Interactions of antioxidants and phenoloxidase reactions in *Manduca sexta* hemolymph

by

Sequoyah Morabito

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Approved by:
Major Professor
Michael R. Kanost
Department of Biochemistry and Molecular Biophysics

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Abstract

Melanization is an important component of the insect innate immune response. Melanization occurs when an insect is exposed to pathogens such as bacteria or fungi. Phenoloxidase (PO) is a key enzyme in the synthesis of melanin. Recognition of bacterial peptidoglycan initiates a cascade of serine proteases, which activates PO. PO then hydroxylates tyrosine and oxidizes DOPA and dopamine, derived from tyrosine, to ortho-quinones. These participate in redox reactions that finally result in synthesizing melanin. Ortho-quinone formation and the redox reactions produce reactive oxygen species (ROS), which may help in eliminating pathogens but may also be harmful to the host insect. Insects may regulate oxidative damage to themselves. In this study, PO in hemolymph was activated similarly when plasma was exposed to either Lys type or DAP type bacterial peptidoglycans. However, lower amounts of Lys type peptidoglycans were able to stimulate PO more when compared to DAP type peptidoglycans. Colorimetric measurements revealed that *M. sexta* plasma contains a high concentration of ascorbate that significantly decreases when plasma PO is stimulated with peptidoglycan. Unexpectedly, PO activation also resulted in an increase in the total antioxidant capacity in plasma, which may be caused by the increased concentrations of DOPA produced during the melanization response, since DOPA can act as a reducing agent. Mass spectrometry, performed on *M. sexta* whole hemolymph and plasma, was used to reproducibly determine concentrations of total thiols, total reduced thiols, reduced glutathione, oxidized glutathione, cysteine, cystine, and glutathione-ss-cysteine, revealing that antioxidants are predominantly located in plasma and not hemocytes. Mass spectrometry was used to determine changes in concentrations of the following antioxidants and metabolites in plasma after stimulation of PO

activation with peptidoglycan: total reduced thiols, total oxidized thiols, percent oxidized thiols, total glutathione, total cysteine, total homocysteine, total cysteine-glutathione, free glutathione, oxidized glutathione (GSSG), free cysteine, cystine, glutathione-ss-cysteine, ascorbate, dehydroascorbate, tyrosine, DOPA, dopamine, and tyrosine glucoside. Stimulated plasma had a significant decrease in concentrations of glutathione, cysteine, ascorbate, and tyrosine. Concentrations of DOPA significantly increased in stimulated plasma samples while the concentration of dopamine did not change, demonstrating the hydroxylation of tyrosine and suggesting that DOPA is the predominant diphenol used during melanization. These results show that the redox environment in *M. sexta* plasma is highly reducing and suggests that shifts in redox potentials and regulation of the redox environment may not only regulate PO activity but may also prevent oxidative damage to the host.

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I also dedicate this thesis to my previous advisor, professor, and mentor, the late Dr. Gerald R. Reeck (Jerry). Jerry provided me numerous opportunities and exposure when I was a young and naive undergraduate. He showed me what it means to be a scientist and how a scientist should think. The kind words and confidence that Jerry had for me, and openly communicated with others, helped inspire me even after he was gone. Jerry played a significant role in my life and career and his sudden loss greatly affected me and will continue to affect me.

Literature Review

Insect Immunity:

Insects possess a hemocoel cavity that suspends their organs in an open circulatory system. Insects lack an adaptive immune response and rely on the expression of innate immune mechanisms (Rosales, 2017). These mechanisms consist of physical barriers, humoral responses, and cellular responses. Physical barriers such as the integument and peritrophic matrix assist in preventing infection and foreign invasion. Cellular immune responses in insects are mediated by circulating hemocytes. The primary mechanisms involved in this response are encapsulation, phagocytosis, and nodulation (Lavine & Strand, 2002; Liu et al., 2017). Hemocytes identify foreign pathogens through direct interactions between hemocyte surface receptors and the foreign pathogen. Additionally, hemocytes can identify pathogens through indirect recognition of humoral receptors that have bound to the surface of the pathogen (Lavine & Strand, 2002). Pattern recognition receptors (PRR) use binding of common molecules found only in microorganisms such as cell wall carbohydrates, lipids, and peptides (Hoffmann et al., 1999). Nodulation and encapsulation are often associated with melanization (Kanost et al., 2004).

The production of antimicrobial peptides, enzymatic complexes, melanin, and reactive oxygen species are primary mechanisms of humoral immune responses (Rosales, 2017). In *Drosophila melanogaster,* two pathways responsible for induced synthesis of antimicrobial peptides are the Toll pathway and the immune deficiency (Imd) pathway (Liu et al., 2017). Phenoloxidase is activated during foreign invasion and is a common insect humoral response enzyme that is important for pathogenic melanization (Kanost & Gorman, 2008). Fat body and hemocytes synthesize and secrete various plasma proteins with anti-microbial activity or indirectly function during an immune response (Kanost et al., 2004). The next sections focus on

humoral immune reactions in *Manduca sexta*, a model organism in insect immunity research, and the experimental organism for this thesis.

Melanization:

Melanin is a phenolic biopolymer that serves many functions in both mammals and insects. In mammals, it is a protective pigment found in hair, skin, and eyes. It is also an effective shield that protects organisms from harmful ionizing radiation. In invertebrates, melanization has three physiologically important uses: immunity, wound healing, and sclerotization (Sugumaran, 2002). Insects use melanin as a defense mechanism against microbial infections (Gillespie and et al., 1997).

In vertebrates, melanin synthesis occurs when tyrosine is hydroxylated by tyrosinase to form 3,4-dihydroxyphenylalanine (DOPA), which is then oxidized to form dopaquinone (Hearing & Tsukamoto, 1991). Insects initiate melanin biosynthesis using tyrosinases and monooxygenases (Gorman, An, et al., 2007). Tyrosine hydroxylase and phenoloxidase (PO) hydroxylates tyrosine to DOPA. PO then oxidizes DOPA to form dopaquinone. The PO oxidation reactions are quicker than the tyrosine hydroxylation reaction (Gorman, An, et al., 2007). DOPA decarboxylase can also convert the DOPA to dopamine, which then can be oxidized by PO to dopamine quinone. Dopaquinone and dopamine quinone then undergo further redox reactions, some also catalyzed by PO, to promote eumelanin generation (Figure 1) (Kanost & Gorman, 2008; Sugumaran, 1998). Insects contain large amounts of catecholamines that are necessary for sclerotization of cuticle. These catecholamines are synthesized by decarboxylating DOPA (K. J. Kramer & Hopkins, 1987). Insect development can significantly impact melanization, as seen by the ability of *M. sexta* to quickly metabolize Tyr in later stages of the

fifth instar when compared to insects with pupation delayed by treatment with the juvenile hormone analog Methoprene (Clark, 2015). While Tyr is the physiological substrate of melanization, it seems that dopamine is more rapidly used as a substrate for PO (Clark, 2015).

