TGF-β Signaling Interferes With the Drosophila Innate Immune and Metabolic Response to Parasitic Nematode Infection

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The common fruit fly, Drosophila melanogaster, is an outstanding model to study the molecular basis of anti-pathogen immunity. The parasitic nematode Heterorhabditis gerrardi, together with its mutualistic bacteria Photorhabdus asymbiotica, infects a wide range of insects, including D. melanogaster. Recently, we have shown that transforming growth factor-β (TGF-β) signaling in D. melanogaster is regulated in response to parasitic nematode infection. In the current study, we investigated the contribution of two TGF-β signaling branches, the activin and the bone morphogenetic protein (BMP), to D. melanogaster immune function against H. gerrardi. We used D. melanogaster larvae carrying mutations in the genes coding for the TGF-β extracellular ligands daw and dpp.

We have demonstrated that the number of circulating hemocytes in uninfected daw and dpp mutants decreases twofold compared to background controls, yet no significant changes in hemocyte numbers and survival of the TGF-β mutants are observed upon nematode infection. However, we have shown that nematode-infected daw mutants express Dual oxidase at higher levels and phenoloxidase activity at lower levels compared to their background controls. To elucidate the contribution of TGF-β signaling in the metabolic response of D. melanogaster to parasitic nematodes, we estimated lipid and carbohydrate levels in daw and dpp mutant larvae infected with H. gerrardi. We have found that both nematode-infected mutants contain lipid droplets of larger size, with daw mutant larvae also containing elevated glycogen levels. Overall, our findings indicate that the regulation of activin and BMP branches of TGF-β signaling can alter the immune and metabolic processes in D. melanogaster during response to parasitic nematode infection. Results from this study shed light on the molecular signaling pathways insects activate to regulate mechanisms for fighting potent nematode parasites, which could lead to the identification of novel management strategies for the control of damaging pests.

Keywords: D. melanogaster, Heterorhabditis, immunity, parasitism, TGF-β signaling
INTRODUCTION

The fruit fly, *Drosophila melanogaster*, is an established insect model to study innate immune responses against pathogenic infection due to the availability of a wide range of genetic tools (Rämet, 2012). The nematode parasite *Heterorhabditis* forms an excellent experimental tool to dissect the molecular basis of nematode parasitism and mutualism in relation to the insect immune system (Hallem et al., 2007). The nematodes live in mutualistic relationship with the Gram-negative bacteria *Photorhabdus* and together they can infect a variety of insect species (Gerrard et al., 2006; Plichta et al., 2009). *Heterorhabditis* nematodes infect their insect hosts at the infective juvenile stage. Upon entering the insect body cavity, the nematode regurgitates its mutualistic bacteria into the hemolymph to overcome the insect immune response (Stock and Blair, 2008; Castillo et al., 2011).

Investigation of the dynamic interaction between *Heterorhabditis* and *Photorhabdus* species in relation to key aspects of the insect immune system has been facilitated in recent years by the establishment of the tripartite system that involves the fruit fly *D. melanogaster* as the model insect host (ffrench-Constant et al., 2007; Hallem et al., 2007). *D. melanogaster* has evolved certain immune mechanisms to fight against parasitic nematode infection (Castillo et al., 2011). The anti-nematode immunity of *D. melanogaster* includes both humoral and cellular responses in addition to the phenoloxidase cascade that results in melanin formation (Eleftherianos et al., 2016a). Nematode infection also induces stress signaling cascades that result in the synthesis of nitric oxide (NO) and differential regulation of genes involved in the production of reactive oxygen species (ROS) (Castillo et al., 2015; Yadav et al., 2017).

