Molecular Immunology 109 (2019) 88–98

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

The prophenoloxidase system in *Drosophila* participates in the anti-nematode immune response

Dustin Cooper, Caitlin Wuebbolt, Christa Heryanto, Ioannis Eleftherianos*

Infection and Innate Immunity Lab, Department of Biological Sciences, The George Washington University, Washington DC, USA

ARTICLE INFO

Keywords:
Prophenoloxidase
Parasitic nematode
Innate immunity
Drosophila
Steinernema

ABSTRACT

Drosophila melanogaster relies on an evolutionarily conserved innate immune system to protect itself from potentially deadly pathogens. One of the earliest pathways activated after injury or infection is the melanization pathway, which is responsible for synthesizing and depositing melanin at the site of injury, or onto invading microbes. Three genes, *PPO1-3,* encoding prophenoloxidase (PPO), an inactive precursor of phenoloxidase (PO), are responsible for the production of melanin after their activation via immune challenge. One pathogen capable of infecting *D. melanogaster* are entomopathogenic nematodes. *Steinernema carpocapsae* nematodes exist in a mutualistic relationship with *Xenorhabdus nematophila* bacteria and are an important biological control agent for controlling insect pests. The nematode-bacteria complex (symbiotic nematodes) can be separated, creating "axenic" nematodes, devoid of their associated bacteria, which are still capable of infecting and killing *D. melanogaster*. In order to investigate how the *D. melanogaster* melanization pathway contributes to the anti-nematode immune response, symbiotic and axenic *S. carpocapsae* were used to study *D. melanogaster* survival, *PPO* gene expression, and activation of PPO to PO. Our research suggests that the expression of all three *D. melanogaster PPO* genes contributes to survival, however only *PPO1* or *PPO3* appear to be up-regulated during axenic or symbiotic nematode infection. Additionally, we present data suggesting that a complex regulatory system exists between PPOs, potentially allowing for the compensation of PPOs by one another. Further, we found that axenic nematode infection leads to higher levels of PO, suggesting that *X. nematophila* suppresses this activation. We also report for the first time the differentiation of lamellocytes, a specialized type of hemocytes in *D. melanogaster*, in response to symbiotic *S. carpocapsae* nematode infection. Our results suggest an important role played by the melanization pathway in response to nematode infection, and demonstrate how this response can be manipulated by *S. carpocapsae* nematodes and their mutualistic *X. nematophila* bacteria.

1. Introduction

Like all insects, the fruit fly *Drosophila melanogaster* is under the constant threat of injury or infection. In order to protect itself from potentially deadly pathogens, it must rely on the evolutionarily conserved innate immune system to clear invading microbes. The *D. melanogaster* innate immune system comprises a number of immune pathways, which rapidly and efficiently protect the host in the event of microbial challenge (Buchon et al., 2014; Ferrandon et al., 2007). One of the earliest pathways activated upon injury or infection is the melanization pathway, which leads to the synthesis and deposition of melanin at openings in the cuticle to prevent loss of hemolymph, and to restrict the entry of microbes into the insect body (Eleftherianos and Revenis, 2011; Tang, 2009). During microbial infection, melanin is also deposited on and around the invading microbe to promote the encapsulation response. Reactive oxygen species generated as a by-product of melanin synthesis are then responsible for killing the immobilized microbe (Kim and Lee, 2014).

The melanization pathway begins with the recognition of microbes via pattern recognition receptors, which trigger the activation of a serine protease cascade. This cascade ends in the activation of prophenoloxidase-activating enzyme (PPOAE), which is negatively regulated by serpin 27a (De Gregorio et al., 2002). PPOAE is responsible for the cleavage of the inactive prophenoloxidase (PPO) zymogen into the active phenoloxidase (PO) enzyme. PO mediates the oxidation of mono- and diphenols to quinones, which then polymerize to form melanin (Loof et al., 2011). Reactive oxygen species are generated during this conversion. The entire reaction is regulated by a number of serpins in

* Corresponding author at: Department of Biological Sciences, The George Washington University Science and Engineering Hall, 800 22ndStreet NW, Washington DC, 20052, USA.

E-mail address: ioannise@gwu.edu (I. Eleftherianos).

https://doi.org/10.1016/j.molimm.2019.03.008
Received 5 September 2018; Received in revised form 19 March 2019; Accepted 19 March 2019
Available online 22 March 2019

order to prevent erroneous melanin synthesis (Reichhart, 2005; Veillard et al., 2016).

In *D. melanogaster*, PPO is encoded by three genes; *PPO1*, *PPO2*, and *PPO3*. *PPO1* and *PPO2* are each pro-enzymes, activated differentially depending on the agent triggering the initial immune response, while *PPO3* is probably produced in an active form (Tang, 2009). Both PPO1 and PPO2 arise from crystal cells, specialized hemocytes which make up ˜5% of the hemocyte population (Honti, 2013). Crystal cells produce and release PPO1 into the circulating hemolymph to provide rapid melanization upon infection or injury, while PPO2 is produced and stored in the crystals of crystal cells for late-phase melanization (Bingeli, 2014). PPO3 is believed to be produced only in lamellocytes, another lineage of specialized hemocyte, which differentiate in response to parasitic wasp infection (Dudzic, 2015). Lamellocytes are responsible for adhering to, engulfing, and ultimately melanizing parasitic wasp eggs through the production of PPO3. PPO3 is likely produced in its active form, as it does not require cleavage to begin converting mono- and diphenols. This may be because lamellocytes already require an infection signal to begin the differentiation process, and further signaling to activate PPO3 would therefore be redundant. It has also been proposed that PPOs are capable of working in pairs: PPO1 and PPO2 cooperate to contain microbial infections, while PPO2 and PPO3 act to deposit melanin upon and encapsulate large non-self objects, such as parasitoid wasp eggs (Dudzic, 2015). *D. melanogaster* is a convenient genetic model, and strains carrying loss-of-function mutations in one or more *PPO* genes have already been generated (Binggeli et al., 2014; Dudzic et al., 2015).

Despite the importance of the PPO system in antimicrobial and anti-parasitoid wasp immunity, little is known about the specific roles of each PPO in the immune response of *D. melanogaster* to nematode infection. The parasitic nematode *Steinernema carpocapsae* is capable of infecting and killing numerous species of insects, including *D. melanogaster* (Dillman et al., 2015; Peña, 2015; Yadav et al., 2018). During infection of *D. melanogaster* larvae, *S. carpocapsae* nematodes and the mutualistic *X. nematophila* bacteria they harbor induce distinct immune responses such as the expression of a suite of antimicrobial peptides and the activation of the melanization response (Goodrich-Blair, 2007; Goodrich-Blair and Clarke, 2007). Determining the role of PPO in response to parasitic nematode infection requires that we differentiate between immune responses to the bacterial pathogen *X. nematophila*, and those specific to *S. carpocapsae* nematodes. In the lab, it is possible to separate the two partners of this mutualistic relationship, a process that generates axenic nematodes which are devoid of their associated bacteria, but still capable of successfully infecting and killing *D. melanogaster* larvae (Yadav et al., 2015). Previous studies demonstrated *S. carpocapsae* axenic nematode infection leads to a greater melanization response, suggesting that the mutualistic bacteria might be capable of suppressing melanization (Hwang et al., 2013; Peña et al., 2015). This idea is further supported by observations of decreased phenoloxidase activity in other species of insects during *X. nematophila* infection (Darsouei et al., 2017a; Eom et al., 2014). Also in *Manduca sexta*, infection with *Heterorhabditis* axenic nematodes failed to elicit high levels of PO activity in the hemolymph compared to infection with their mutualistic bacteria *Photorhabdus* (Eleftherianos et al., 2010).

Because of its similarity to the mammalian clotting system, the use of insect models to dissect innate immune mechanisms, such as the melanization response, may prove useful when examining conserved clotting factors for their role in mammalian immunity (Theopold et al., 2014). A homologue for the human transglutaminase clotting factor XIIIa has been identified in *D. melanogaster* (Lindgren et al., 2008; Theopold et al., 2002). Further, transglutaminases have been found associated with PO during the clot formation on the surface of pathogenic microbes, suggesting a role in pathogen clearance in insects (Wang et al., 2010). Parasitic nematodes constitute a major threat to human health, and so the use of entomopathogenic nematodes to study host anti-nematode immune mechanisms, such as the melanization response, may contribute to the development of novel means for the efficient control of important human parasitic nematode infections.

In this study, we investigated the involvement of each of the three *D. melanogaster PPO* genes in the innate immune response to *S. carpocapsae* symbiotic and axenic nematodes. We demonstrate that all three *PPOs* contribute to the survival of *D. melanogaster* larvae during parasitic nematode infection. This suggests a novel role, which we further investigate, for *PPO3;* previously only ascribed to the innate immune response to parasitoid wasp infection.

2. Materials and methods

2.1. Nematode cultures

Steinernema carpocapsae infective juveniles (IJs) were used to infect fourth instar *Galleria mellonella* larvae and were allowed to develop and multiply for ˜14 days on a moistened piece of filter paper placed in a petri dish. After several cycles of growth, *G. mellonella* corpses were transferred to a new piece of filter paper positioned above a water trap. Subsequently, *S. carpocapsae* IJs were allowed to emerge, and were then collected from the water traps and stored before use in experiments.

To generate axenic nematodes (worms lacking their mutualistic *Xenorhabdus nematophila* bacteria), *S. carpocapsae* were processed using a previously established protocol (Yadav et al., 2015).