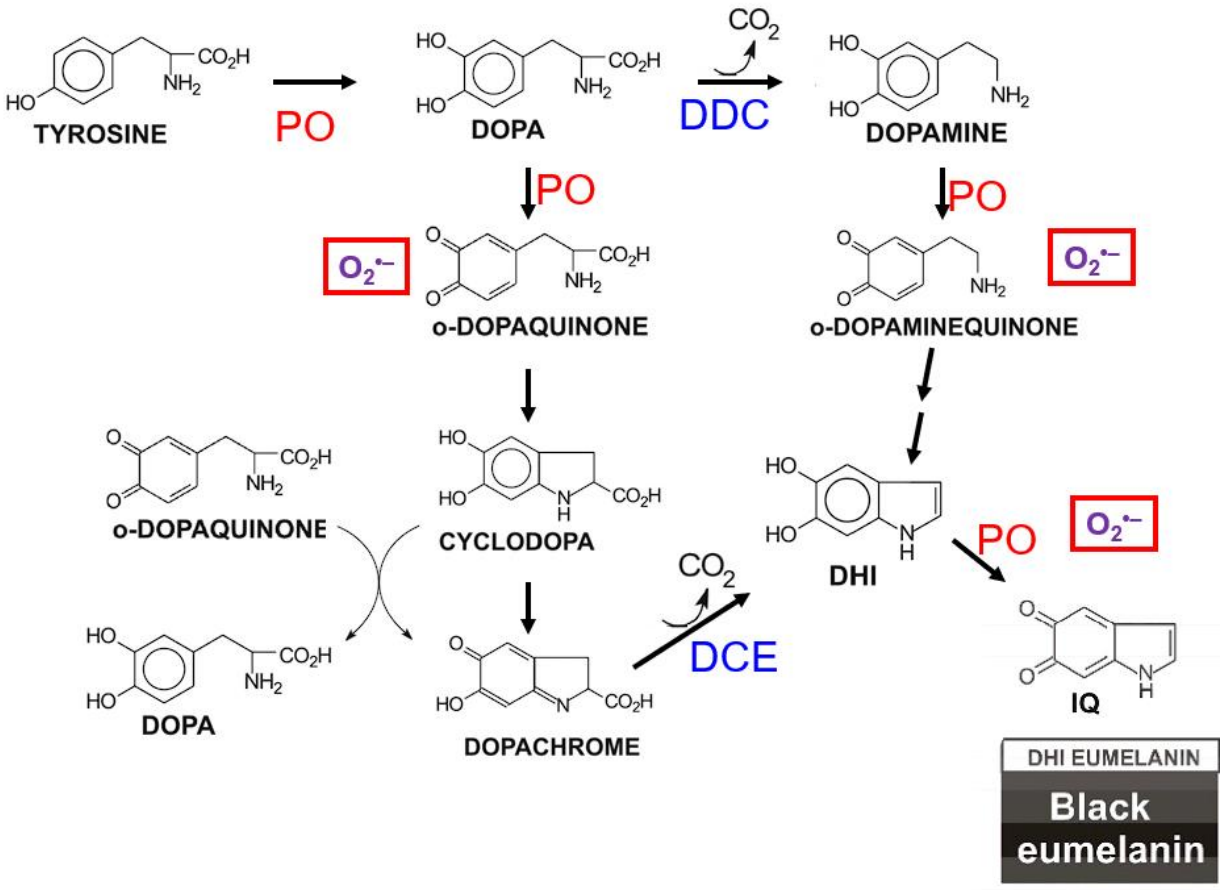

Figure 1 The redox reactions and ROS generation involved in melanin synthesis from tyrosine hydroxylation and diphenol oxidation by PO. Tyrosine is hydroxylated by PO to form DOPA. DOPA decarboxylase can convert DOPA to dopamine. PO oxidizes DOPA and dopamine into quinones which produces ROS. Additional redox reactions and PO oxidation of 5,6-dihydroxyindole (DHI) promotes formation of melanin.

Phenoloxidase Activity:

Arthropods synthesize PO as a zymogen that is present in the plasma of some insects (Cerenius & Söderhäll, 2004; Kanost & Gorman, 2008). Immune stimulation by recognition of pathogenic molecular patterns activates various serine proteases in hemolymph. In *M. sexta*, the proPO-activating proteinase (PAP) cleaves proPO at a specific peptide bond near the amino-terminal end to generate active PO (An et al., 2009). However, the PO product produced by PAP has low activity. (Jiang, Wang, Yu, & Kanost, 2003; Zou et al., 2005). Purified PAP-1 from *M. sexta* was shown to be ineffective in activating proPO by itself and required the addition of serine proteinase homologs (SPHs), which act as co-factors (Yu et al., 2003) for efficient PO activation. This phenomenon was also observed when using purified *M. sexta* PAP-3 in conjunction with *M. sexta* SPH-1 and SPH-2 (Jiang, Wang, Yu, Zhu, et al., 2003). The need for both PAP and SPH for effective proPO cleavage and PO activation demonstrates a mechanism for regulation of proPO activation; for proper melanin synthesis to occur, all components must be localized at the site of melanization. (Gupta et al., 2005; Yu et al., 2003).

The serine protease cascade, responsible for PO activation, is initiated when fungal β-1,3-glucan binds to *M. sexta* β-1,3-glucan recognition proteins (βGRPs) or bacterial peptidoglycan binds to peptidoglycan recognition proteins (PGRPs) and microbe binding protein (MBP), leading to autoactivation of a cascade-initiating protease, proHP14 (Figure 2) (Y. Wang et al., 2011, 2022). In *M sexta,* Active HP14 can trigger the Toll immunity pathway or melanin synthesis through activation of serine protease cascades. HP14 can activate proHP2 and proHP21. HP2 cleaves proPAP2, while HP21 cleaves both proPAP2 and proPAP3 (Gorman, Wang, et al., 2007; He et al., 2018; Jiang, Wang, Yu, Zhu, et al., 2003; Y. Wang et al., 2014). The activated PAPs then cleave and activate co-factors SPH1 and SPH2, which promote the

cleavage of proPO. HP21 can also cleave proHP5, and active HP5 can cleave proHP6 (Y. Wang et al., 2020). HP6 can then cleave proHP8, which activates proSpätzle or proPAP1, which can activate proPO (An et al., 2009; He, Wang, Yang, et al., 2017; Yang et al., 2016).

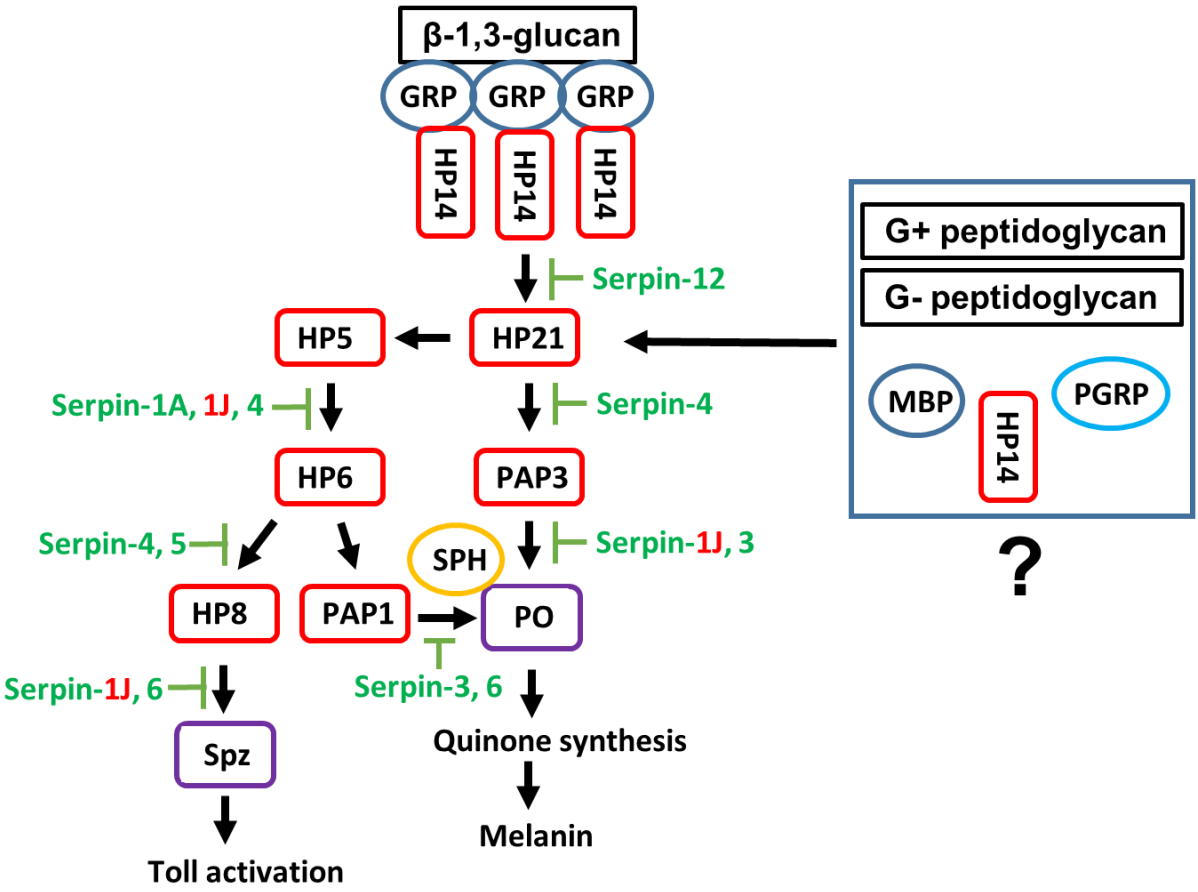

Figure 2. General overview of PO activation by the serine protease cascade triggered by the initiating protease proHP14. HP14 is activated upon recognition of fungal β-1,3-glucan via βGRP or bacterial peptidoglycan via proHP14 (autoactivation), MBP, and/or PGRP. HP14 activates HP21 activates both HP5 or PAP3. HP5 activates HP6 which can trigger melanization by activating PAP1 or the Toll pathway by activating HP8. HP8 activates Spätzle which then activates the Toll pathway. In addition to PAP1 or PAP3, the co-factor SPH is needed to properly activate PO for synthesis of melanin.