Transforming growth factor-β (TGF-β) signaling pathway is pivotal in cell-cell communication and is involved in several cellular processes, including cell proliferation and differentiation as well as tissue homeostasis and regeneration in mammals (Harradine and Akhurst, 2009). In *D. melanogaster*, it regulates developmental mechanisms including axis formation, body patterning, and morphogenesis (Masucci et al., 1990; Lecuit et al., 1996; Dobens and Raftery, 1998). Similar to vertebrates, the TGF-β pathway in *D. melanogaster* is composed of two signaling branches: the bone morphogenetic protein (BMP) and the activin branches. The TGF-β signaling pathway is initiated by the binding of an extracellular ligand to a transmembrane receptor complex of serine/threonine kinases (Raftery and Sutherland, 1999; Shi and Massagué, 2003). BMP signaling includes three ligands: decapentaplegic (dpp), glass-bottom boat (gbb), and screw (scw); and the activin subfamily ligands include activin-β (actβ), dawdle (daw), and myoglobin (myo; Peterson and O’Connor, 2014). Following the activation of the receptor through ligand binding, receptor complex phosphorylates downstream transcription factors that regulate the activation of target genes (Zi et al., 2012).

Recently, a link between TGF-β signaling pathway activity and interaction with parasitic nematode infection has been found in *D. melanogaster* (Eleftherianos et al., 2016b; Patrnogic et al., 2018a,b). More precisely, both activin and BMP branches of TGF-β signaling pathway are involved in the immune response to sterile injury and *Micrococcus luteus* bacterial infection in flies (Clark et al., 2011). Also, gene transcript levels of both *dpp* and *daw* are upregulated by *Heterorhabditis gerrardi* and *H. bacteriophora* nematode infection in flies (Eleftherianos et al., 2016b). In addition, inactivation of *dpp* increases fly survival and activates humoral immunity in response to *H. bacteriophora* assault (Patrnogic et al., 2018a).

In the current study, we investigated the potential contribution of activin and BMP branches of TGF-β signaling in *D. melanogaster* immunity against *H. gerrardi* infection. For this, we infected larvae carrying loss-of-function mutations in *daw* or *dpp* with *H. gerrardi* infective juveniles to estimate their survival ability, cellular immune activity including changes in hemocyte numbers, ROS and NO activation, and melanization response. In addition, in order to understand whether TGF-β signaling regulates the *D. melanogaster* metabolic response to nematode parasites, we measured metabolic processes, including lipid and carbohydrate metabolism in *H. gerrardi*-infected larvae with inactivated *daw* or *dpp* genes. Similar studies in insect model hosts are expected to facilitate our understanding of the link between activation of conserved signaling pathways and their components and host immune capacity in response to potent nematode parasites.

MATERIALS AND METHODS

Fly and Nematode Stocks

All flies were reared on instant *D. melanogaster* diet (Formula 4–24 *D. melanogaster* medium) supplemented with yeast (Carolina Biological Supply), maintained at 25°C, and a 12:12-h light-dark photoperiodic cycle. A fly line with spontaneous *dpp* mutation and a line carrying P-bac insertion Pbac{XP}daw<sup>6680</sup> were used. Line w<sup>1118</sup> was used as the background control in all experiments. All lines were obtained from Bloomington Drosophila Stock Center. Validation of mutant lines was performed using quantitative RT-PCR (Supplementary Figure S1). *H. gerrardi* nematodes were amplified in the larvae of the wax moth *Galleria mellonella* using the water trap technique (White, 1927). Nematodes were used 1–4 weeks after collection.

Larval Infection

Infections of *D. melanogaster* late 2nd instar larvae with nematodes were performed in microtiter 96-well plates containing 100 µl of 1.25% agarose in each well. Infected juveniles were washed and adjusted to the final density of 100 nematodes in 10 µl of sterile distilled water. Nematodes were pipetted into the wells of the microtiter plate and a single larva was transferred to each well. The plate was covered with a Masterclear real-time PCR film (Eppendorf) and holes were pierced for ventilation. Sterile distilled water was used as negative control. Control larvae maintained with water were able to survive, grow normally, and eventually pupate during the course of the experiment. Infected and uninfected larvae were kept at room temperature in the 96-well plate. At 3- and 24-h time point, infected and uninfected larvae were collected and frozen.
at −80°C or immediately used in experiments. Each infection was performed three times with biological duplicates. For survival experiments, the survival of larvae kept in nematode-free solution or in nematode solution was counted every 12 h for 60 h. Four independent survival experiments were conducted.

RNA Analysis
Total RNA was extracted from 5 to 10 D. melanogaster larvae, using TRIzol® reagent according to manufacturer's protocol. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 350 ng RNA. Quantitative RT-PCR (qRT-PCR) experiments were carried out with gene-specific primers (Table 1) and 3.5 ng cDNA, using iQ SYBR Green Supermix (Bio-Rad Laboratories) and a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories), following the manufacturer's instructions. Each experiment was run in biological duplicates and repeated three times.