2.2. Fly stocks

Flies were maintained at 25°C and 60% humidity in a 12:12 light:dark photoperiod, and fed a standardized cornmeal-soy diet supplemented with a few granules of active-dry yeast. All Δ*PPO* single-, double-, and triple-mutant stocks were acquired from the Lemaitre lab and have been described previously (Dudzic et al., 2015). *Leptopilina boulardi*-infested *D. melanogaster* larvae (w^{1118} strain) were obtained from the Govind lab. *Collier* $Col^1/CyO(tb)$ mutant flies were acquired from the Govind lab, and the Col^1 mutation has previously been described (Crozatier and Vincent, 1999). $\Delta Spn27A^1/CyO$ mutant flies were purchased from the Bloomington Stock Center (55,720) and have been previously described (De Gregorio et al., 2002). *Drosophila* w^{1118} flies were purchased from the Bloomington Stock Center and served as the background controls for all experiments.

2.3. Larval infection

Late second- to early third-instar *D. melanogaster* larvae were collected from vials, washed with sterile water, and kept on moistened filter paper until use.

S. carpocapsae symbiotic IJs were collected from stock flasks and prepared for infection by ˜15 s of centrifugation at 13,000 rpm in an accuSpin Micro 17 centrifuge to form a loose pellet. Supernatant was removed and replaced with fresh, sterile water. Nematodes were then counted and the concentration was adjusted to ˜1/µl. *S. carpocapsae* axenic nematodes were centrifuged as above, but were additionally washed with a 1% bleach solution for 20 s and rinsed with sterile water five times before being counted.

To infect *D. melanogaster* larvae with nematodes, 100 µl of 1.25% agarose were deposited into each well of a clear 96-well microplate. Once cooled and solidified, a single larva was added to each well along with 10 µl of the nematode suspension. Wells were then sealed with PCR film and two small holes for air transfer were made per well. Microplates were stored in a cool, dark cabinet while not being observed.

2.4. Survival assay

To assess the survival of *D. melanogaster* larvae to *S. carpocapsae* infection, 10 larvae of each Δ*PPO* mutant strain, together with w^{1118}

background strain individuals, were infected with either symbiotic or axenic nematodes, or treated with sterile water as negative control. Larvae were then observed every 8 h for a total of 70 h post infection under a Tritech™ stereoscope, and survival was recorded. Larvae that reached the pupal stage during the experiment were discounted from the assay. Survival assays were performed three times with two technical replicates for each experimental treatment, each involving 10 s- to early third-instar *D. melanogaster* larvae.

2.5. PPO gene expression

To determine relative expression of *PPO* genes after *S. carpocapsae* infection, *D. melanogaster* larvae were infected with nematodes, as previously described. At 6, 12, or 24 h post infection, four larvae per treatment were removed from the microplate well with a sterile pipette tip or paint brush and subsequently frozen in a 1.5 ml centrifuge tube at -84°C.

Total RNA was extracted using the TRIzol™ Reagent according to the manufacturer's protocol, and concentration was measured using a NanoDrop 2000c. Extracted RNA was then used in cDNA synthesis via reverse transcription using the iScript™ cDNA Synthesis Kit according to the manufacturer's protocol, in a Bio-Rad C1000 Touch Thermal Cycler. The amount of RNA used was standardized to 212.5 ng/µl during this step. All centrifugation steps were carried out at 4°C in an Eppendorf 5430 R centrifuge. The qRT-PCR was then performed using the Bio-Rad CFX96™ Real-Time System with iTaq™ Universal SYBR® Green Supermix and gene-specific primer pairs (Table 1). All samples were run in technical duplicates, and all experiments were performed three times.

2.6. Phenoloxidase activity assay

To measure levels of activated phenoloxidase (PO) in nematode infected *D. melanogaster*, larvae were infected with *S. carpocapsae* IJs as previously described and removed from the microplates at six hours post infection. PO activity was then measured as described before with slight modifications (Bingeli et al., 2014).

To extract hemolymph from infected *D. melanogaster*, ten larvae were bled into 20 µl of 2.5x protease inhibitor. Hemolymph was collected and added to 10 µl of additional protease inhibitor on ice. Samples were added to a Pierce® Spin Column and centrifuged at 4 °C and 13,000 rpm for 10 min. Protein concentrations were measured using a Synergy HTX Multi-Mode Reader and the Pierce™ BCA Protein Assay Kit. A sample volume of 40 µl, containing a mixture of 15 µg of protein, 5 mM $CaCl_2$, and 2.5x protease inhibitor, was added to 160 µl L-DOPA solution in phosphate buffer (pH 6.6) in a clear microplate well, and incubated at 29 °C for 20 min. PO activity was measured via kinetic reads at 29 °C at 492 nm for 60 min. The OD value of a blank control was subtracted from each averaged biological duplicate. Each experiment was performed three times with two technical replicates per experimental treatment.

2.7. Hemocyte staining and microscopy

To examine the presence of lamellocytes in hemolymph, hemocyte staining was performed. Ten larvae were infected with nematodes as described above and collected 27 h post infection. Larvae were washed in 75% EtOH and rinsed in 1x PBS, and then bled into 40 µl of 1x PBS on a poly-lysine coated microscope slide and allowed to air dry for 20 min. Slides were washed with 1x PBS between each of the following treatments. Hemocytes were fixed in 4% paraformaldehyde for 15 min, permeabilized in PBST (0.3% Triton in 1x PBS) for 20 min, and subsequently blocked in PBSTB (8% BSA in PBST) for 15 min. Hemocytes were incubated with 20 µl diluted phalloidin TRITC (1:100) for 20 min before a final wash in 1x PBS. Hemocytes were mounted and covered with ProLong® Gold Antifade reagent with DAPI and stored at 4°C until they could be viewed.

Stained hemocytes were viewed at 40x using an Olympus BX53 fluorescent microscope and an X-Cite® 120 LED *Boost* light source. DAPI and TRITC single-channel images were captured with an Olympus DP80 camera, and subsequently merged using the Olympus cellSens Standard imaging software.

Table 1
Primers and their sequences used in quantitative RT-PCR experiments.

Gene	Forward 5' - 3'	Reverse 5' - 3'
PPO1	CAACTGGCTTCGTTGAGTGA	CGGGCAGTTCCAATACAGTT
PPO2	CCCGCCTATACCGAGA	CGCACGTAGCCGAAAC
PPO3	GGCGAGCTGTTCTACT	GAGGATACGCCCTACTG
RP49	GATGACCATCCGCCCAGCA	CGGACCGACAGCTGCTTGGC

2.8. Statistical analysis

GraphPad Prism 7 was used to construct all graphs and figures, as well as to perform all statistical analysis. P values of < 0.05 were considered significant. Survival analysis was performed with a log-rank (mantel-cox) test. Gene expression and PO activity analyses were performed using ordinary one-way ANOVA and the unpaired two-tailed t-test.

3. Results

3.1. Drosophila melanogaster PPO1 and PPO3 genes are differentially regulated after parasitic nematode infection

Previous research has demonstrated a potential involvement of the prophenoloxidase cascade in the *D. melanogaster* anti-nematode innate immune response during infection with either symbiotic or axenic *S. carpocapsae* (Peña et al., 2015). While reported as being activated during nematode infection, it has not been determined which of the *PPO* genes, specifically, is contributing to this response. To assess the contribution of each *PPO* gene to the *D. melanogaster* innate immune response to nematode infection, we first examined the expression of each *PPO* gene in w^{1118} background control *D. melanogaster* larvae upon infection with axenic or symbiotic *S. carpocapsae* (Fig. 1 and S1). At 12 h post-infection, larvae infected with symbiotic *S. carpocapse* nematodes expressed significantly higher levels of *PPO1* than those infected with axenic *S. carpocapsae* (Fig. 1A). Axenic nematode-infected larvae expressed however, significantly higher levels of *PPO3* than their symbiotic nematode-infected counterparts (Fig. 1B). This suggests that both *PPO1* and *PPO3* may contribute to the *D. melanogaster* anti-nematode innate immune response, and likely play specialized roles during nematode infection. *PPO2* expression was also measured, although no significant differences were observed between axenic and symbiotic nematode-infected larvae (Fig. S1). While *PPO2* may still play an active role during nematode infection, it does not appear to be differentially regulated between these two forms of nematode infection. Together, these results suggest that *PPO1* and *PPO3*, but not *PPO2*, are either capable of acting specifically against, or they are capable of being inhibited by, some component of *S. carpocapsae* infection.

3.2. Drosophila melanogaster PPO genes contribute to the survival against parasitic nematode infection

To further assess the role played by each *PPO* gene during *S. carpocapsae* infection in *D. melanogaster*, we analyzed the survival of Δ*PPO* single-, double-, and triple-mutant larvae alongside that of the w^{1118} background controls. Survival was measured over the course of 70 h of infection with axenic (Fig. 2) or symbiotic (Fig. 3) *S. carpocapsae* nematodes. All Δ*PPO* single-mutant larvae displayed increased sensitivity