Maintenance of PO activity and the serine protease cascade is regulated by serpins. The serpin superfamily has a conserved tertiary structure containing three β-sheets, 8-9 α-helices, and an exposed reactive center loop (RCL) (Silverman et al., 2001). Inhibition by serpins involves the formation of a complex between the protease active site and the P1-P1' residues of the serpin RCL (Ye et al., 2001). Alignment of the P1 side chain with the primary specificity pocket of the protease promotes cleavage of the serpin scissile bond by the protease active site, forming a covalent acyl complex. The serpin undergoes a conformational change in which the RCL inserts into the β-sheet A. The protease is inactivated because the catalytic triad becomes distorted (Gettins & Olson, 2016). Nine serpins have been shown to be experimentally relevant to the PO cascade. Serpin-1 has 14 alternatively spliced isoforms (Li et al., 2018). Serpin-1J inhibits PAP1, PAP2, PAP3, and HP8 (An et al., 2011; Jiang, et al., 2003b; Y. Wang & Jiang, 2004). Serpin-1J and serpin-1A were also shown to inhibit HP5 (Y. Wang et al., 2020). Serpin-3 inhibits PAP1, PAP2, and PAP3 (Christen et al., 2012; Zhu et al., 2003). Serpin-4 inhibits HP1, HP5, HP6, and HP21 (Tong et al., 2005; Y. Wang et al., 2020). Serpin-5 inhibits HP1 and HP6 (An & Kanost, 2010; Tong et al., 2005). Serpin-6 inhibits PAP1, PAP3, and HP8 (Y. Wang & Jiang, 2004; Zou & Jiang, 2005). Serpin-7 inhibits PAP3 (Suwanchaichinda et al., 2013). Serpin-9 and serpin-13 inhibit conformationally active proHP1, HP2, HP6, and HP8 (He, Wang, Zhao, et al., 2017). Serpin-12 inhibits HP14 (Yang et al., 2018).

Hemolymph Redox environment:

Humoral immune activation of melanization by PO helps to protect insects against harmful pathogens. However, this process also generates reactive oxygen species (ROS) that can aid in combating invasion, but can also be harmful to the host (González-Santoyo & Córdoba-

Aguilar, 2012). Injection of nematode *Caenorhabditis elegans* into *Galleria mellonella* larvae increased insect stress, causing mortality (Tonogawa et al., 2021). *G. mellonella* injected with live nematodes exhibited a four-fold increase in ROS levels with accelerated mortality when compared to the control group (Tonogawa et al., 2021). Co-injection of ascorbate or N-acetylcysteine (NAC) with nematodes increased *G. mellonella* survival rate by 25% and 70% respectively (Tonogawa et al., 2021). The regulation of quinone products produced by PO and the redox reactions of melanization could be affected or modulated by the redox environment in insect hemolymph, depending on the concentration of antioxidant molecules.

Insects cannot synthesize ascorbic acid, vitamin C, and must obtain it from their diets (Kramer et al., 1981; Kramer & Seib, 1982). Two insect species have been observed to be capable of synthesizing ascorbate: Diptera, *Drosophila melanogaster,* and Lepidoptera, *Bombyx mori.* However, the synthesis of ascorbate in *B. mori* is insufficient for proper larval development and must be obtained through diet (Duque et al., 2022; Hou et al., 2021). Adequate amounts of ascorbate obtained through diet is important for proper larval development (K. Kramer & Seib, 1982). Insects must be able to detoxify harmful compounds present in plants. Compounds such as glutathione (GSH), uric acid (UA), and ascorbate assist in food detoxification (Jeschke et al., 2016; McMillan et al., 2018).

GSH in insects is involved in disease resistance and detoxification of plant glucosinolates and phototoxic constituents (Jeschke et al., 2016; Stahlschmidt et al., 2015; Timmermann et al., 1999). Glutathione-s-transferases (GSTs) use GSH as a substrate for the elimination of xenobiotics in addition to products of lipid peroxidation and ROS (Awasthi et al., 2017; Habig et al., 1974). GST aids in detoxification of xenobiotics and lipid peroxidation by molecular conjugation of GSH. Conjugation with GSH increase the solubility of neutralized products

allowing for enhanced excretion (Allocati et al., 2018; Jeschke et al., 2016). In the case of ROS, GST can indirectly reduce ROS (Pavlidi et al., 2018) In *Helicoverpa zea*, increasing concentrations of added ascorbate to substrate buffer linearly correlated to increased lag time in the activity of dietary polyphenol oxidase (Felton & Duffey, 1992). The involvement of ascorbate and uric acid in detoxification of dietary oxidants could be affected by the specificity of herbivory of insects. Generalist insects, like *Trichoplusia ni*, may rely more on dietary ascorbate and uric acid for detoxification of xanthotoxin than specialist insects like *Depressiaria pastinacella* (Timmermann et al., 1999).

The use of non-enzymatic antioxidants in the detoxification required for herbivory demonstrates a complex relationship between resource allocation for feeding and insect immunity. Insects given a nutritionally deficient diet had an increase in constitutive immune genes expression and also reduced resistance to oxidative stress, Gram-positive and Gram-negative bacterial infection, and hemolymph GSH concentration (Adamo et al., 2016). Dual challenges with the neurotoxin permethrin and injection of *Serratia marcescens* in *M. sexta* reduced GSH concentrations at a level equal to insects treated with only toxin or only bacteria (McMillan et al., 2018). Injection of GSH increased survival in insect groups with dual immune and toxin challenge and toxin only challenge. Anorexia in insects caused by illness could be a regulatory mechanism to allocate resources between immune defense responses and food detoxification capability (McMillan et al., 2018).

GSH and cysteine redox potential in hemolymph of a cricket, *Gryllus assimilis* shifted during development towards a more oxidized state that may be necessary for maturation (Sadowska-Bartosz et al., 2017). The increased redox potentials during development may be associated with developmental oxidative stress and the progressive oxidation of redox systems

(Sadowska-Bartosz et al., 2017; Sohal & Orr, 2012). Antioxidants in hemolymph may help regulate melanization and prevent damage to the host from melanization and PO activity. Increased PO melanization is accompanied by a decrease in the concentration of GSH in plasma (Clark et al., 2010). Melanization can be inhibited with the addition of exogenous GSH to plasma, this inhibition can be nullified when additional substrate is added. Melanization occurs when the concentration of GSH is less than the concentration of PO substrate. In native plasma melanization occurred when GSH concentrations decreased below 20μM. (Clark et al., 2010).

Antioxidants may prevent damage caused by an immune response. GSH may be consumed to prevent oxidative stress (Bordalo et al., 2020). The encapsulation response in *G. mellonella* decreases as PO activity increased. The initial increase in PO activity results in oxidative stress, depleting hemocytes and which in turn decreases the encapsulation response. The initial oxidative stress is followed by a decrease in both ascorbate and GSH. After mitigation of oxidative stress hemocytes begin to recover and the encapsulation response is stabilized. (Grizanova et al., 2018). There have been few measurements of antioxidant concentrations in *M. sexta* hemolymph. Previous reports done on fifth instar day 2 *M. sexta* hemolymph found that ascorbate concentrations were present at around 2.72 ± 2.27 mM (K. Kramer & Seib, 1982). Concentrations of GSH were also measured in fifth instar day 1-2 *M. sexta* hemolymph and was present in concentrations around 120-280 μM (Adamo et al., 2016; McMillan et al., 2018).

Insect plasma has a much more reducing environment than mammalian plasma, and the regulation of antioxidant concentrations may modulate PO activity and the effects of reactive oxygen species. An understanding of situational and specific conditions that activate PO is important for the context of melanization conditions. The form of bacteria may affect PO activity in hemolymph, depending on if peptidoglycan or whole cell bacteria are detected. I investigated

this and whether different types of bacteria or peptidoglycan linkages elicit different PO responses. Since PO activity is in the plasma, it is important to determine PO substrates present in plasma. In this work, I measured antioxidant concentrations and the redox environment after bacterial stimulation to examine the degree to which antioxidant concentrations change and the change in the capacity for the plasma to be reducing. Measurements of specific substrates and metabolites in stimulated plasma not only show the changes in substrate and metabolite concentrations but also show the relationship between them. To better understand how PO activity in plasma may be modulated by the redox environment, I assessed changes in specific metabolite and substrate concentrations involved in melanization.

Materials and Methods

Insects and bacterial peptidoglycan

M. sexta were cultured at 26˚C on a wheat germ-based artificial diet, with a photoperiod of 16 hours of light and 8 hours of darkness (Bell & Joachim, 1976). Hemolymph was collected from *larvae* in the second day of fifth instar. Bacterial peptidoglycan from *Micrococcus luteus, Staphylococcus aurueus, Bacillus subtilis,* and *Escherichia coli* were obtained from InvivoGen. The peptidoglycans were suspended in endotoxin free water that was provided by InvivoGen, and stored at -20˚C.