Hemolymph Collection and Total Hemocyte Counts
To extract hemolymph from nematode-infected and uninfected D. melanogaster mutant and background control larvae, 10 individuals were bled into 30 μl of 2.5× protease inhibitor cocktail (Sigma P2714). Hemolymph samples were loaded on a hemocytometer and total numbers of cells were counted using 40× magnification of a compound microscope (Olympus CX21). Each experiment was repeated three times.

Phenoloxidase Activity Assay
D. melanogaster larvae were infected with H. gerrardi nematodes as previously described, and five larvae were collected at 24 h post infection. Larvae were washed several times in cold 1 ml 1× PBS and homogenized in either 100 μl of 1× PBS to determine glucose and glycogen levels or 100 μl of cold PBST (1× PBS + 0.05% Tween 20) to measure triglyceride levels, as previously described (Tennessen et al., 2014). Proteins were quantified by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). To determine the amount of triglycerides in infected and uninfected larvae, samples were diluted 1:1 in PBS-Tween and added to 200 μl of the Infinity™ Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific) in a clear microplate well. Covered samples were incubated at 37°C for 30 min, and absorbance was measured at 540 nm. The amount of triglycerides was determined by the glycerol standard curve. To determine the amount of glucose and glycogen, samples were initially diluted 1:3 in PBS and then separated into two sets for further dilutions. The first set of samples was diluted 1:1 in amylglucosidase stock solution (1.5 μl of amylglucosidase in 1 ml of PBS, Sigma) and the second set was diluted 1:1 in PBS. Samples (30 μl) were incubated at 37°C for 60 min in a clear microplate well. Hexokinase (Glucose Assay Reagent, Sigma) reagent (100 μl) was added to each well and samples were incubated at room temperature for 15 min. Absorbance was measured at 340 nm, and the amount of glucose was determined by the second set of samples, which were diluted in PBS. The amount of glycogen was calculated by subtracting the absorbance of glucose from the absorbance of first set of samples (samples diluted with amylglucosidase stock). The amounts of triglycerides, glucose, and glycogen were calculated relative

| Gene | Accession no. | Primer (5′-3′) | Sequence | Tm (°C) |
|------|---------------|----------------|----------|---------|
| Daw  | CG16967       | Forward        | GGTGGATCAGCGAGAAAGCAGT   | 57      |
|      |               | Reverse        | GCGACGATCGAGGTGTTGTA    | 57      |
| Dpp  | CG9885        | Forward        | CCTTGAGCCTGCTGCGAGT    | 60      |
|      |               | Reverse        | TGACACCCTGACTGAGATG     | 60      |
| PPO1 | CG5779        | Forward        | CCACTGCCCTGCTGAGTGA    | 59      |
|      |               | Reverse        | CGGACGTTCAATACAGTT       | 58      |
| PPO2 | CG8193        | Forward        | GGCGGAATGCGTTACT       | 59      |
|      |               | Reverse        | GCGACGTTCAATACAGTT       | 58      |
| PPO3 | CG2952        | Forward        | AAAGTTGAGCAATGCGCAA     | 60      |
|      |               | Reverse        | GTGTGCTGCTGCTGCTCTCT    | 60      |
| Nos  | CG6713        | Forward        | ACGTTGCCCAATGCGACAGAG   | 60      |
|      |               | Reverse        | ACGACGTCGCCATGCAGTG     | 60      |
| Duox | CG3131        | Forward        | GATGACCCATCGCCGCCAAGCA  | 60      |
|      |               | Reverse        | CGGACGAGACGCTGTTGAGC    | 60      |