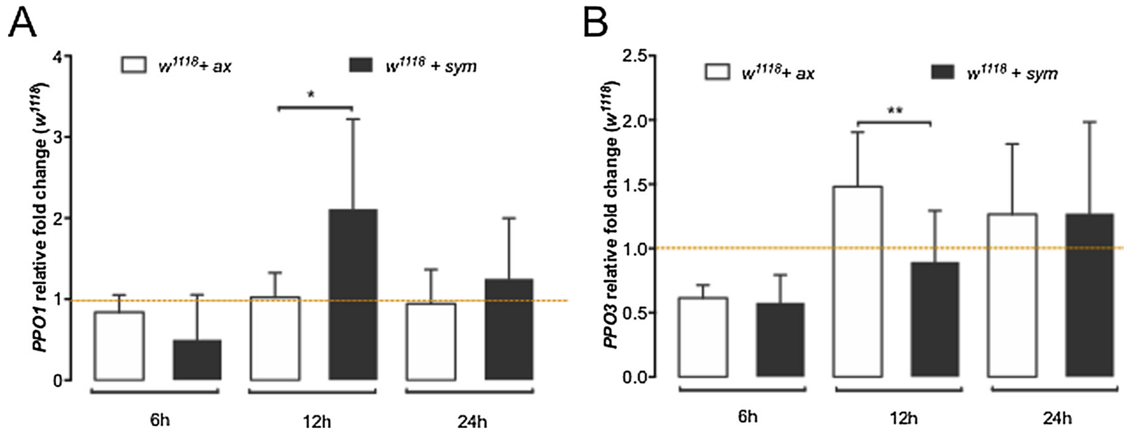

Fig. 1. Relative fold change of *PPO1* and *PPO3* in w^{1118} *Drosophila melanogaster* larvae after infection with *Steinernema carpocapsae* nematodes. At 12 h post-infection, *D. melanogaster* w^{1118} background control larvae infected with *S. carpocapsae* symbiotic (sym) nematodes showed significantly higher levels of *PPO1* transcripts (A) and significantly lower levels of *PPO3* transcripts (B) than larvae infected with *S. carpocapsae* axenic (ax) nematodes. No significant differences in *PPO1* or *PPO3* transcript levels were observed among axenic- and symbiotic-infected larvae at 6 or 12 h post-infection. Four Larvae were collected per treatment and gene expression was estimated by quantitative RT-PCR at 6, 12, and 24 h post infection. Only living larvae were selected for gene expression analysis. Means are shown for three independent experiments. Error bars display standard deviation. *, $p < 0.05$; **, $p < 0.01$.

towards infection with either axenic or symbiotic *S. carpocapsae* nematodes versus that of w^{1118} background control larvae (Figs. 2A and 3A). This suggests that all three *PPO* genes play a role in the survival of *D. melanogaster* to *S. carpocapsae* nematode infection. Next, in order to determine the relative contribution of *PPO* genes to the *D. melanogaster* survival response against parasitic nematodes, we compared the

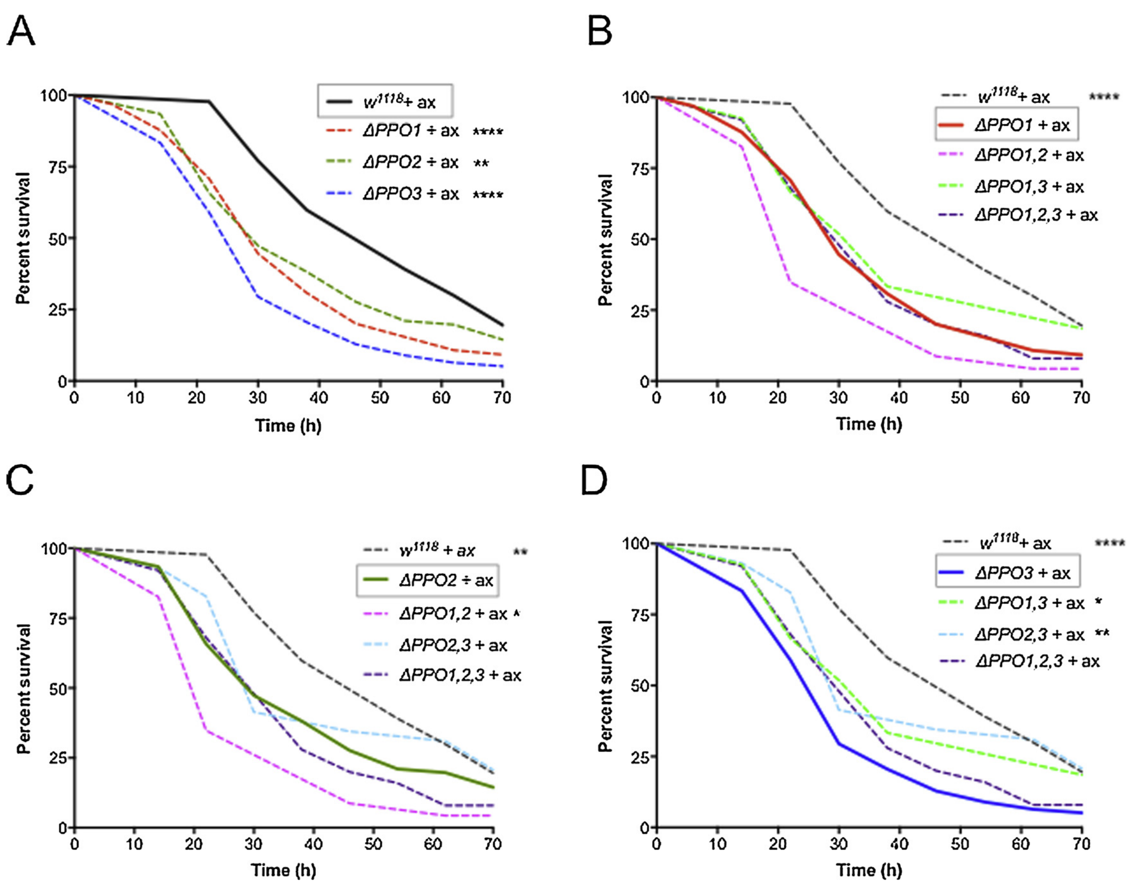

Fig. 2. Survival response of *Drosophila melanogaster* Δ*PPO* loss-of-function mutant larvae to *Steinernema carpocapsae* axenic nematode infection. Through 70 h of infection with *S. carpocapsae* axenic nematodes, *D. melanogaster* w^{1118} background control larvae survive significantly better than Δ*PPO1*, Δ*PPO2*, and Δ*PPO3* loss-of-function mutants (A). Under the same conditions up to 70 h post-infection, Δ*PPO1* mutants survive similarly to Δ*PPO1,2* and Δ*PPO1,3* double-mutants or Δ*PPO1,2,3* triple-mutants (B), Δ*PPO2* mutants survive significantly better than only Δ*PPO1,2* double-mutants (C), and Δ*PPO3* single-mutants are significantly more sensitive than Δ*PPO2,3* and Δ*PPO1,3* double-mutants (D). At least 10 *D. melanogaster* larvae from each of the single (Δ*PPO1*, Δ*PPO2*, Δ*PPO3*), double (Δ*PPO1,2*, Δ*PPO2,3*, Δ*PPO1,3*), and triple (Δ*PPO1,2,3*) mutants as well as the background control (w^{1118}) were individually infected with ˜10 *S. carpocapsae* axenic nematodes. Larvae were observed every 8 h for a total of 70 h. Pupated larvae were discounted from the experiment. Means are shown for three independent experiments. Error bars display standard error. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

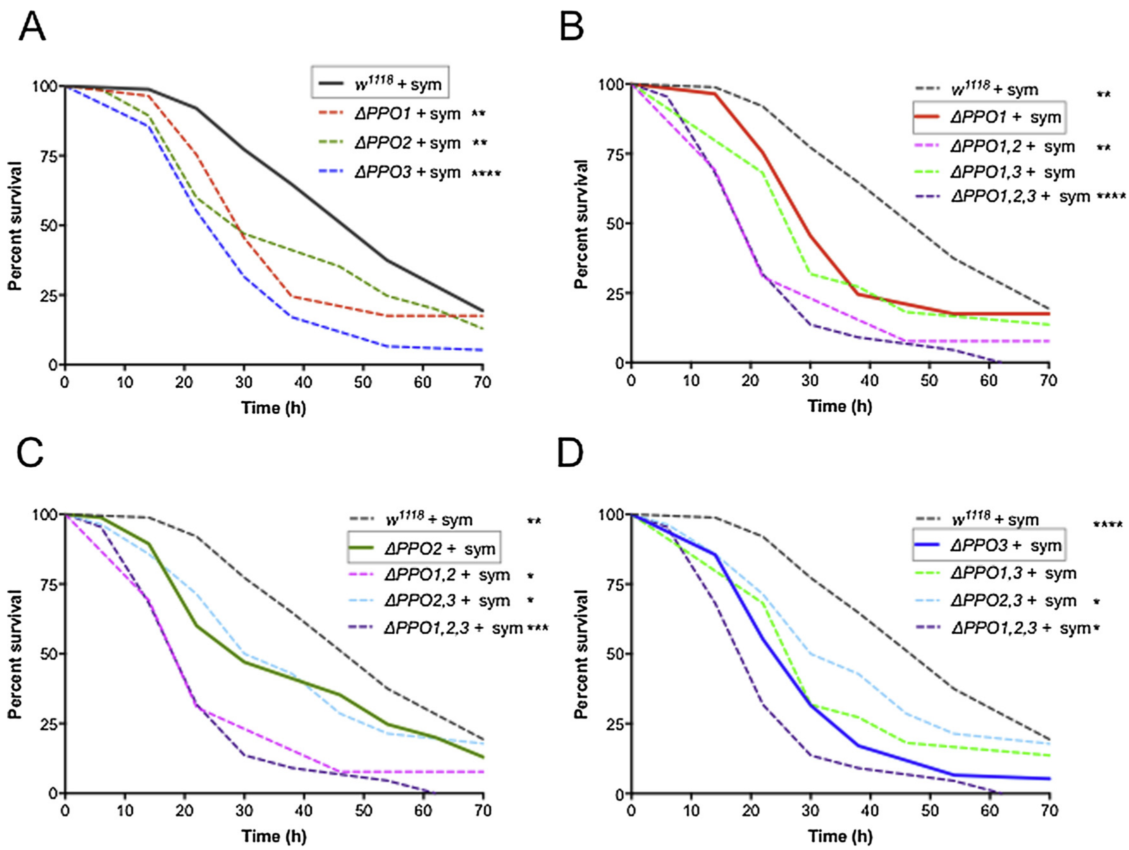

Fig. 3. Survival response of *Drosophila melanogaster* Δ*PPO* loss-of-function mutant larvae to *Steinernema carpocapsae* symbiotic nematode infection. Up to 70 h post-infection with *S. carpocapsae* symbiotic nematodes, w^{1118} background control larvae survive significantly better than Δ*PPO1*, Δ*PPO2*, and Δ*PPO3* loss-of-function mutants (A). Under the same conditions up to 70 h post-infection, Δ*PPO1* mutants survive significantly better than either Δ*PPO1,2* double- or Δ*PPO1,2,3* triple-mutant *D. melanogaster* larvae (B), Δ*PPO2* mutants survive significantly better than both Δ*PPO1,2* and Δ*PPO2,3* double-mutants as well Δ*PPO1,2,3* triple-mutants (C), and Δ*PPO3* mutants survive significantly better than Δ*PPO2,3* double-mutants and Δ*PPO1,2,3* triple mutants (D). At least 10 *D. melanogaster* larvae from each of the single (Δ*PPO1*, Δ*PPO2*, Δ*PPO3*), double (Δ*PPO1,2*, Δ*PPO2,3*, Δ*PPO1,3*), and triple (Δ*PPO1,2,3*) mutants as well as the background control (strain w^{1118}) were individually infected with ˜10 *S. carpocapsae* symbiotic nematodes. Larvae were observed every 8 h for a total of 70 h. Pupated larvae were discounted from the experiment. Means are shown for three independent experiments. Error bars display standard error. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