Hemolymph collection

Hemolymph was collected into a microcentrifuge tube by cutting the proleg of the insect with sterile micro scissors. The hemolymph was centrifuged using a Eppendorf centrifuge 5415D at 4˚C for 18 minutes at 16100 x g. Following centrifugation, the samples were put on ice and the supernatant was transferred into a new microcentrifuge tube. The hemocyte pellet was discarded, and the hemolymph plasma was stored at -80˚C.

Measurement of hemolymph plasma contents using mass spectrometry

Whole hemolymph and hemolymph plasma were analyzed by mass spectrometry at Bloodworks Northwest Research Institute in Seattle, Washington. Hemolymph replicates were prepared with the addition of N-Ethylmaleimide (NEM), to prevent disulfide exchange in the sample, and analyzed. Anti-coagulant solution (0.75 mL), composed of purified water from a Barnstead Nanoure Diamond at 18.2MΩ-cm, 4mM NaCl, 40mM KCl, 8mM EDTA, 9.5mM

citric acid monohydrate, and 27mM sodium citrate was added to a microcentrifuge tube. Hemolymph was collected directly into the microcentrifuge tube in a 1:1 dilution for a final volume of 1.5 mL. Each hemolymph sample was used to prepare two different conditions for analysis. One microcentrifuge tube contained 10µL of 100 mM N-Ethylmaleimide (NEM), to prevent disulfide exchange added to 100µL of the diluted hemolymph, mixing while pipetting. The other microcentrifuge tube contained only 100µL of the diluted hemolymph. These microcentrifuge tubes were sealed then flash-frozen in liquid nitrogen and stored at -80°C. The original diluted hemolymph tubes were centrifuged at 16,100 x g at 4˚C for 18 minutes and then placed on ice. The supernatants, called plasma samples, were also prepared for analysis. Two new microcentrifuge tubes were prepared for each sample. One tube contained 5 µL of NEM added to 100µL of plasma, mixing while pipetting, and the other tube contained only 100µL of plasma. The remaining hemolymph and the prepared sample tubes were sealed then flash-frozen in liquid nitrogen and stored at -80°C.

Hemolymph plasma samples treated by addition of *M. luteus* peptidoglycan were also analyzed by mass spectrometry at Bloodworks Northwest Research Institute. These hemolymph plasma samples were prepared similarly to the samples described above except that the anticoagulant buffer did not contain EDTA. Two experimental samples were made from each plasma sample. One sample was a control that included the addition of LAL water, and the other sample included the addition of *M. luteus* peptidoglycan suspended in LAL water. The final volume of the samples prepared was increased to 265 µL to accommodate for additional dilution caused from peptidoglycan addition. A microcentrifuge tube had 0.5 mL of anticoagulant solution added to it. Then 0.5 mL of *M. sexta* whole hemolymph was directly added to the tube for a final volume of 1 mL. The samples were then centrifuged at 16,100 x g at 4˚C for 18

minutes. After centrifugation, the samples were placed on ice, and the supernatant was transferred to a new microcentrifuge tube. In two new microcentrifuge tubes, 200µL of the plasma was transferred to each tube. The remaining plasma in the tube was snap-frozen in liquid nitrogen and stored at -80˚C. In one of the sample tubes, 50µL of LAL water was added. In the remaining tube, 50µL of 2µg/µL *M. luteus* peptidoglycan was added and mixed by pipetting. Both sets of samples were incubated for 30 minutes at room temperature. Then 12.5µL of 100mM NEM was added and mixed by pipetting. The tubes are then flash-frozen in liquid nitrogen and stored at -80˚C. The samples prepared for MS analysis were placed in a small box with a lid and shipped using an insulated container containing dry ice.

Quantification of glutathione (GSH), cysteine and the oxidized metabolites

Manduca sexta hemolymph plasma was treated with N-ethylmaleimide (NEM) as described above to block the free thiols and stop further disulfide exchange before the sample was frozen. After mixing with the isotopically labeled internal standards, the diluted plasma was used for mass spectrometry measurements of GSH, cysteine and the oxidized metabolites measurements as described below. For individual small molecule thiols and disulfides, the samples were treated with NEM. For total thiols including reduced and oxidized forms, a parallel aliquot of plasma was reduced with dithiothreitol (DTT) and then blocked the thiols with NEM. The two sets of samples were individually extracted with methanol and analyzed by liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (LC-MS/MS-MRM) as described previously (Fu et al., 2019).

Quantification of metabolites by LC-MS/MS-MRM

Metabolites (ascorbate, dehydroascorbate, tyrosine, dopa, and dopamine) in hemolymph plasma were measured after mixing with isotopically labeled internal standards (including L-tyrosine(U-13C9-15N), Dopamine-d4 and α-Ketoglutaric acid (13C5)), extracted with methanol and analyzed by LC-MS/MS-MRM analysis.

LC-MS/MS analysis was performed using a Waters ACQUITY I-Class ultra-performance liquid chromatograph coupled to a SCIEX QTRAP 6500 mass spectrometer with standard electrospray ionization source (ESI). Analytes were separated using a Waters HSS T3 column (2.1 mm × 100 mm × 1.8 µm). Tyr, Dopamine, Dopa and Tyrosine glucoside were monitored in positive mode using L-tyrosine(U-13C9-15N) or Dopamine-d4 as internal standards. Ascorbic acid and Dehydroascorbic acid were monitored in negative mode using α-Ketoglutaric acid (13C5) as an internal standard. All LC-MS/MS systems were controlled by Analyst® software version 1.6.2 (SCIEX) and MRM data were acquired using the same software. LC-MS/MS-MRM data was processed using MultiQuant 2.1 software (SCIEX). The peak area was used to quantify the concentration of analytes.

Effect of whole cell bacteria and different peptidoglycans on PO activity

PO activity was measured using replicates of plasma that were collected from individual insects and stored at -80˚C. For each insect sample 10µL of hemolymph plasma was added to wells of a 96-well plate with and without the addition of 10 µL of 0.1 µg/µL *M. luteus* freeze dried whole cells, peptidoglycan, or 10 µL water as a control. The plate was then incubated at room temperature for 15 minutes. 50 mM sodium phosphate buffer (pH 6.5) was then added to each well to reach a final volume of 50µL. 50µL of sodium phosphate buffer containing 2 mM

dopamine was added to each well and mixed by pipetting. Absorbance was measured at 470 nm for 30 minutes with one read per minute. The data for each well was then graphed and five-minute intervals were selected at the earliest times where linear increases in absorbance were observed, and these slopes calculated as the PO rate. PO activity was defined as one unit = 0.001 ΔA_{470}/min.

Additional PO activity experiments were performed using bacterial peptidoglycans from *M. luteus, S. aureus, B. subtilis,* and *E. coli* that were prepared at 0.1 µg/µL in purified water and stored at -20˚C. Hemolymph plasma samples stored at -80˚C were thawed, and 10 µL of plasma were added to five wells that are adjacent in the same row. In four of the wells, 10 µL of different bacterial peptidoglycan types were added to the plasma. Plasma in the remaining control well was mixed 10 µL water. The samples were incubated for 15 minutes at room temperature and then diluted with 50 mM sodium phosphate buffer (pH 6.5) to make the final volume 50 µL. 50 µL of sodium phosphate buffer containing 2mM dopamine was added to each well and mixed by pipetting. PO activity was then measured as described above.

Concentration dependence of bacterial peptidoglycan on PO activation

Different concentrations of peptidoglycan were added to hemolymph plasma to measure peptidoglycan concentration efficacy on PO activation. For each peptidoglycan type, a control, containing no peptidoglycan, and six different peptidoglycan concentrations were used. Each peptidoglycan stock solution was diluted in endotoxin free water so that final amounts were 1, 10, 100, 1000, and 10000 ng when the 40 µL of the dilution was added to the plasma samples. The peptidoglycan stock solutions were stored at -20˚C and the dilutions were kept on ice while the hemolymph plasma was collected from -80˚C storage and thawed at room temperature. 10

µL of plasma was added to every experimental and control well of a 96-well plate. Using a multichannel pipette, 40 µL of the peptidoglycan dilutions were added to the respective rows, mixing by pipetting. The samples were incubated at room temperature for 15 minutes. Following incubation, 50 µL of sodium phosphate buffer containing 2 mM dopamine was added to each well, and the contents were mixed by pipetting before measurement of PO activity.