Metabolic Assays
D. melanogaster larvae were infected with H. gerrardi nematodes as previously described, and five larvae were collected at 24 h post infection. Larvae were washed several times in cold 1 ml 1× PBS and homogenized in either 100 μl of 1× PBS to determine glucose and glycogen levels or 100 μl of cold PBST (1× PBS + 0.05% Tween 20) to measure triglyceride levels, as previously described (Tennessen et al., 2014). Proteins were quantified by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). To determine the amount of triglycerides in infected and uninfected larvae, samples were diluted 1:1 in PBS-Tween and added to 200 μl of the Infinity™ Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific) in a clear microplate well. Covered samples were incubated at 37°C for 30 min, and absorbance was measured at 540 nm. The amount of triglycerides was determined by the glycerol standard curve. To determine the amount of glucose and glycogen, samples were initially diluted 1:3 in PBS and then separated into two sets for further dilutions. The first set of samples was diluted 1:1 in amylglucosidase stock solution (1.5 μl of amylglucosidase in 1 ml of PBS, Sigma) and the second set was diluted 1:1 in PBS. Samples (30 μl) were incubated at 37°C for 60 min in a clear microplate well. Hexokinase (Glucose Assay Reagent, Sigma) reagent (100 μl) was added to each well and samples were incubated at room temperature for 15 min. Absorbance was measured at 340 nm, and the amount of glucose was determined by the second set of samples, which were diluted in PBS. The amount of glycogen was calculated by subtracting the absorbance of glucose from the absorbance of first set of samples (samples diluted with amylglucosidase stock). The amounts of triglycerides, glucose, and glycogen were calculated relative

TABLE 1 | Primers and their sequences used in quantitative RT-PCR experiments.
to the amount of proteins in each sample. Each experiment was run in biological duplicates and repeated three times.

**Lipid Droplet Staining**

*D. melanogaster* larvae were infected with *H. gerrardi* nematodes as previously described, and 15 larvae were collected at 24 h post infection. Fat body tissues of larvae were dissected and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. Tissues were washed two times in PBS and then incubated in the dark for 30 min in 0.05% Nile red diluted 1:1,000 in 1 mg/ml of methanol. Tissues were mounted in ProLong™ Diamond AntiFade Mountant with DAPI (Life Technologies). Images were taken by Zeiss LSM 510 confocal microscope. Quantification of lipid droplet size was performed by selecting the area of the five largest lipid droplets per cell from 20 fat body cells. ImageJ software (National Institutes of Health) was used for quantifications. The experiment was repeated three times.

**Statistical Analysis**

GraphPad Prism7 was used for data plotting and statistical analyses. Log-rank (Mantel-Cox) test was used for statistical analysis of the survival results. Statistical analyses of all other experiments were performed using unpaired *t*-test.

**RESULTS**

**Nematode Infection Does Not Alter the Survival of TGF-β Mutants**

We assessed the ability of *daw* and *dpp* mutant larvae to survive the infection by *H. gerrardi* symbiotic nematodes. For this, we monitored larval survival every 12 h and up to 60 h post nematode infection. We found no significant differences in survival between uninfected TGF-β mutants and their background control (Figure 1). Also, we did not observe any significant changes in survival between nematode-infected TGF-β mutants and control individuals. These results indicate that activin and BMP branches of TGF-β signaling do not contribute to the survival ability of *D. melanogaster* larvae to infection by *H. gerrardi* nematodes.

**Uninfected *daw* and *dpp* Mutants Contain Fewer Circulating Hemocytes**

In *D. melanogaster*, circulating hemocytes play a major role in immune surveillance, and their number can change drastically during pathogenic or non-pathogenic bacterial infection (Eleftherianos et al., 2014; Vlisidou and Wood, 2015; Shokal et al., 2017). To investigate whether inactivating the activin or BMP branches of TGF-β signaling alters the total number of circulating hemocytes in uninfected *D. melanogaster* or those infected with nematode parasites, we counted hemocytes in larvae carrying loss-of-function mutations in *daw* or *dpp* following treatment with water (control) or infection with *H. gerrardi*. We used two time points to examine changes in hemocyte numbers over time: 3 h post infection as an early time point and 24 h post infection as a later point when nematode infection is established. Both uninfected *daw* and *dpp* mutants contained significantly reduced numbers of hemocytes relative to their *w*1118 background control at the 3-h time point (*daw*, *p* = 0.0014 and *dpp*, *p* = 0.0078; Figure 2). Similarly, at 24 h, we observed that uninfected *daw* mutants contained significantly fewer hemocytes compared to *w*1118 larvae (*p* = 0.0119). We then estimated the total