survival of each Δ*PPO* single-mutant to that of the Δ*PPO* double-mutants which shared a common mutation as well as the Δ*PPO1,2,3* triple-mutant. Significant differences in survival among these mutants would suggest which *PPO* genes are more important to the overall survival response.

After infection with axenic *S. carpocapsae* nematodes, Δ*PPO1 D. melanogaster* larvae survived similarly to the Δ*PPO1,2* and Δ*PPO1,3* double-mutants, as well as the Δ*PPO1,2,3* triple-mutant (Fig. 2B), suggesting that additional mutations do not significantly alter the survival response. Δ*PPO1,2* double-mutants, but not Δ*PPO2,3*, survived significantly worse than the Δ*PPO2* single-mutants (Fig. 2C). This suggests that *PPO1* contributes more strongly than *PPO3* to the *D. melanogaster* survival against axenic *S. carpocapsae.* Interestingly, the Δ*PPO1,2,3* triple-mutants survived similarly to all single-mutants. This may suggest a compensatory effect in the case of inactivation of all three *PPO* genes by another function of the *D. melanogaster* innate immune response. Both Δ*PPO1,3* and Δ*PPO2,3* double-mutants survived significantly better than the Δ*PPO3* single-mutants after infection with axenic *S. carpocapsae* (Fig. 2D). However, there was no statistical difference between the survival curves of Δ*PPO3* single-mutant larvae and the Δ*PPO1,2,3* triple-mutants. These results suggest that Δ*PPO3* is a particularly debilitating mutation, so much so that mutation of either remaining *PPO*, but not both, partially rescues *D. melanogaster* survival during axenic *S. carpocapsae* nematode infection.

After infection with symbiotic *S. carpocapsae* nematodes, the Δ*PPO1,2,3* triple-mutant larvae survived significantly worse than any Δ*PPO* single-mutant (Fig. 3B, C, D). This suggests that any compensatory effect seen against axenic *S. carpocapsae* in the absence of functional *PPO* genes is not active, or not sufficient to protect against the nematode-bacteria complex. During symbiotic nematode infection, Δ*PPO1,2* double-mutant larvae survived significantly worse than the individual Δ*PPO1* or Δ*PPO2* single-mutants (Fig. 3B, C). Conversely, Δ*PPO1,3* double-mutants survived no differently than either the Δ*PPO1* or Δ*PPO3* single-mutants (Fig. 3B, D). These results suggest that mutation of *PPO1* and *PPO2* together is significantly more debilitating than the mutation of *PPO1* and *PPO3* together. Additionally, Δ*PPO2,3* mutants survived significantly better than the Δ*PPO3* single-mutant, further purporting that *PPO1* and *PPO2* are capable of compensating for the loss of the other, but not for the loss of *PPO3* (Fig. 3D).

3.3. Drosophila melanogaster PPO1 is up-regulated during symbiotic Steinernema carpocapsae infection as well as upon the simultaneous absence of PPO2 and PPO3

Results from the survival experiments suggested a significant amount of interplay between *D. melanogaster PPO* genes. Previous research has also proposed that *D. melanogaster PPO* genes work in tandem with one another (Dudzic et al., 2015). In order to further investigate how these genes cooperate to contribute to the *D. melanogaster* anti-nematode innate immune response, we examined the expression of *PPO1* and *PPO3* in both the w^{1118} background control larvae and Δ*PPO* double-mutant larvae carrying mutations in the two *PPO* genes not

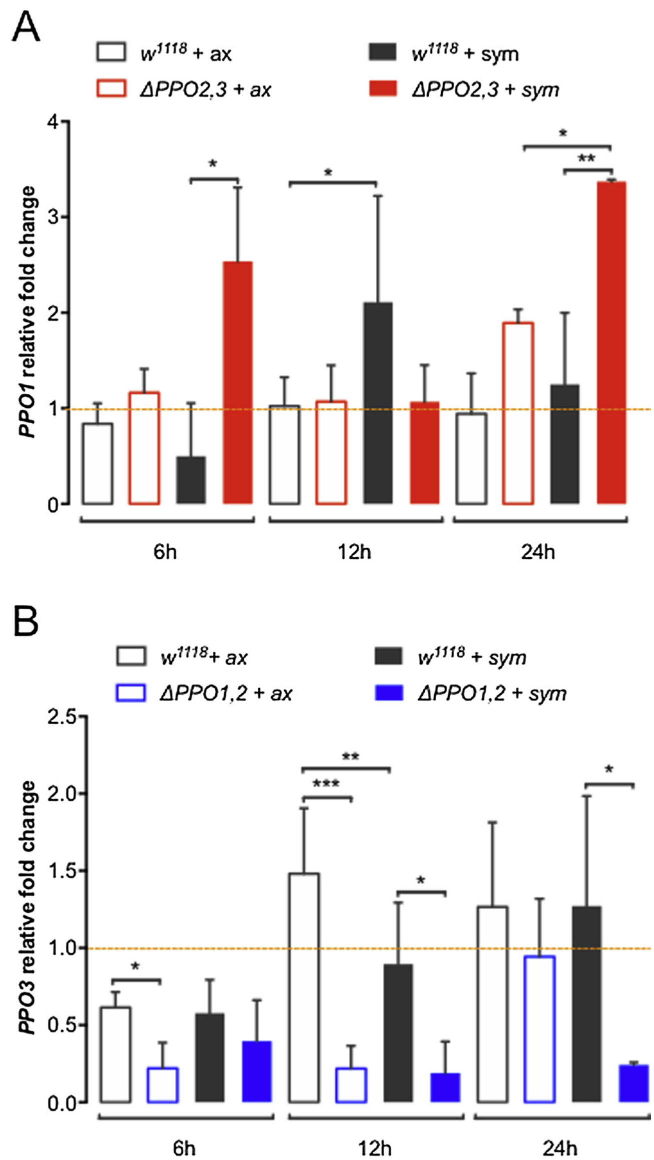

Fig. 4. Differential expression of *PPO1* and *PPO3* in *Drosophila melanogaster* Δ*PPO2,3* and Δ*PPO1,2* double-mutants during *S. carpocapsae* nematode infection. After infection with *S. carpocapsae* symbiotic (sym) nematodes, *PPO1* transcript levels in *D. melanogaster* Δ*PPO2,3* double-mutant larvae were significantly higher than those of w^{1118} background controls at 6 and 24 h. *PPO1* transcript levels were also significantly higher in Δ*PPO2,3* double-mutants at 24 h post-infection with symbiotic *S. carpocapsae* versus those infected with axenic (ax) nematodes. Symbiotic nematode infection led to significantly higher levels of *PPO1* than axenic nematode infection of w^{1118} background control larvae at 12 h (A). At 6 and 12 h post-infection with axenic nematodes, *PPO3* transcript levels were significantly higher in w^{1118} background control larvae when compared to Δ*PPO1,2* double-mutants. At 12 and 24 h post-infection with symbiotic nematodes, *PPO3* transcript levels were significantly higher in w^{1118} background control larvae when compared to Δ*PPO1,2* double-mutants. Axenic nematode infection led to significantly higher *PPO3* levels than symbiotic nematode infection in w^{1118} background controls at 12 h (B). Four larvae per treatment were collected and gene expression was estimated by quantitative RT-PCR at 6, 12, and 24 h post infection. Only living larvae were selected for gene expression analysis. Means are shown for three independent experiments. Error bars display standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

being measured.

At 6 and 24 h post-infection with symbiotic *S. carpocapsae* nematodes, *PPO1* was significantly up-regulated in Δ*PPO2,3* double-mutant larvae versus w^{1118} background control larvae (Fig. 4A). Further, we compared the expression of *PPO1* in *D. melanogaster* larvae infected with axenic nematodes and those infected with symbiotic nematodes. At 12 h post-infection, w^{1118} background control larvae infected with symbiotic *S. carpocapsae* expressed significantly more *PPO1* than those infected with axenic *S. carpocapsae*. At 24 h post-infection, Δ*PPO2,3* larvae infected with symbiotic nematodes expressed significantly more *PPO1* than those infected with axenic nematodes. These results suggest that *PPO1* is up-regulated significantly more during symbiotic *S. carpocapsae* infection than it is during axenic *S. carpocapsae* infection. Additionally, it appears that in the absence of the other two *PPO* genes, *PPO1* is up-regulated during infection with symbiotic *S. carpocapsae* nematodes.