Ascorbic acid measurements

Quantification of ascorbate was done using the Oxiselect™ Ascorbic Acid Assay Kit (FRASC), catalog number: STA-860 from Cell Biolabs. For each insect plasma sample four wells were used. A standard curve was made using ascorbic acid powder in purified water to a concentration of 200 mM, which was then diluted with 1X assay buffer to 2 mM. The 2 mM solution was further diluted to the concentrations listed in Table 1, and then placed on ice. The reaction reagent and ascorbate oxidase (AO) were prepared as directed in the kit manual and kept on ice. Hemolymph plasma was obtained from -80˚C storage and thawed at room temperature. For each sample, 5 µL of plasma was added to two adjacent wells and mixed with 5 µL water. In two adjacent wells, 5 µL of plasma was mixed with 5 µL of 0.1µg/µL freeze-dried *M. luteus*. The sample wells were then incubated at room temperature for 8 minutes. Following incubation, a multichannel pipette was used to add 1X assay buffer to make the final volume of the wells 100µL. 10µL of purified water was then added one of each paired sample wells and 10 µL of 1X ascorbate oxidase (AO) solution was added to the other paired sample well and mixed by pipetting. The samples were then incubated for 15 minutes at room temperature. After incubation, 100 µL of the reaction reagent was added to every well, including the ascorbic acid

standards and mixed by pipetting. The plate was then incubated at room temperature for 2.5 minutes, and the absorbance at 540 nm was measured.

The concentration of ascorbate in the plasma was determined from the difference in absorbance between the samples with and without ascorbate oxidase. The ascorbate standard curve was plotted to generate a line of best fit equation that was used to calculate the concentration of ascorbate present in each plasma sample.

Table 1. Ascorbic acid standard curve concentrations.

Standard Tubes	2 mM Ascorbic Acid (µL)	1X Assay Buffer (µL)	Final Ascorbic Acid Concentration (µM)
1	105	395	420
2	250 of #1	250	210
3	250 of #2	250	105
4	250 of #3	250	52.50
5	250 of #4	250	26.25
6	250 of #5	250	13.13
7	250 of #6	250	6.56
8	250 of #7	250	3.28
9	0	250	0.00

Total antioxidant concentration measurements

Total antioxidant concentration was determined using the OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit, Catalog Number: STA-360 from Cell Biolabs. Uric acid powder was dissolved in 1 N NaOH to make a 60 mM stock solution, which was then diluted further to make

a 2 mM uric acid solution. The 2 mM uric acid solution was further diluted to the concentrations listed in Table 2 to make a standard curve. The standards were placed on ice, and plasma samples were collected from -80˚C storage and thawed at room temperature. 5 µL of plasma was added to wells of a 96-well plate, with or without the addition of 5 µL of 0.1µg/µL freeze dried *M. luteus.* The plate was then incubated for 10 minutes at room temperature. During incubation, 14 µL of the uric acid standards were added to wells of a 96-well plate. Following incubation, autoclaved PBS (pH 7.4) was added to dilute the plasma wells to 14 µL. Using a multichannel pipette, 120 µL of 1X reaction buffer from the kit was added to each well and mixed by pipetting. The initial absorbance of the plate was then read at 490 nm. Using a multichannel pipette, 33 µL of 1X Copper Ion Reagent, from the kit, was added to each well and the plate was incubated for 5 minutes using the spectrophotometer's orbital shaker. Following incubation, a multichannel pipette was used to add 33 µL of 1X Stop Solution, from the kit, to each well and mixed by pipetting. The plate was then read again at 490 nm.

Net absorbance was calculated by subtracting the initial absorbance readings from the final absorbance readings. The uric acid standards were plotted to generate a line of best fit linear regression equation. The equation was used with the sample values to calculate the total antioxidant capacity in terms of uric acid equivalents (UAE), in mM. UAE values were then converted to copper reducing equivalents (CRE) by multiplying the values by 2189 µM Cu^{++}/mM uric acid. Sample CRE values are proportional to total antioxidant capacity.

Table 2. Uric acid standard curve concentrations.

Standard Tubes	2 mM Uric Acid Standard (µL)	Water (µL)	Uric Acid Concentration (µM)
1	0	0	2000
2	375 of #1	125	1500
3	250 of #1	250	1000
4	250 of #3	250	500
5	250 of #4	250	250
6	250 of #5	250	125
7	250 of #6	250	62.50
8	250 of #7	250	31.25
9	250 of #8	250	15.60

Statistical analysis

One-way ANOVA post hoc Tukey and paired t-test was performed using RStudio version 4.3.1 (2023-06-16) R Core Team (2023). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. One-way ANOVA post hoc Tukey and paired t-test was also performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Generation of tables and figures were done using RStudio and Prism.

Results

Bacterial Peptidoglycan stimulates activation of prophenoloxidase in *M. sexta* plasma

Insects respond to infections with innate immune responses stimulated after recognition of pathogens. Cell wall components of microbes are recognized as signals of infection. I investigated the activation of phenoloxidase (PO) after adding bacterial peptidoglycan to *M. sexta* larval plasma. PO activation was stimulated by either freeze-dried *Micrococcus luteu*s cells or purified peptidoglycan from *M. luteus* (Figure 3). At the doses used, there was no significant difference between the PO activity stimulated by whole bacteria or peptidoglycan.

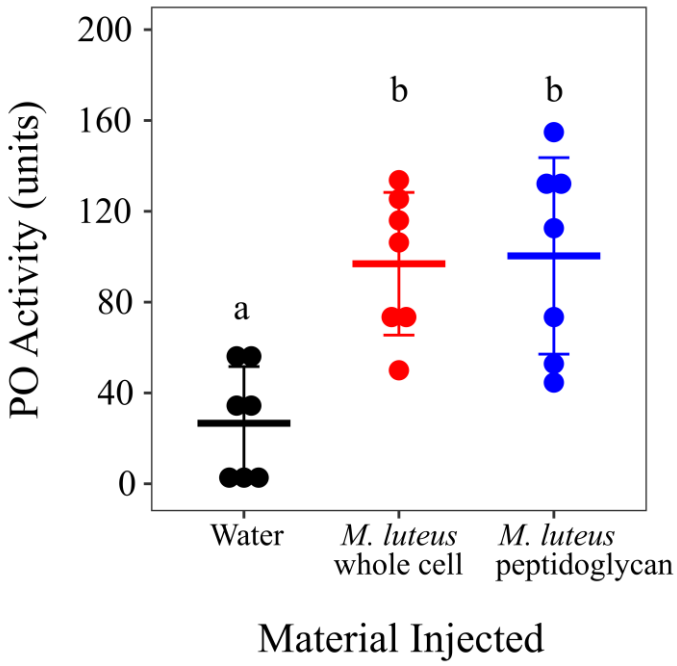

Figure 3 *M. luteus* freeze dried cells and *M. luteus* peptidoglycan stimulate PO activation in *M. sexta* plasma. 10 µL of plasma from day 2 fifth instar larvae were mixed with 1 µg freeze dried *M. luteus* or 1 µg *M. luteus* peptidoglycan, or with 10 µL water as a control. The samples were incubated for 15 minutes at room temperature, and the PO activity was measured as described in Materials and Methods. Each point indicates activity from an individual larva. *M. luteus* whole cell and peptidoglycan significantly stimulated PO equally. Cross bars and error bars represent the mean and standard deviation. Differing letters denote significantly different means at $p < 0.05$ (ANOVA post hoc Tukey).

To examine stimulation of PO activation by peptidoglycans from different bacterial species, PO activity was measured after mixing plasma with peptidoglycans from, *M. luteus, S. aureus, B. subtilis,* or *E. coli* (Figure 4). PO activity significantly increased regardless of peptidoglycan type when compared to the water control. At this peptidoglycan concentration there was no significant difference in the increased PO activity between the different types of peptidoglycans added.