![Figure 1](image-url)
A significant number of hemocytes in response to *H. gerrardi* and found that at 3 h post nematode infection, hemocyte numbers in *daw* mutants and *w*¹¹¹⁸ control larvae were significantly lower relative to uninfected controls (*daw*, *p* = 0.0008; control, *p* = 0.0023). However, at the same time point, *dpp* mutants did not show any significant changes in hemocytes numbers in response to *H. gerrardi* infection. We also did not observe any differences in hemocyte numbers between infected or uninfected mutants and *w*¹¹¹⁸ larvae at the 24-h time point. These results indicate that both activin and BMP branches of TGF-ß signaling in *D. melanogaster* are potentially involved in regulating the number of circulating hemocytes in the absence of infection.

**Nematode-Infected *daw* Mutants Express Duox at Higher Levels**

The production of reactive oxygen species (ROS) and nitric oxide (NO), mediated by dual oxidase (Duox) and nitric oxide synthase (Nos) enzymes, respectively, constitutes an essential regulator of diverse biological processes that include the immune response against bacterial infection (Marletta, 1994; Kuraishi et al., 2013; Eleftherianos et al., 2014). In addition, in mammals in the absence of infection, TGF-ß signaling is potentially regulated by ROS and NO responses (Saura et al., 2005; Jain et al., 2013). However, ROS and NO responses in *D. melanogaster* in the context of parasitic nematode infection and whether TGF-ß signaling participates in the regulation of these processes have not been examined yet. To investigate a potential link between these responses and TGF-ß signaling, we used qRT-PCR and gene-specific primers to determine the transcript levels of *Nos* and *Duox* in *daw* and *dpp* mutant larvae 24 h after infection with *H. gerrardi* nematodes. We found no statistically significant differences in *Nos* transcript levels between nematode-infected *daw* or *dpp* mutants and their *w*¹¹¹⁸ background controls. However, the expression of *Duox* in infected *daw* mutants was upregulated compared to the background controls (*p* = 0.00419, Figure 3B) and *dpp* mutants (*p* = 0.0022, Figure 3B). These results suggest a link between the ROS response and the activin branch of TGF-ß signaling in *D. melanogaster* upon response to parasitic nematode infection.

**FIGURE 2** Total number of circulating hemocytes in *Drosophila melanogaster* TGF-ß mutant larvae upon infection with the parasitic nematodes *Heterorhabditis gerrardi*. Hemolymph samples were collected at 3 and 24 h after infection. Numbers of hemocytes in uninfected *daw* and *dpp* mutants are significantly reduced at 3 h relative to their background control (*w*¹¹¹⁸). *Daw* mutants contain significantly reduced numbers of hemocytes upon nematode infection. Asterisks indicate significant differences between experimental treatments (*p* < 0.05, **p** < 0.01, ***p*** < 0.001; non-significant differences are not indicated).

**FIGURE 3** Expression of *Nos* and *Duox* in *Drosophila melanogaster* TGF-ß mutant larvae at 24 h after infection with the parasitic nematodes *Heterorhabditis gerrardi*. *Nos* and *Duox* gene transcript levels in infected larvae are shown as relative fold change normalized to uninfected controls. (A) There is no significant change in the expression of *Nos* between the TGF-ß mutants relative to their background control (*w*¹¹¹⁸). (B) Expression of *Duox* in *daw* mutants is upregulated compared to the background controls (**p** = 0.00419 and ***p*** = 0.0022, respectively; non-significant differences are not indicated).
The Activin Signaling Suppresses the Phenoloxidase Response in Response to *H. gerrardi*