At 6 and 12 h post-infection with axenic *S. carpocapsae* nematodes, *PPO3* was significantly up-regulated in w^{1118} background control larvae versus the Δ*PPO1,2* double-mutant larvae (Fig. 4B). At 12 and 24 h post-infection with symbiotic *S. carpocapsae*, *PPO3* was significantly up-regulated in w^{1118} background control larvae than in Δ*PPO1,2* double-mutant larvae. Further, at 12 h post-infection, w^{1118} background larvae expressed significantly more *PPO3* during axenic nematode infection than during symbiotic nematode infection. Taken together, these results suggest that *PPO3* is not up-regulated in the absence of both *PPO1* and *PPO2*.

3.4. *Steinernema carpocapsae* infection activates phenoloxidase in *Drosophila melanogaster*

To further investigate the involvement of *PPO* genes in the *D. melanogaster* anti-nematode immune response, we estimated PO activity in w^{1118} background strain larvae after 6 h of infection with axenic or symbiotic *S. carpocapsae*. Relative to water-treated *D. melanogaster* larvae, those infected with axenic or symbiotic nematodes demonstrated significantly increased PO activity (Fig. 5). While both axenic and symbiotic *S. carpocapsae* infections led to elevated PO activity, there also existed a significant difference between the two. *D. melanogaster* larvae infected with axenic *S. carpocapsae* demonstrated slightly

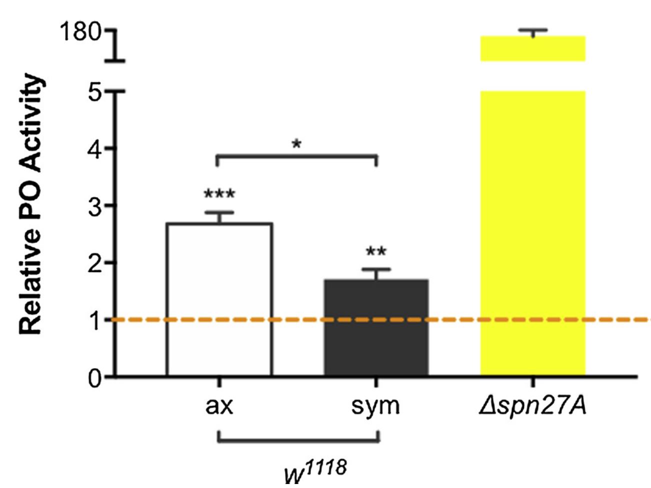

Fig. 5. Phenoloxidase (PO) activity in *Drosophila melanogaster* larvae after *Steinernema carpocapsae* nematode infection. Background control w^{1118} larvae infected with either *S. carpocapsae* symbiotic (sym) and axenic (ax) nematodes exhibited significantly higher levels of PO activity compared to water-treated larvae (orange dashed line, set to 1) at 6 h post infection. Larvae infected with axenic nematodes displayed significantly higher PO activity compared to those infected with symbiotic nematodes. Spontaneously melanizing Δ*Spn27A* mutant larvae were used as a positive control for PO activity. Ten larvae per treatment were used for hemolymph extraction. Only living larvae were selected for PO activity measurement. Means are shown for three independent experiments. Error bars display standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

increased PO activity versus those infected with symbiotic nematodes. PO activity of *S. carpocapsae*-infected w^{1118} larvae was compared to that of Δ*Spn27A* mutant larvae, which spontaneously activate PO, in order to ensure accurate measurement. These results suggest that infection with *S. carpocapsae* nematodes containing or lacking their mutualistic *X. nematophila* bacteria can induce PO activity in *D. melanogaster* larvae. The results also suggest that this induction is stronger among axenic *S. carpocapsae*-infected larvae than it is in symbiotic *S. carpocapsae*-infected larvae.

3.5. Infection with symbiotic, but not axenic Steinernema carpocapsae nematodes, leads to differentiation of lamellocytes in Drosophila melanogaster

Results from the survival experiments indicated that *PPO3* plays a role in the survival response of *D. melanogaster* larvae against axenic and symbiotic *S. carpocapsae* (Fig. 2A, 3A). Because *PPO3* is believed to only be produced in lamellocytes (Nam et al., 2008), a specialized type of hemocyte (Irving et al., 2005), we examined *D. melanogaster* hemocyte populations after infection with axenic or symbiotic *S. carpocapsae*. Hemocytes were collected from nematode-infected larvae and compared to those removed from *D. melanogaster* infested with *L. boulardi* parasitoid wasp eggs (Fig. 6B, 8D), as lamellocyte differentiation in response to this type of infection is well documented (Anderl et al., 2016). Hemocytes collected from *D. melanogaster* larvae treated with only water (Fig. 6A) or infected with axenic *S. carpocapsae* (Fig. 6C), did not contain any lamellocytes (Fig. 8D). Hemocytes collected from *D. melanogaster* infected with symbiotic *S. carpocapsae*, however, contained a large number of lamellocytes (Fig. 6D, 8D). These results suggest that lamellocytes differentiate in response to *S. carpocapsae* nematodes when present with their associated *X. nematophila* bacteria, but are either incapable of differentiating, or not required to differentiate, during infection with *S. carpocapsae* lacking *X. nematophila*.

3.6. Drosophila melanogaster PPO3 is expressed early during Steinernema carpocapsae axenic nematode infection, despite the lack of differentiated lamellocytes

While examining the hemocyte populations among nematode-infected *D. melanogaster* larvae, we noted the curious lack of lamellocytes during axenic *S. carpocapsae* nematode infection (Fig. 6C). This, combined with the survival results of Δ*PPO3* single-mutant larvae after axenic nematode infection (Fig. 2A) raises a new question: If lamellocytes are not differentiated during axenic nematode infection, and *PPO3* is produced in lamellocytes, why do Δ*PPO3* mutants display reduced survival during axenic nematode infection when compared to w^{1118} background control larvae during the same type of infection? To investigate this, we examined the expression of *PPO3* in *Collier* (*Col*) *D. melanogaster* mutant larvae, which are incapable of differentiating lamellocytes (Crozatier and Meister, 2007). At 6 h post-infection with axenic *S. carpocapsae* nematodes, increases in *PPO3* expression are significantly higher in *Col* mutant larvae than in w^{1118} background control larvae (Fig. 7A). This suggests that, despite the inability to differentiate lamellocytes, *D. melanogaster Col* mutants are capable of producing *PPO3* during axenic nematode infection. In order to confirm that lamellocytes were not differentiated among *Col* mutant larvae, we stained hemocytes collected from either axenic or symbiotic *S. carpocapsae*-infected *Col* mutant larvae (Fig. 8B, C).

Lamellocytes were not detected in any of those samples, demonstrating that these larvae were not capable of lamellocyte differentiation (Fig. 8D). In order to better display the effect of nematode infection on hemocyte composition, quantification of all visualized hemocytes was performed and displayed as a percentage of either plasmatocytes or lamellocytes of the total cells for all treatments of w^{1118} and *Col* mutant larvae (Fig. 8D). Plasmatocytes made up 100% of hemocytes in water-treated and axenic-infected w^{1118} larvae, as well as 100% of all *Col* mutant larvae examined. Lamellocytes were present among hemocytes in both *L. boulardi*-infected (~25% lamellocytes) and symbiotic nematode-infected w^{1118} background control larvae (~75% lamellocytes). The percent composition of hemocyte types found between these two treatments suggest that symbiotic *S. carpocapsae* nematodes may elicit a stronger lamellocyte differentiation response than that of *L. boulardi*

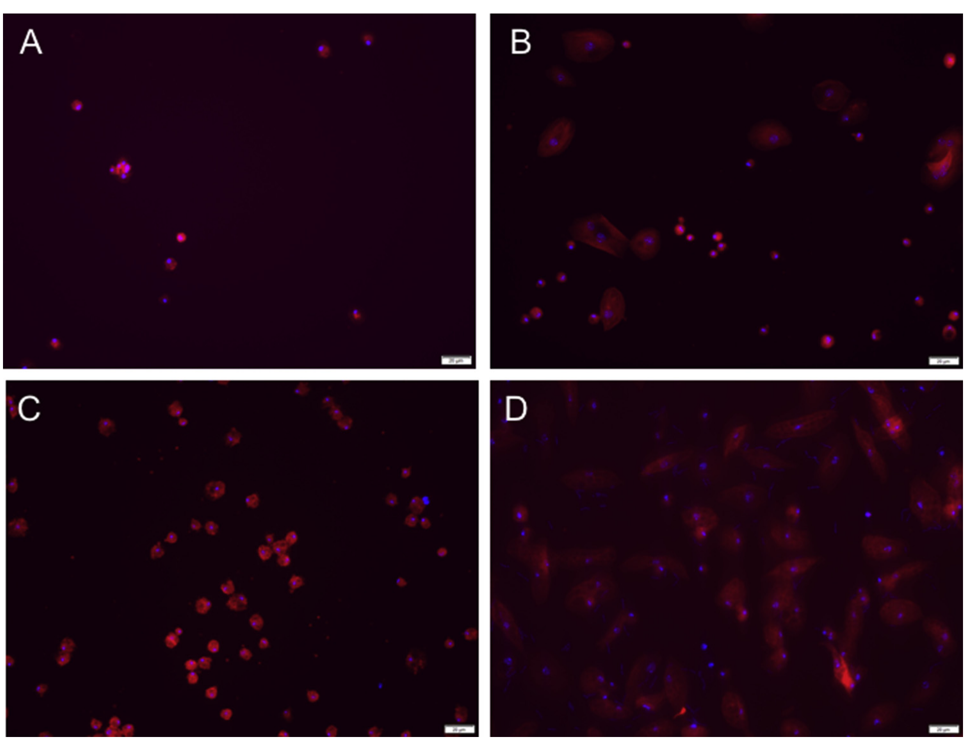

Fig. 6. Hemocyte types in *Drosophila melanogaster* larvae in response to infection with *Steinernema carpocapsae* nematodes. Hemocytes collected from *D. melanogaster* water-treated w^{1118} control larvae are composed entirely of plasmatocytes (A). Hemocytes collected from *Leptopilina boulardi* parasitoid-infested larvae contain both plasmatocytes and lamellocytes (B). Hemocytes collected from w^{1118} larvae infected with *S. carpocapsae* axenic (ax) nematodes contain only plasmatocytes (C). Hemocytes collected from w^{1118} larvae infected with symbiotic (sym) nematodes are predominantly composed of lamellocytes. Stained bacteria are present among the extracted hemocytes (D). Ten larvae per experimental treatment were bled 27 h post-infection. Hemocytes were collected only from living larvae. Representative fluorescent microscopy images are shown, magnified 40x, and are composite images of individual DAPI and TRITC-filter images.