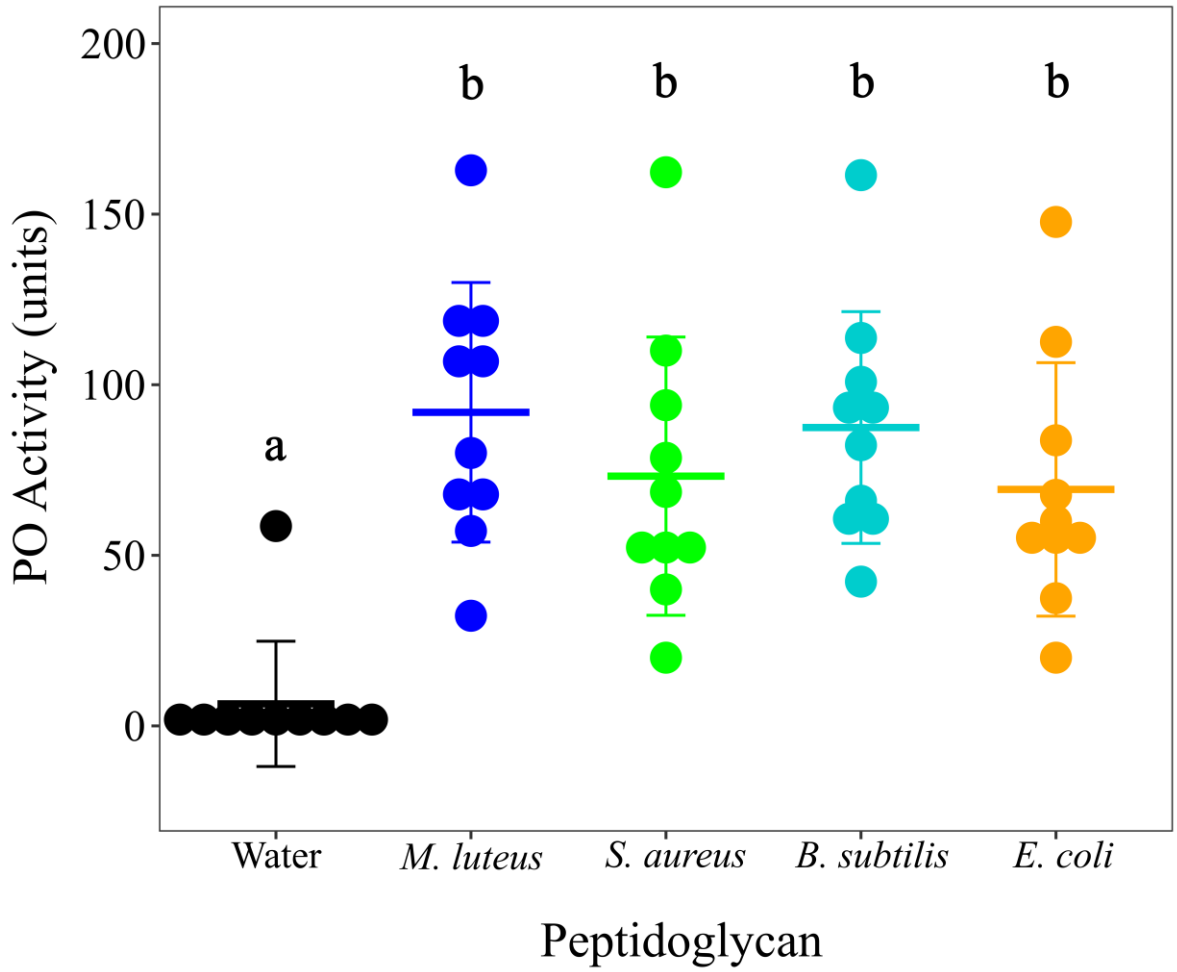

Figure 4. Stimulation of PO in *M. sexta* plasma with peptidoglycan addition from different bacteria. Plasma (10 µL) from second day fifth instar larvae was mixed 1 µg of peptidoglycan from Gram-positive *M. luteus*, *S. aureus*, and *B. subtilis*, or Gram-negative *E. coli*. The samples were incubated for 15 minutes at room temperature and then PO activity was measured as described in Materials and Methods. Plasma treated with peptidoglycan significantly increased PO activity, with similar increases observed irrespective of peptidoglycan type. Each point indicates PO activity of plasma from an individual larva. Cross bars and error bars indicate the statistical mean and standard deviation. Differing letters denote significantly different means at $p < 0.05$ (ANOVA post hoc Tukey).

PO Activation is dependent on peptidoglycan concentration

To determine if different concentrations of peptidoglycan from the bacterial species tested are required for proPO activation, peptidoglycans from *M. luteus, S. aureus, B. subtilis,* and *E. coli,* were added from 1 to 10,000 ng to *M. sexta* plasma (Figure 5). I observed that only one ng of peptidoglycan from *B. subtilis* could stimulate a significant increase in PO activity. In contrast, 1000 ng of *E. coli* peptidoglycan was required to elicit a significant increase in PO activity. Peptidoglycans from *M. luteus* and *S. aureus* required 10 ng or 100 ng, respectively, to elicit a significant increase in PO activity. For the three Gram-positive peptidoglycans there was a maximum PO activity at 1000 ng peptidoglycan. Amounts of peptidoglycans added at concentrations higher than 1000 ng showed a decrease in PO activity. With Gram-negative peptidoglycan from *E. coli,* PO activity increases from 1000 to 10,000 ng. These results suggest that difference in sensitivity to peptidoglycan with differing structures may affect the degree of activation of proPO as an immune response to different types of bacteria.

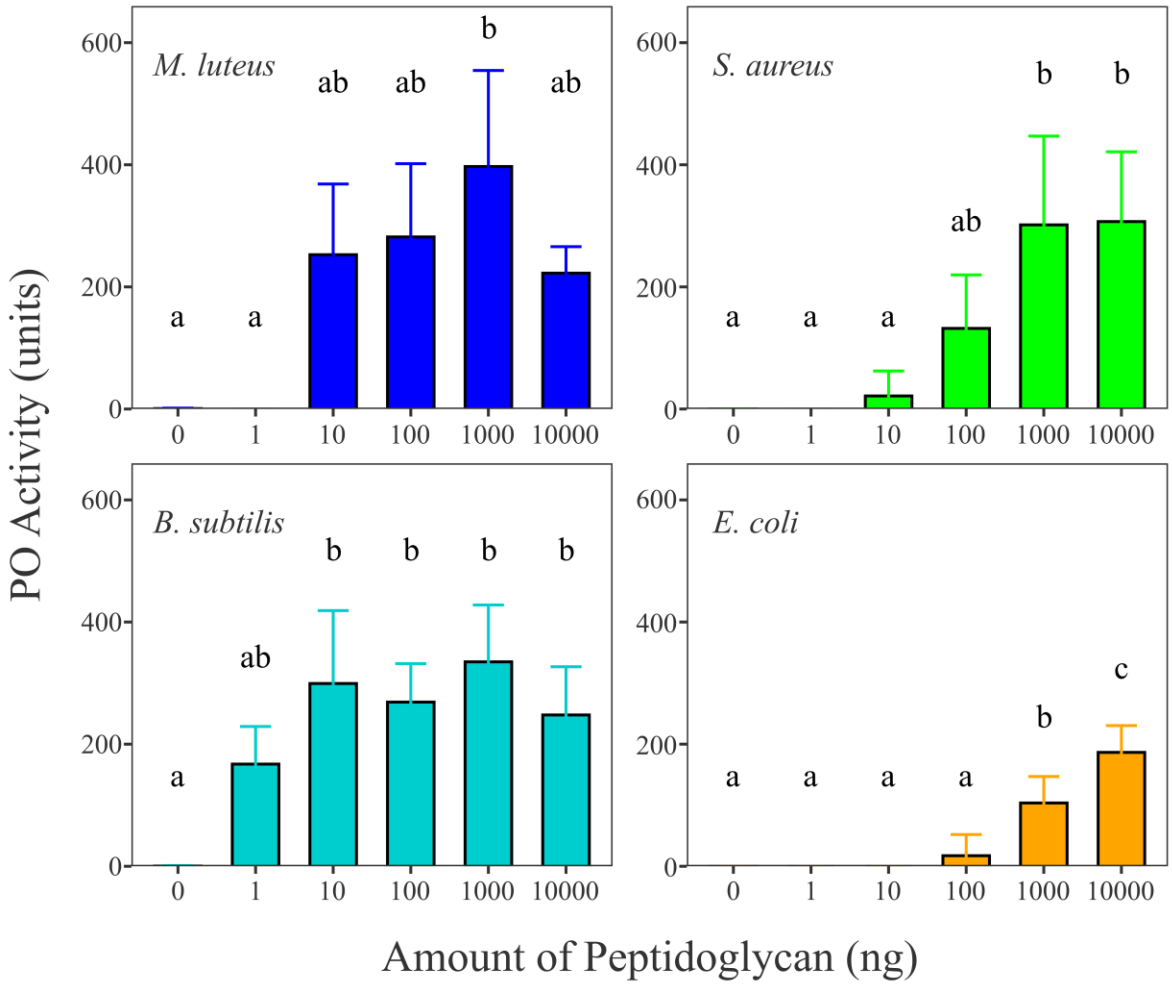

Figure 5. Effect of peptidoglycan concentration on stimulation of PO activation by different types of peptidoglycans. 10 µL of insoluble peptidoglycan from *M. luteus, S. aureus, B. subtilis,* and *E. coli* was added to 10 µL of plasma from *M. sexta* insects in the second day of fifth instar. Peptidoglycan amounts ranging from 1 ng to 10000 ng were added to each plasma sample group. The samples were incubated for 15 minutes at room temperature and the PO activity was measured as described in the Methods and Materials. At higher amounts of peptidoglycan PO activity was increased irrespective of peptidoglycan type. Lower amounts of Gram-positive peptidoglycan were able to significantly increase PO activity compared to Gram-negative peptidoglycan. The statistical averages and standard deviation of each peptidoglycan type amount is indicated using columns and error bars. Differing letters denote significantly different means at $p<0.05$ (ANOVA post hoc Tukey, n=4).