Previous results indicate that ubiquitous knockdown of *daw* in *D. melanogaster* adult flies results in the formation of melanotic tumors suggesting an association between the TGF-β activin branch and regulation of the melanization response (Clark et al., 2011). To investigate whether inactivation of TGF-β signaling in *D. melanogaster* modifies the phenoloxidase response in the context of nematode infection, we challenged BMP and activin loss-of-function mutant larvae with *H. gerrardi* parasites, and 24 h later, we estimated the expression of prophenoloxidase genes *PPO1*, *PPO2*, and *PPO3* using qRT-PCR and gene-specific primers (Tang, 2009). We found no statistically significant differences in the transcript levels of *PPO1* and *PPO2* in *daw* or *dpp* mutants relative to *w*1118 control larvae upon *H. gerrardi* infection (Figures 4A,B). However, *PPO3* transcript levels were significantly reduced in nematode-infected *daw* (*p = 0.0140*) and *dpp* mutants (*p = 0.0169*) compared to *w*1118 controls (Figure 4C). We then determined the phenoloxidase enzyme activity in the hemolymph of *daw* and *dpp* mutant larvae infected with *H. gerrardi*. We found that phenoloxidase activity in *daw* mutant larvae was significantly reduced upon nematode infection relative to uninfected counterparts (*p = 0.0008*, Figure 4D). These results imply that the activin branch of TGF-β signaling in *D. melanogaster* might be involved in suppressing phenoloxidase response in response to *H. gerrardi* nematode infection.

Size of Lipid Droplets Increases in Nematode-Infected *daw* and *dpp* Mutants

Lipid droplets are vital energy storage organelles found in many organisms. Recent findings suggest that lipid droplets increase in size in *D. melanogaster* infected with *Steinernema carpocapsae* nematodes, which implies a participation in the interaction with certain nematode parasites (Yadav et al., 2018). To determine lipid droplet status in the fat body of TGF-β-deficient larvae, we stained lipid droplets with Nile red (red) and DAPI (blue) and measured lipid droplet sizes in *daw* and *dpp* loss-of-function mutant larvae (Figures 5A–C). We found that lipid droplets in uninfected *dpp* mutants significantly increased in size compared to *w*1118 controls (Figure 5B, *p = 0.0458*). However, uninfected *daw* mutants had significantly smaller lipid droplets relative to *w*1118 larvae (Figure 4B, *p < 0.0001*). Then, we determined lipid droplet sizes 24 h post *H. gerrardi* infection in *daw* and *dpp* mutants. Size of lipid droplets significantly increased in nematode-infected *daw* and *dpp* mutants compared to uninfected larvae (Figure 5C; *p < 0.0001*). Also, nematode infected *w*1118 controls contained significantly smaller lipid droplets relative to uninfected individuals (Figure 5C; *p = 0.0221*). To further assess changes

![FIGURE 4](image-url) | Expression of PPO genes and activity of phenoloxidase in *Drosophila melanogaster* TGF-β mutant larvae at 24 h after infection with the parasitic nematodes *Heterorhabditis gerrardi*. Transcript levels of *PPO1*, *PPO2*, and *PPO3* in nematode-infected larvae are shown as relative fold change normalized to uninfected controls. (A,B) There is no significant change in the expression of *PPO1* and *PPO2* between nematode-infected *daw* and *dpp* mutants relative to their background control (*w*1118). (C) Expression of *PPO3* is reduced in nematode-infected *daw* (*p = 0.0140*) and *dpp* mutants (*p = 0.0169*) compared to their background control (*w*1118). (D) Phenoloxidase activity in the hemolymph of *daw* mutants is significantly reduced upon *H. gerrardi* nematode infection compared to uninfected individuals (**p = 0.0022**, ***p = 0.0008**, non-significant differences are not indicated).
in lipid metabolism in TGF-β-deficient D. melanogaster larvae in the context of nematode infection, we estimated triglyceride concentrations in daw and dpp mutant larvae challenged with H. gerrardi (Figure 5D). Triglyceride levels in dpp mutants infected with the parasitic nematodes were significantly elevated compared to uninfected larvae ($p = 0.0193$), but there were no statistically significant changes in daw mutants relative to uninfected individuals. These findings suggest that both BMP and activin branches of TGF-β signaling in D. melanogaster regulate fat body lipid droplet size during response to nematode infection.