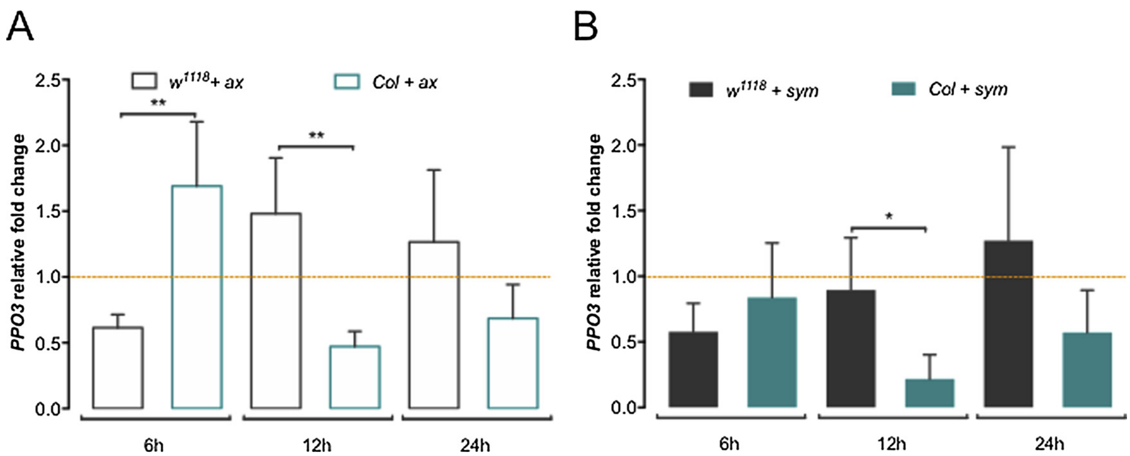

Fig. 7. Differential expression of *PPO3* between w^{1118} and Col^1 mutant *Drosophila melanogaster* larvae during *Steinernema carpocapsae* nematode infection. At 6 h post-infection with *S. carpocapsae* axenic (ax) nematodes, *PPO3* transcript levels are significantly higher in *D. melanogaster* Col^1 mutant larvae when compared to w^{1118} background controls. At 12 h post axenic nematode infection, *PPO3* transcript levels are significantly higher in the w^{1118} larvae compared to Col^1 mutants (A). Background control w^{1118} larvae infected with symbiotic (sym) nematodes display significantly higher levels of *PPO3* than the Col^1 mutants at 12 h (B). Four larvae per treatment were collected and gene expression was estimated by quantitative RT-PCR at 6, 12, and 24 h post nematode infection. Only living larvae were selected for gene expression analysis. Means are shown for three independent experiments. Error bars display standard deviation. *, $p < 0.05$; **, $p < 0.01$.

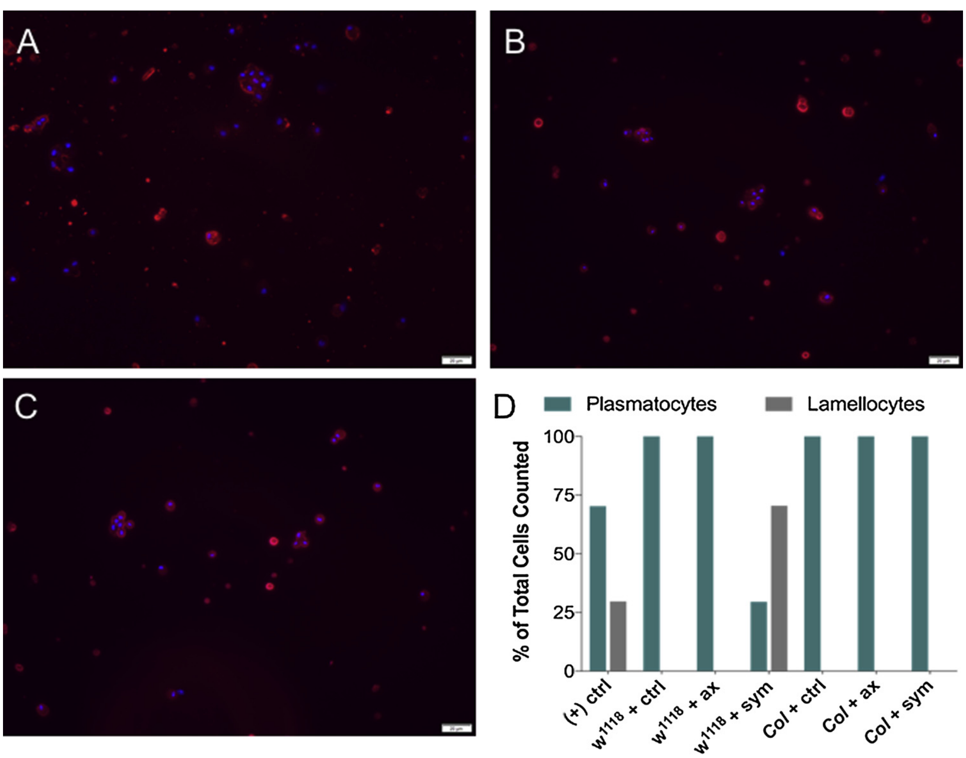

Fig. 8. Hemocyte types collected from Col^1 mutant *Drosophila melanogaster* after infection with *S. carpocapsae* axenic or symbiotic nematodes. Hemocytes collected from water-treated *D. melanogaster* Col^1 mutant larvae are composed entirely of plasmatocytes (A). Hemocytes collected from Col^1 mutant larvae infected with *S. carpocapsae* axenic (ax) nematodes (B) and symbiotic (sym) nematodes (C) contain only plasmatocytes. Percent-composition quantification of hemocytes collected from w^{1118} and Col^1 mutant larvae (D) is 100% plasmatocytes among uninfected (w^{1118} + ctrl) and axenic-infected (w^{1118} + ax) background larvae as well as all among uninfected and nematode-infected Col^1 mutant larvae (*Col* + ctrl and *Col* + ax, *Col* + sym, respectively). Lamellocytes make up just over 25% and nearly 75% of all visualized hemocytes collected from L. *boulardi*-infected (+ ctrl) and symbiotic *S. carpocapsae*-infected w^{1118} larvae (w^{1118} + sym), respectively. Ten larvae per treatment were bled approximately 27 h post-infection. Hemocytes were collected only from living larvae. Representative fluorescent microscopy images are shown, magnified 40x, and are composite images of individual DAPI and TRITC-filter images.

among *D. melanogaster* larvae. Further, we compared background levels of *PPO3* between *Col* mutants and w^{1118} background control larvae and found significantly higher background levels of *PPO3* among *Col* mutants (Fig. S2). These observations suggest that *PPO3* is potentially produced in alternative locations and is not limited to production in just lamellocytes during *S. carpocapsae* parasitic nematode infection.

4. Discussion

Steinernema carpocapsae nematodes are capable of infecting and killing *D. melanogaster* larvae with or without their associated bacteria (Yadav et al., 2015). Two *D. melanogaster PPO* genes (*PPO1* and *PPO2*) have been ascribed crucial roles in wound healing and microbe encapsulation (Binggeli et al., 2014), while a third (*PPO3*) has been implicated in the anti-parasite melanization response (Dudzic et al., 2015). *Drosophila melanogaster* is a convenient genetic model, and strains carrying loss-of-function mutations in one or more *PPO* genes have already been generated without compromising larval viability (Binggeli et al., 2014; Dudzic et al., 2015). Taken together with the ease of separating *S. carpocapsae* nematodes from their mutualistic *X. nematophila* bacteria, we have developed an elegant system, designed to further understand the interaction between entomopathogenic nematode parasites, their associated bacteria, and the participation of *D. melanogaster PPO* genes in the immune response against the nematode-bacteria complex.

Our results suggest that all three *PPO* genes contribute to the *D. melanogaster* larval survival against nematode infection, *S. carpocapsae* infection is capable of modifying the transcription of *PPO* genes in *D.*

melanogaster larvae, *PPO1* is strongly up-regulated in the absence of *PPO2* and *PPO3* during symbiotic nematode infection, *S. carpocapsae* infection leads to increased levels of active PO, and that the *S. carpocapsae-X. nematophila* complex is capable of inducing the differentiation of lamellocytes. Ultimately, these findings demonstrate a complex and key role for all three *D. melanogaster PPO* genes in the anti-nematode innate immune response. Previous studies in *M. sexta* larvae and *Heterorhabditis* have revealed that *PPO-2* was transcribed at undetectable levels in the fat body and PO activity remained low in the hemolymph after challenge with axenic nematodes suggesting that these parasites are not detected by the host PO-activating system or they actively produce molecules to suppress this early insect immune response (Eleftherianos et al., 2010).