Quantitative analysis of selected plasma metabolites by mass spectrometry

Peptidoglycan stimulated activation of PO, which is expected to hydroxylate tyrosine to form DOPA, and to oxidize the diphenols DOPA and dopamine to their corresponding quinones (Kanost & Gorman, 2008). These reactions can produce reactive oxygen species such as superoxide and hydrogen peroxide (Komarov et al., 2005, 2009; Slepneva et al., 1999),which may react with antioxidant molecules present in hemolymph. To investigate how stimulation of PO activity affects concentration of PO substrates and products as well as antioxidants such as ascorbate, cysteine, and glutathione, we used methods to analyze plasma samples by mass spectrometry. In a preliminary experiment, we analyzed selected metabolite concentrations in plasma and in whole hemolymph (containing hemocytes) (Table 3. Concentration (μM) of selected metabolites in *M. sexta* hemolymph and plasma measured by mass spectrometry.). The results indicated that these metabolites are predominantly in the plasma fraction, and because the phenoloxidase reaction is expected to occur in plasma, we focused further analyses on changes of concentration of metabolites in plasma. Plasma contained relatively high concentrations of reduced thiols, including glutathione and cysteine, which may act as antioxidants. In contrast, the concentration of oxidized forms of glutathione and cysteine accounted for less than 10% of the total for these thiol compounds. Two trials carried out at different times, with different sets of plasma samples, indicated that the measurements were consistent and reproducible. In the first trial, we also included a set of duplicate plasma samples that were immediately mixed with the PO inhibitor phenylthiourea (PTU), to assess whether spontaneous PO activation during sample collection, storage, or shipment might affect the concentration of the thiol compounds. We found no significant difference between the plasma samples with and without PTU, indicating that any

spontaneous, low PO activation of these control samples did not affect concentration of the thiol compounds.

Table 3. Concentration (µM) of selected metabolites in *M. sexta* hemolymph and plasma measured by mass spectrometry.

Metabolite	hemolymph Trial 1 (n=8)	plasma trial 1 (n=8)	plasma + PTU trial 1 (n=8)	plasma trial 2 (n=9)
Total thiols	558 ± 160	451 ± 97	453 ± 90	446 ± 71
Total reduced thiol	422 ± 116	311 ± 66	306 ± 61	327 ± 52
Reduced glutathione	96 ± 23	105 ± 13	102 ± 14	69 ± 11
Oxidized glutathione	6 ± 2	10 ± 2	10 ± 3	6 ± 1
Cysteine	318 ± 95	205 ± 63	202 ± 57	253 ± 46
Cystine	25 ± 9	14 ± 8	15 ± 8	24 ± 7
Glutathione–ss-Cys	12 ± 4	17 ± 6	19 ± 7	12 ± 3
Ascorbate				586 ± 167
Dehydroascorbate				512 ± 175
Tyrosine				2,184 ± 714
DOPA				51 ± 47
Dopamine				1 ± 0.5

M. sexta hemolymph and plasma from the second day of 5th instar were analyzed for selected metabolites by mass spectrometry.

In Trial 2, we had available standards for measuring concentration of additional metabolites, including the PO substrates tyrosine, DOPA, and dopamine, as well as ascorbate and dehydroascorbate. Tyrosine was at much higher concentration in plasma (~2000 µM) compared with the diphenol PO substrates DOPA (~50 µm) and dopamine (~1 µm). In this trial

we also found that ascorbate, an antioxidant compound, and its oxidized product deoxyascorbate were present at relatively high concentration, both ~500 µM.

Addition of peptidoglycan to plasma results in conversion of tyrosine to DOPA and decreased concentration of ascorbate and free thiol compounds.

We carried out assays to investigate how activation of PO may affect concentration of PO substrates, products, and antioxidants in plasma. Because treatment of plasma with 1 µg of *M. luteus* peptidoglycan resulted in maximal PO activation, we used this treatment to stimulate PO activation and measured changes in metabolite concentration after 15 minutes.

We initially measured ascorbate concentration by a colorimetric assay that depends on oxidation of copper in the presence and absence of ascorbate oxidase. The concentration of ascorbate in plasma decreased about 1.5-fold after treatment with peptidoglycan (Figure 6A). We tested the effect of diluting a plasma with an anticoagulant buffer that contained citrate but not EDTA, a copper chelator, and observed similar decreased concentration of ascorbate after PO activation (Figure 6B).

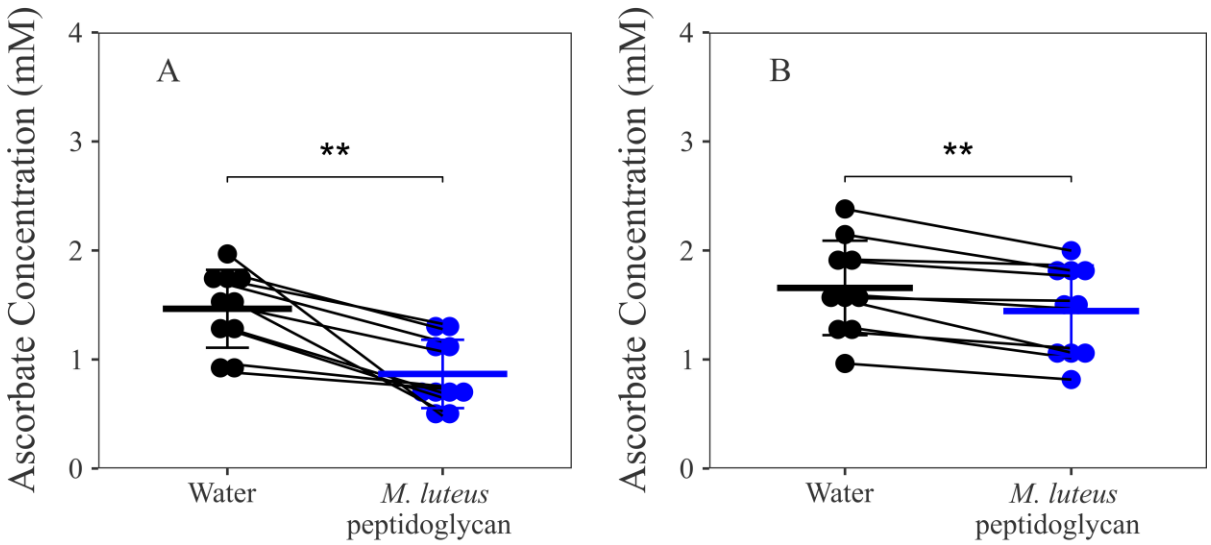

Figure 6. Activation of PO after addition of *M. luteus* peptidoglycan results in decreased concentration of ascorbate in plasma. PO activity was stimulated by adding 1 µg of *M. luteus* peptidoglycan to 5 µL plasma. After 15 minutes ascorbate concentration was determined by colorimetric assay as described in Materials and Methods. Plasma treated with added peptidoglycan (A) exhibited significantly lower ascorbate concentration compared to the paired controls of plasma from the same larva. Similarly, a different set of plasma samples diluted with anticoagulant solution (B) also showed significantly lower ascorbate concentration than the paired controls. (A). Lines connecting the symbols indicate change in concentration in the plasma from an individual larva. Note that ascorbate concentration decreased in each sample after peptidoglycan treatment. Asterisks denote a significance level of $p < 0.05$ (paired t-test, n=10).

PO Activation stimulated increased Total Antioxidant Concentration in Plasma

The same plasma samples were used to measure the concentration of total antioxidants. Plasma samples treated with *M. luteus* peptidoglycan to activate PO had significantly higher concentration of total antioxidants than the control plasma treated with water (Figure 7). The total antioxidant concentration in plasma that was not diluted with anticoagulant solution (Figure 7A) was lower in both the control and peptidoglycan groups when compared to the control and peptidoglycan groups in plasma diluted with anticoagulant solution (Figure 7B), perhaps due to citrate in the anticoagulant solution chelating copper that is the basis for the assay.