**Nematode-Infected dpp Mutants Have Elevated Glycogen Levels**

In D. melanogaster, glucose is an essential resource for energy production. Glycogen is synthesized and stored in several tissues and is required for energy metabolism (Mattila and Hietakangas, 2017). In mammals, there is a direct link between regulation of carbohydrate homeostasis and TGF-β signaling in the absence of an infection (Yadav et al., 2011). To investigate whether TGF-β signaling affects carbohydrate metabolism in D. melanogaster anti-nematode response, we estimated glucose and glycogen levels 24 h post infection with H. gerrardi. We found that upon nematode infection, infected $w^{1118}$ control larvae had significantly increased glucose levels compared to uninfected individuals ($p = 0.0381$). However, we did not observe any statistically significant changes in infected daw and dpp mutant larvae relative to uninfected controls. We found that only nematode-infected daw mutants had significantly elevated levels of glycogen relative to uninfected larvae ($p = 0.0482$). These results indicate that the activin branch of TGF-β signaling in D. melanogaster might participate in modulating glycogen metabolism in the context of nematode infection.

**DISCUSSION**

In this study, we explored the contribution of activin and BMP branches of TGF-β signaling in regulating immune activity in D. melanogaster. For this, we analyzed changes in larval survival capacity, hemocyte numbers, activation of ROS and NO, and melanization response in uninfected daw or dpp loss-of-function mutant larvae as well as in larvae infected with H. gerrardi parasitic nematodes. We have found a significant
decrease in the number of circulating hemocytes in uninfected *daw* and *dpp* mutants compared to their background controls, but no significant change in hemocyte numbers following nematode infection. However, *daw* mutants have higher expression of *Duox* and decreased phenoloxidase activity in response to nematode infection compared to their background controls. We further examined the metabolic activity of *daw* and *dpp* mutant larvae in the presence or absence of *H. gerrardi* infection and found an increase in the size of lipid droplets in both mutants as well as elevated glycogen levels in *daw* mutants upon nematode challenge.

Hemocytes are the central regulators of the cellular immune response against microbial infection in insects, and previous information supports the notion that total number of circulating hemocytes constitutes a robust indication for the level of activation of the cellular arm of the insect innate immune system to act against foreign invaders (Parsons and Foley, 2016). To investigate the contribution of activin and BMP signaling on cellular immune activity in the context of parasitic nematode infection, we estimated changes in hemocyte numbers in uninfected *D. melanogaster* *daw* and *dpp* mutant larvae and larvae infected with *H. gerrardi* nematodes. Our results indicate that both *daw* and *dpp* uninfected mutants contain significantly fewer hemocytes relative to their background controls. Interestingly, a recent study has shown that the activin branch extracellular ligand Actβ is expressed in sensory neurons, and silencing of Actβ results in fewer hemocyte numbers in *D. melanogaster* larvae in the absence of infection (Makhijani et al., 2017). In agreement with this recent study, our results support the concept that the activin branch of TGF-β signaling participates in the regulation of hemocyte population at the larval stage of *D. melanogaster*. In contrast, we did not detect any significant changes in hemocyte numbers between nematode-infected *daw* or *dpp* mutants and their background controls, which probably explains the lack of alteration in the survival of the TGF-β mutants in response to *H. gerrardi*. This result further implies that *H. gerrardi* infection has no effect on the total number of circulating hemocytes in *D. melanogaster* larvae. Therefore, current findings suggest that *H. gerrardi* infection does not alter the dynamics of hemocyte numbers in *D. melanogaster* and that the activin and BMP branches of TGF-β signaling modulate the amount of hemocytes in the uninfected state of the larval stage.

The phenoloxidase enzyme in the melanization cascade regulates the formation of melanin at wound sites and around invading pathogens in the insect hemolymph (Eleftherianos and Revenis, 2011). Previous findings signify that ubiquitous silencing of *daw* in the adult fly causes melanotic tumors mostly in the abdomen, indicating that activin signaling controls the inhibition of the melanization response (Clark et al., 2011). Similarly, here we have found that uninfected *daw* mutants contain significantly higher levels of hemolymph phenoloxidase compared to *dpp* mutants and their background controls. This might suggest a direct or indirect interaction between phenoloxidase activity and activin signaling in response to nematode infection, which will form a subject of our future studies. It is also important to consider that *Photobacteria* bacteria released from *Heterorhabditis* nematodes into the insect hemolymph secrete molecules, such as rhabduscin and hydroxystilbene, that interfere with the melanization cascade and suppress phenoloxidase activity in the infected insects (Eleftherianos et al., 2007; Crawford et al., 2012). Here, we have found that symbiotic *H. gerrardi* nematodes (containing mutualistic *P. asymbiotica* bacteria) fail to alter phenoloxidase activity in background control and *dpp* mutant larvae, but they are able to suppress the activity of the enzyme in *daw* mutants. This implies that phenoloxidase activity in the hemolymph of *D. melanogaster* larvae during infection with *H. gerrardi* symbiotic nematodes is regulated by the activin signaling of the TGF-β pathway.