In order to first validate our research endeavor, we examined changes in *D. melanogaster PPO* gene expression following *S. carpocapsae* infection that might suggest a role against parasitic nematodes. We explored expression of *PPO* genes at different time-points because their regulation appears highly time-dependent and can fluctuate substantially over the course of infection (Binggeli et al., 2014; Javar et al., 2015; Lu et al., 2014; Valadez-Lira et al., 2012). Significant changes in expression of *PPO1* and *PPO3* were observed 12 h post infection, which suggests that these *PPO* genes are somehow involved in the anti-nematode immune response. We also observed differences in *PPO* gene expression between axenic and symbiotic *S. carpocapsae* infection. This suggests that either the PPO system discriminates and acts differently when challenged by a complex of pathogens, or one member of the nematode-bacteria complex is capable of interfering or altering the *D. melanogaster* anti-nematode immune response. In some cases, we also found that *PPO* gene expression in larvae infected with axenic or symbiotic nematodes was reduced to the level of the water-treated controls. Again, as described before, these results indicate the ability of *S. carpocapsae* and *X. nematophila* to modify the host immune response during infection (Ahmed and Kim, 2018; Darsouei et al., 2017a, b; Hwang et al., 2013; Ji and Kim, 2004; Peña et al., 2015). Additionally, the lack of change in *PPO2* expression suggests that it is either 1) not necessary to protect against nematode infection, 2) is interfered with by either one or both members of the *S. carpocapsae-X. nematophila* complex, or 3) PPO2 found within crystal cells is sufficient and increased *PPO2* gene transcription would be unnecessary. We took advantage of the availability of *D. melanogaster* Δ*PPO* mutants in order to answer some of the questions raised here, as well as to better describe the series of immunological events taking place in *D. melanogaster* following axenic or symbiotic *S. carpocapsae* nematode infection.

In order to rapidly evaluate the role played by each *PPO* gene during *S. carpocapsae* infection of *D. melanogaster*, we analyzed the survival response of larvae during nematode infection. The availability of Δ*PPO* mutant *D. melanogaster* of every combination allowed us to explore this response with only a single *PPO* active (in *PPO* double-mutants), or how two *PPO* genes can work in tandem during *S. carpocapsae* infection. In addition, the Δ*PPO* triple-mutant allowed us to examine the survival response in the absence of any functional *PPO* genes. Further, the use of symbiotic as well as axenic *S. carpocapsae* nematodes allowed us to examine the effect that *X. nematophila* have on *D. melanogaster* survival.

Our results demonstrate that all three Δ*PPO* single-mutants are significantly more susceptible than the w^{1118} strain to both axenic and symbiotic *S. carpocapsae* infection. This suggests that all three PPOs are employed, directly or indirectly, at some stage during the course of *S. carpocapsae* infection, and their absence is detrimental to *D. melanogaster*. Interestingly, the *PPO1,2,3* triple-mutant was significantly more susceptible than any Δ*PPO* single-mutant during symbiotic *S. carpocapsae* infection, but not during axenic nematode infection. This result suggests that, in the absence of all three functional *PPO* genes, there may be another, possibly unknown, immune factor employed by *D. melanogaster* larvae to counter *S. carpocapsae*, but not *X. nematophila*, infection. Indeed, our recent RNAseq analysis of the *D. melanogaster* larval response to *S. carpocapsae* axenic or symbiotic nematodes revealed the significant up-regulation of several known immune-related genes as well as genes encoding chitin binding proteins, factors involved in lipid metabolic functions, and neuroactive ligand receptors (Yadav et al., 2017). The survival data of Δ*PPO* double-mutants suggest that a significant amount of cross-talk occurs between *PPO* genes.

During infection with *S. carpocapsae*, Δ*PPO* double-mutants survived significantly worse, or similarly to, Δ*PPO* single-mutants, except in the case of Δ*PPO3* single-mutants. Here, we observed Δ*PPO1,3* and Δ*PPO2,3* double-mutants surviving significantly *better* than the Δ*PPO3* single-mutants. This unexpected result may be explained through two assumptions. The first assumption is that *PPO1* and *PPO2* contribute the bulk of the activity against *S. carpocapsae*. Inactive PPO1 and PPO2 circulate in *D. melanogaster* larval hemolymph and is believed to be activated very soon after the detection of a wound or pathogen (Binggeli et al., 2014). The early anti-nematode activity supplied by these PPOs may interfere with *S. carpocapsae* infection such that further PO activity may be contributed; without them, invading nematodes are given ample time to further their infective process.

The second assumption is that for any given Δ*PPO* double-mutant, the third and only functional *PPO* is heavily over-expressed in order to compensate for the lack of the other two. In this way, any Δ*PPO* double-mutant capable of transcribing functional *PPO1* or *PPO2* would do so in excess, allowing it to survive better than the Δ*PPO3* single-mutant, despite the single-mutant's ability to transcribe both functional *PPO1* and *PPO2*. Conversely, the Δ*PPO1,2* double-mutant may be capable of over-expressing *PPO3*. However, because *PPO3* is probably only transcribed in lamellocytes (Tang, 2009), which require up to 30 h post-infection to differentiate and begin producing active PO (Andrl, 2016), the importance of this PPO is diminished, and even *PPO3* over-expression is not capable of compensating for the lack of both *PPO1* and *PPO2* together.

In order to further investigate the possibility of such a compensatory system, we analyzed expression of *PPO1* and *PPO3* among w^{1118} background controls and Δ*PPO* double-mutant *D. melanogaster* strains after infection with axenic or symbiotic *S. carpocapsae.* In fact, *PPO1* gene expression results suggest that *PPO1* is capable of being up-regulated in Δ*PPO1,2* double-mutants during symbiotic *S. carpocapsae* infection. It is possible that the level of *PPO1* up-regulation seen during symbiotic nematode infection is not necessary during axenic nematode infection, or that the nematode is capable of interfering with its transcription in the absence of its mutualistic partner. Opposite to these results, *PPO3* is only up-regulated in the w^{1118} control larvae during nematode infection. It is possible that PPO1 and PPO2 are the only PPOs capable of signaling, potentially as a result of their cleavage and activation, a step that PPO3 is thought to potentially not require (Tang, 2009).

Our survival and gene expression results suggest that *S. carpocapsae* infection is capable of moderately altering the regulation of *PPO* genes in *D. melanogaster* larvae, and that *PPO* genes play an important role in the anti-nematode immune response. PPO1 is produced and secreted into the hemolymph by crystal cells, while PPO2 is produced and kept stored within the crystal cells (Binggeli et al., 2014). It is possible that PPO1 and PPO2 are produced prior to the onset of infection and this "pre-produced" PPO is responsible for contributing the bulk of the anti-nematode immune response during nematode challenge. We then investigated whether or not *S. carpocapsae* nematode infection leads to an increase in levels of activated PO in *D. melanogaster* and found that both axenic and symbiotic nematode infection leads to significantly higher levels of activated PO in larvae of the background strain w^{1118}. Further, infection with axenic *S. carpocapsae* leads to significantly higher levels of active PO compared to levels after infection with symbiotic nematodes. These findings demonstrate that PPO is capable of being activated in *D. melanogaster* during *S. carpocapsae* infection, and that pre-produced PPO may contribute to the anti-nematode immune response. This also suggests, and supports previous findings, that *X. nematophila* bacteria may be capable of suppressing the *D. melanogaster* melanization response (Peña et al., 2015). Once again, due to the 6-hour time-point used to measure levels of activated PO, the probable lack of differentiated lamellocytes means that our results most likely do not include active PO contributed by *PPO3*. Further, if *PPO3* does in fact contribute to PO levels at this time-point, it is important to remember that PPO3 may be produced in an active form (Tang, 2009), and that these results would not accurately reflect levels of PO activated via the melanization cascade.

Our survival data suggest an important role played by *PPO3* in the *D. melanogaster* anti-nematode innate immune response. *PPO3* is thought to only be expressed in lamellocytes (Irving et al., 2005), a hemocyte type primarily differentiated after parasitic wasp infection (Honti et al., 2014). Until now, no previous research has demonstrated the differentiation of lamellocytes in *D. melanogaster* larvae following nematode infection. We visualized *D. melanogaster* hemocytes 27 h after axenic or symbiotic *S. carpocapsae* infection and found that upon symbiotic, but not axenic nematode infection, lamellocytes have differentiated and are present within *D. melanogaster* hemolymph. These results suggest that the *S. carpocapsae-X. nematophila* complex is sufficient enough to drive the differentiation of lamellocytes in vivo, and that lamellocytes are not required during axenic *S. carpocapsae* infection, or that the nematode alone is not sufficient to differentiate them. It is also possible that *S. carpocapsae* is capable of interfering with the process of lamellocyte differentiation, and that *X. nematophila*, in turn, interferes or inhibits this cellular response. The absence of lamellocytes in axenic nematode-infected larvae raises its own set of new questions. If *PPO3* is expressed exclusively in lamellocytes, and these are not differentiated in response to axenic *S. carpocapsae* infection, why then do we see a reduction in survival of *PPO3* single-mutant larvae in response to this type of nematode? It is possible that another source exists for the expression of *PPO3* that is not inhibited during axenic *S. carpocapsae* infection. Alternatively, the loss-of-function mutation of *PPO3* may affect another immune factor employed during the *D. melanogaster* anti-nematode innate immune response. A system whereby the cleavage and activation of circulating, "pre-produced" PPOs is capable of signaling the up-regulation of PPO3, expression of which only comes in conjunction with the parasite-driven differentiation of lamellocytes is quite elegant, and would provide an efficient tool at the disposal of the *D. melanogaster* innate immune system for dealing with complex parasites such as *S. carpocapsae* entomopathogenic nematodes. All these different possibilities will form the basis of future research to dissect the role of the PO response in the *D. melanogaster* anti-nematode immunity.

