015), *Bombyx mori* (Hu et al., 2006; Sasibhushan et al., 2013), *Spodoptera exigua* (Park and Kim, 2012), *Spodoptera frugiperda* (Barat-Houari et al., 2006), and *Helicoverpa armigera* (Shelby and Popham, 2009). Bacterial and viral infection has been reported to enhance expression of GBP-BP genes in the hemocytes of some lepidopteran larvae, which supports the proposed immunological functional role of this protein. Moreover, expression of GBP-BP is dependent on the dependent stage of the insect and is enhanced by 20-hydroxyecdysone (20E), which together suggests that there are other consequences for the interaction of GBP with GBP-BP (Zhuo et al., 2018). For example, GBP and its binding protein may exert metabolic regulation during metamorphosis; down-regulation of metabolic levels by clearance of hemolymph GBP by GBP-BP would help the normal process of metamorphosis because it is well known that insects become inactive during metamorphosis. Furthermore, it has been shown that there are sharp GBP peaks in the hemolymph during each larval molt (Ohnishi et al., 1995). It is possible that GBP-BP contributes toward purging hemolymph GBP after the shut-off of its gene expression, which could make the sharp GBP peaks during molt periods.

FUTURE INVESTIGATIONS OF GBP SIGNALING

There remain many important questions regarding GBP multifunctionality and their regulation. For example, it has been demonstrated that GBP serves its immunological and metabolic functions as described above. Furthermore, GBP functions as a cell growth factor (Hayakawa and Ohnishi, 1998; Matsumoto et al., 2012). It has been reported that *Ms*GBP acts as a bipolar growth regulator: high concentrations (over several 10 pmol/ml) suppress larval growth but low concentrations (several pmol/ml) enhance larval growth and cell proliferation (Hayakawa and Ohnishi, 1998). In fact, several pmol/ml of *Ms*GBP enhances proliferation of human keratinocytes and of SF-9 insect cells in a manner similar to mammalian EGF (Hayakawa and Ohnishi, 1998). It is not yet known if Mthl10 contributes to *Dm*GBP-dependent cell proliferation. Indeed, based on the prior results obtained by structural (Aizawa et al., 1999) and kinetic studies (Hayakawa and Ohnishi, 1998; Ohnishi et al., 2001), it is reasonable to expect that stimulation of cell growth by GBP requires another type of the receptor similar to the EGF family of receptor tyrosine kinases. The speculation that GBP could activate multiple receptor types has arisen from the demonstration that different minimal peptide sequences of *Ms*GBP are required for cell growth and cellular immune activities: residues 2–23 in GBP are required for the former activity and 1–22 in GBP for the latter (Aizawa et al., 2001).

When *Dm*GBP (CG15917) was first identified, four other *Drosophila* genes encoding the proGBP-like peptide were also found: CG11395, CG12517, CG14069, and CG17244. Koyama and Mirth recently found that the CG11397 gene product regulated the release of ILPs from the brain in the similar manner of GBP (CG15917) and they named CG15917 and CG11397 for GBP1 and GBP2, respectively (Koyama and Mirth, 2016). The role of these two *Dm*GBPs in metabolic regulation has been demonstrated, but it has not yet been checked whether GBP2 also shares similar immune regulatory functions with GBP1. Moreover, it remains to be seen if the other candidate genes described above (CG12517, CG14069, and CG17244) will turn out to expand the functionality of the *Dm*GBP family.

CONCLUSIONS

Although the multiple functionalities of *Ms*GBP and *Dm*GBP have been clearly demonstrated, it remains unclear to what extent the GBP-signaling pathways and functionalities are conserved in other insects. For example, it is not yet known if *Mthl10* orthologous gene occurs in the armyworm. Moreover, SRP and GBP-BP have been examined only in the armyworm. It will be important to identify all these essential components for GBP-signaling function and regulation in broad insect species, so as to identify species-, development-, and stage-specific expression of such components. Elucidating commonality and difference of such GBP-associated factors in insects may hint at the conservation of some of these important homeostatic mechanisms in mammals.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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