Immune cells are required to maintain their cellular metabolism to function efficiently in combating pathogens (Loftus and Finlay, 2016). During infection, *Staphylococcus aureus* induces changes in the host extracellular environment by reducing oxygen and nutrient availability, which generates significant metabolic stress in the mammalian host (Vitko et al., 2015). In the current study, we aimed at understanding the contribution of activin and BMP branches of TGF-β signaling to metabolic changes in uninfected *D. melanogaster* larvae as well as during nematode infection. It has been previously shown...
that the BMP ligand gbb is essential in the fat body of uninfected D. melanogaster larvae to maintain lipid homeostasis and metabolism. Gbb loss-of-function mutants also display abnormalities in fat body morphology (Ballard et al., 2010). Here, we have also found a significant increase in the size of lipid droplets in uninfected dpp mutants compared to background controls indicating the contribution of BMP signaling in maintaining lipid metabolism. However, uninfected daw mutants contain significantly smaller lipid droplets suggesting the disruption of lipid metabolism in these larvae. A previous study reported that in D. melanogaster embryos histones bound to cytosolic lipid droplets can eliminate both Gram-positive and Gram-negative bacteria in vitro. (Anand et al., 2012). In addition, infection with the intracellular bacteria Mycobacterium tuberculosis, M. bovis, and M. leprae leads to the accumulation of lipid droplets in macrophages and Schwann cells in mammalian hosts (D’Avila et al., 2006; Russell et al., 2009; Mattos et al., 2011). Also, infection with the intracellular parasite Trypanosoma cruzi in rats induces an increase in the size of lipid droplets in macrophages (Melo et al., 2003). In the fat body of D. melanogaster, size of lipid droplets increases in response to infection with the parasitic nematode S. carpocapsae carrying the mutualistic bacteria Xenorhabdus nematophilus (Yadav et al., 2018). In contrast, here we have demonstrated that upon infection with H. gerrardi nematodes, which contain the mutualistic bacteria P. asymbiotica, size of lipid droplets in the fat body of background control larvae significantly decreases compared to uninfected individuals, suggesting reduced lipid accumulation in this tissue. Such alterations in host lipid metabolism might be an indication of pathogen-specific immune or metabolic responses (Govind, 2008). In our experiments, infection with H. gerrardi causes a significant increase in the size of lipid droplets in both daw and dpp mutant larvae, suggesting that both activin and BMP branches might be involved in the regulation of lipid metabolism in D. melanogaster during response to nematode insult.

The current findings highlight the overlapping interactions between the two TGF-ß signaling pathway branches activin and BMP with immune activity and maintenance of lipid and carbohydrate metabolism in uninfected D. melanogaster larvae as well as during infection with potent parasitic nematodes.

Future research to examine the molecular and functional details of these interactions will contribute toward clarifying the exact role of activin and BMP branches in the host anti-nematode immune response. Due to conservation of innate immune signaling and function in humans, the identification of key immune signaling components will create the basis for identifying novel antihelminth treatment strategies. Alternatively, a better understanding of how parasitic nematodes interact with the immune and metabolic processes of model insects host could potentially lead to the development of innovative tactics for the effective management of agricultural insect pests and vectors of human diseases.

**AUTHOR CONTRIBUTIONS**

YO designed and conducted the experiments, analyzed the data, constructed the figures, interpreted the results, and wrote drafts of the manuscript. IE designed the experiments, interpreted the results, and revised the manuscript.

**FUNDING**

This research is funded by the National Institute of Allergy and Infectious Diseases (grants 1R01AI110675 and 1R56AI110675).

**ACKNOWLEDGMENTS**

We thank Kyle Devine for maintaining and amplifying the laboratory fly lines and members of the Department of Biological Sciences at George Washington University for providing feedback to manuscript drafts.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00716/full#supplementary-material

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