Because of its similarity to the mammalian clotting system, the use of insect models to dissect innate immune mechanisms and answer questions like those posed herein may prove useful when examining conserved clotting factors for their role in mammalian immunity (Theopold et al., 2014). A homologue for the human transglutaminase clotting factor XIIIa has been identified in *D. melanogaster* (Theopold et al., 2002). Further, transglutaminases have been found associated with PO during the clot formation on the surface of pathogenic microbes, suggesting a role in pathogen clearance in insects (Wang et al., 2010). Parasitic nematodes constitute a major threat to human health, and so the use of entomopathogenic nematodes as models to study host anti-nematode immune mechanisms, such as the melanization response, may contribute to the development of novel means for the efficient control of important human parasitic nematode infections.

Acknowledgements

We thank Dr. Bruno Lemaitre (Ecole Polytechnique Federale de Lausanne, Switzerland) for kindly sharing the *Drosophila melanogaster PPO* mutant stocks and Dr. Shubha Govind (The City College of New York, USA) for providing parasitized *D. melanogaster* larvae. We also thank Kyle Devine for maintaining the fly stocks and members of the Department of Biological Sciences at GWU for critical reading of the manuscript. Research in the Eleftherianos lab is funded through the National Institute of Allergy and Infectious Diseases.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.03.008.

References

Ahmed, S., Kim, Y., 2018. Differential immunosuppression by inhibiting PLA_2 affects virulence of *Xenorhabdus hominickii* and *Photorhabdus temperata* temperata. J. Invertebr. Pathol. 157, 136–146.

Anderl, I., Vesala, L., Ihalainen, T.O., Vanha-Aho, L.M., Andó, I., Rämet, M., Hultmark, D., 2016. Trans differentiation and proliferation in two distinct hemocyte Lineages in *Drosophila melanogaster* Larvae after Wasp Infection. PLoS Pathog. 12, e1005746.

Binggeli, O., Neyen, C., Poidevin, M., Lemaitre, B., 2014. Prophenoloxidase activation is required for survival to microbial infections in Drosophila. PLoS Pathog. 10, e1004067.

Buchon, N., Silverman, N., Cherry, S., 2014. Immunity in *Drosophila melanogaster*–from microbial recognition to whole-organism physiology. Nat. Rev. Immunol. 14, 796–810.

Crozatier, M., Meister, M., 2007. *Drosophila* haematopoiesis. Cell. Microbiol. 9, 1117–1126.

Crozatier, M., Vincent, A., 1999. Requirement for the *Drosophila* COE transcription factor *Collier* in formation of an embryonic muscle: transcriptional response to notch signalling. Development 126, 1495–1504.

Darsouei, R., Karimi, J., Dunphy, G.B., 2017a. The role of pilin protein of *Xenorhabdus nematophila* against immune defense reactions of insects. J. Insect Physiol. 101, 82–90.

Darsouei, R., Karimi, J., Ghadamyari, M., Hosseini, M., 2017b. Differential change patterns of main antimicrobial peptide genes during infection of entomopathogenic nematodes and their symbiotic bacteria. J. Parasitol. 103, 349–358.

De Gregorio, E., Han, S.J., Lee, W.J., Baek, M.J., Osaki, T., Kawabata, S., Lee, B.L., Iwanaga, S., Lemaitre, B., Brey, P.T., 2002. An immune-responsive Serpin regulates the melanization cascade in Drosophila. Dev. Cell 3, 581–592.

Dillman, A.R., Macchietto, M., Porter, C.F., Rogers, A., Williams, B., Antoshechkin, I., Lee, M.M., Goodwin, Z., Lu, X., Lewis, E.E., Goodrich-Blair, H., Stock, S.P., Adams, B.J., Sternberg, P.W., Mortazavi, A., 2015. Comparative genomics of *Steinernema* reveals deeply conserved gene regulatory networks. Genome Biol. 16, 200.

Dudzic, J.P., Kondo, S., Ueda, R., Bergman, C.M., Lemaitre, B., 2015. *Drosophila* innate immunity: regional and functional specialization of prophenoloxidases. BMC Biol. 13, 81.

Eleftherianos, I., Revenis, C., 2011. Role and importance of phenoloxidase in insect hemostasis. J. Innate Immun. 3, 28–33.

Eleftherianos, I., Joyce, S., Ffrench-Constant, R.H., Clarke, D.J., Reynolds, S.E., 2010. Probing the tri-trophic interaction between insects, nematodes and Photorhabdus. Parasitology 137, 1695–1706.

Eom, S., Park, Y., Kim, Y., 2014. Sequential immunosuppressive activities of bacterial secondary metabolites from the entomopahogenic bacterium Xenorhabdus nematophila. J. Microbiol. 52, 161–168.

Ferrandon, D., Imler, J.L., Hetru, C., Hoffmann, J.A., 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. Nat. Rev. Immunol. 7, 862–874.

Goodrich-Blair, H., 2007. They've got a ticket to ride: *xenorhabdus nematophila-Steinernema carpocapsae* symbiosis. Curr. Opin. Microbiol. 10, 225–230.

Goodrich-Blair, H., Clarke, D.J., 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Mol. Microbiol. 64, 260–268.

Honti, V., Csordás, G., Kurucz, É., Márkus, R., Andó, I., 2014. The cell-mediated immunity of *Drosophila melanogaster*: hemocyte lineages, immune compartments, microanatomy and regulation. Dev. Comp. Immunol. 42, 47–56.

Hwang, J., Park, Y., Kim, Y., 2013. An Entomopathogenic Bacterium,*Xenorhabdus nematophila*, Suppresses expression of antimicrobial peptides controlled by toll and IMD pathways by blocking eicosanoid biosynthesis. Arch. Insect Biochem. Physiol. 83, 151–169.

Irving, P., Ubeda, J.M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J.A., Hetru, C., Meister, M., 2005. New insights into *Drosophila* larval haemocyte functions through genome-wide analysis. Cell. Microbiol. 7, 335–350.

Javar, S., Mohamed, R., Sajap, A.S., Lau, W.H., 2015. Expression profiles of lysozyme- and prophenoloxidase-encoding gene in *Spodoptera* species challenged with entomopathogenic fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin using qRT-PCR. Invertebr. Reprod. Develop. 59, 230–236.

Ji, D., Kim, Y., 2004. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits the expression of an antibacterial peptide, cecropin, of the beet armyworm, *Spodoptera exigua*. J. Insect Physiol. 50, 489–496.

Kim, S.H., Lee, W.J., 2014. Role of DUOX in gut inflammation: lessons from *Drosophila* model of gut-microbiota interactions. Front. Cell. Infect. Microbiol. 3, 116.

Lindgren, M., Riazi, R., Lesch, C., Wilhelmsson, C., Theopold, U., Dushay, M.S., 2008. Fondue and transglutaminase in the *Drosophila* larval clot. J. Insect Physiol. 54, 586–592.

Loof, T.G., Schmidt, O., Herwald, H., Theopold, U., 2011. Coagulation systems of invertebrates and vertebrates and their roles in innate immunity: the same side of two coins? J. Innate Immun. 3, 34–40.

Lu, A., Zhang, Q., Zhang, J., Yang, B., Wu, K., Xie, W., Luan, Y.X., Ling, E., 2014. Insect

prophenoloxidase: the view beyond immunity. Front. Physiol. 5, 252.
Nam, H.J., Jang, I.H., Asano, T., Lee, W.J., 2008. Involvement of pro-phenoloxidase 3 in lamellocyte-mediated spontaneous melanization in Drosophila. Mol. Cells 26, 606–610.
Peña, J.M., Carrillo, M.A., Hallem, E.A., 2015. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. Infect. Immun. 83, 1130–1138.
Reichhart, J.M., 2005. Tip of another iceberg: *drosophila* serpins. Trends Cell Biol. 15, 659–665.
Tang, H., 2009. Regulation and function of the melanization reaction in *Drosophila*. Fly 3, 105–111.
Theopold, U., Li, D., Fabbri, M., Scherfer, C., Schmidt, O., 2002. The coagulation of insect hemolymph. Cell. Mol. Life Sci. 59, 363–372.
Theopold, U., Krautz, R., Dushay, M.S., 2014. The *Drosophila* clotting system and its messages for mammals. Dev. Comp. Immunol. 42, 42–46.
Valadez-Lira, J.A., Alcocer-Gonzalez, J.M., Damas, G., Nuñez-Mejía, G., Oppert, B., Rodriguez-Padilla, C., Tamez-Guerra, P., 2012. Comparative evaluation of phenoloxidase activity in different larval stages of four lepidopteran pests after exposure to Bacillus thuringiensis. J. Insect Sci. 12, 80.
Veillard, F., Troxler, L., Reichhart, J.M., 2016. *Drosophila melanogaster* clip-domain serine proteases: structure, function and regulation. Biochimie 122, 255–269.
Wang, Z., Wilhelmsson, C., Hyrsl, P., Loof, T.G., Dobes, P., Klupp, M., Loseva, O., Mörgelin, M., Iklé, J., Cripps, R.M., Herwald, H., Theopold, U., 2010. Pathogen entrapment by transglutaminase–a conserved early innate immune mechanism. PLoS Pathog. 6, e1000763.
Yadav, S., Shokal, U., Forst, S., Eleftherianos, I., 2015. An improved method for generating axenic entomopathogenic nematodes. BMC Res. Notes 8, 461.
Yadav, S., Daugherty, S., Shetty, A.C., Eleftherianos, I., 2017. RNAseq Analysis of the*Drosophila* Response to the Entomopathogenic Nematode *Steinernema*. G3 7, 1955–1967.
Yadav, S., Gupta, S., Eleftherianos, I., 2018. Differential regulation of immune signaling and survival response in *Drosophila melanogaster* larvae upon *Steinernema carpocapsae*. Nematode Infect. Insects 9, E17.