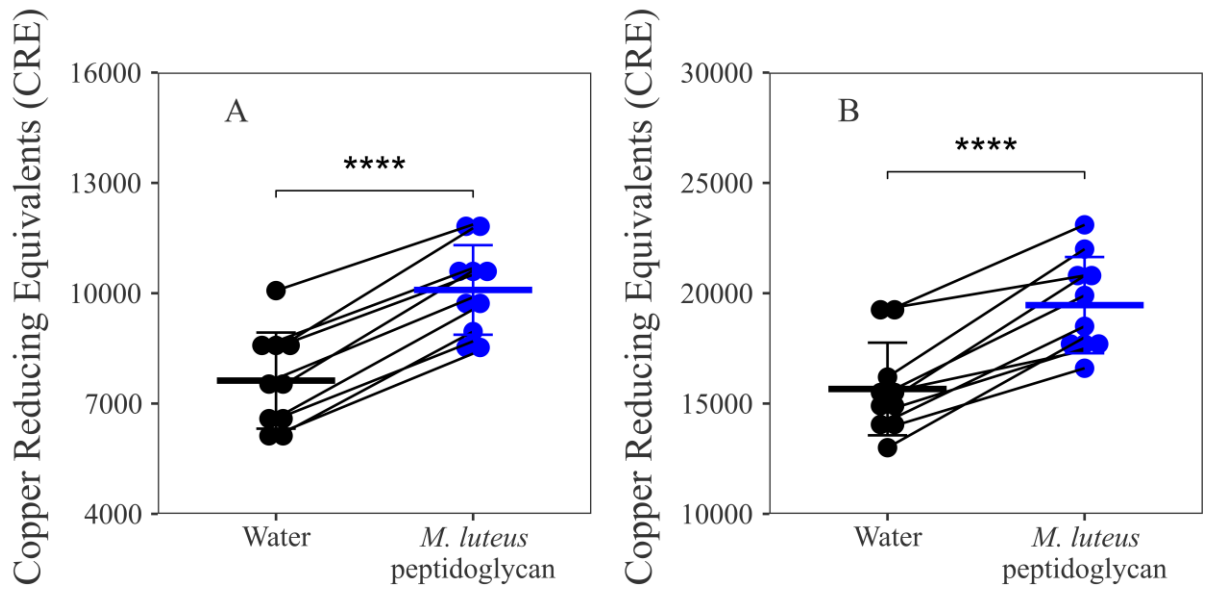

Figure 7. Activation of PO after addition of *M. luteus* peptidoglycan resulted in increased concentration of total antioxidants in plasma. PO activity was stimulated by adding 1 µg of *M. luteus* peptidoglycan to 5 µL of plasma. The samples were incubated for 15 minutes, and the total antioxidant concentration was determined using the Oxiselect™ Total Antioxidant Capacity Assay Kit (TAC). Plasma with added peptidoglycan (A) showed a significantly higher total antioxidant concentration compared to the paired controls. Similarly, plasma diluted with an anticoagulant solution (B) also had a significantly higher total antioxidant concentration than the paired controls. Paired replicates are linked by connecting lines. Asterisks denote the level of significance at $P < 0.05$ (paired t-test).

Activation of proPO by peptidoglycan stimulates changes in concentration of antioxidants and metabolites of PO.

To further investigate the effect of PO activation on concentration of PO substrates, products, and antioxidants, plasma samples were analyzed by mass spectroscopy. The samples were prepared in paired replicates, containing added *M. luteus* peptidoglycan or water as a control, as described in Materials and Methods.

The amount of total thiols and the amounts of thiols in different redox states were measured in plasma treated with or without peptidoglycan (Table 4). The concentration of total thiols and total reduced thiols was significantly lower in plasma treated with peptidoglycan.

There was no significant difference in the concentration of total oxidized thiols, and the concentrations detected were nearly the same in both the control and the peptidoglycan samples. However, the percentage of oxidized thiols present in the plasma treated with peptidoglycan was statistically significant compared to the controls.

Table 4. Mass spectroscopy analysis of total thiols and redox state thiols in plasma with or without *M. luteus* peptidoglycan.

Metabolite	Control mean ± SD (µM)	Peptidoglycan mean ± SD (µM)	p-value	significance
Total thiols	526.9 ± 108.3	284.7 ± 66.01	0.00	****
Total reduced thiols	367.6 ± 78.05	125 ± 47.67	0.00	****
Total oxidized thiols	159.3 ± 33.31	159.7 ± 34.81	0.93	NS
% Oxidized Thiols	0.3 ± 0.02	0.57 ± 0.09	0.00	****

Peptidoglycan or water were added to paired replicate samples of M. sexta plasma diluted with anticoagulant solution containing no EDTA (n = 10). Asterisks indicate the level of significance at $P < 0.05$ (paired t-test).

We examined the concentrations of the individual thiols: glutathione (GSH), cysteine (Cys), and homocysteine (Hcy), and cysteine-glycine (CG) (Table 5). The concentration of all these thiols was lower in the plasma treated with peptidoglycan. The total concentration of Cys had the largest difference between the control and the peptidoglycan-treated samples.

Table 5. Mass spectroscopy analysis of plasma thiols with or without addition of *M. luteus* peptidoglycan.

Metabolite	Control mean ± SD (µM)	Peptidoglycan mean ±_SD (µM)	p-value	significance
GSH-total	124.5 ± 45.69	92.27 ± 32.93	0.00	****
Cys-total	394.4 ± 77.65	185.4 ± 43.59	0.00	****
Hcy-total	5.19 ± 1.49	4.6 ± 1.32	0.00	**
CG-total	2.7 ± 0.97	2.31 ± 0.68	0.01	**

Peptidoglycan or water were added to paired replicate samples of M. sexta plasma diluted with anticoagulant solution containing no EDTA (n = 10). Asterisks indicate the level of significance at $P < 0.05$ (paired t-test).

We measured the concentrations of reduced and oxidized forms of glutathione and cysteine. The plasma samples treated with peptidoglycan had significantly lower concentrations of the reduced forms of these metabolites (Table 6). The largest difference of reduced thiols was the decreased concentration of free Cys in plasma samples treated with peptidoglycan. Samples with peptidoglycan also had higher concentrations of the oxidized forms GS-ss-Cys and GSSG but lower concentrations of cysteine

Table 6. Mass spectroscopy analysis of reduced and oxidized thiols in plasma with or without *M. luteus* peptidoglycan.

Metabolite	Control mean ± SD (µM)	Peptidoglycan mean ± SD (µM)	p-value	significance
GSH-free	80.74 ± 32.31	42.91 ± 17.93	0.00	****
GSSG	7.01 ± 4.16	10.43 ± 5.72	0.00	***
Cys-free	283.2 ± 55.97	80.11 ± 34.54	0.00	****
Cystine	35.4 ± 11	25.46 ± 8.15	0.00	****
GS-ss-Cys	19.04 ± 4.44	22.06 ± 5.87	0.01	**

Peptidoglycan or water were added to paired replicate samples of M. sexta plasma diluted with anticoagulant solution containing no EDTA (n = 10). Asterisks indicate the level of significance at $P < 0.05$ (paired t-test).

PO hydroxylates tyrosine to form DOPA and oxidizes DOPA and dopamine to their quinone forms (Kanost & Gorman, 2008). We measured the concentrations of these metabolites, and a storage form of tyrosine, tyrosine glucoside (K. J. Kramer et al., 1980; Lu et al., 1982), as well as the antioxidant ascorbate and its oxidized form dehydroascorbate to determine if their concentrations change after activation of PO by addition of peptidoglycan (Table 7). Addition of peptidoglycan led to decreased concentration of the PO substrate tyrosine and a corresponding increase in its product DOPA, consistent with a conclusion that activation of PO converted plasma tyrosine to DOPA. Concentration of dopamine did not change significantly, suggesting that little dopamine was converted to its quinone form by PO during the incubation. Tyrosine glucoside concentration did not change with PO activation, indicating that mobilization of this form of tyrosine did not significantly contribute to the tyrosine pool available as a PO substrate.

The concentration of ascorbate in samples treated with peptidoglycan decreased about 5-fold. This suggests that the oxidative conditions elicited by PO activity may consume the

antioxidant ascorbate. However, the concentration of dehydroascorbate did not increase as expected if ascorbate were being oxidized.

Table 7. Mass spectroscopy analysis of metabolites in plasma with or without *M. luteus* peptidoglycan.

Metabolite	Control mean ± SD (µM)	Peptidoglycan mean ± SD (µM)	p-value	significance
Tyr	1833 ± 562.6	937 ± 496.9	0.00	****
DOPA	84.89 ± 54.17	745.6 ± 230.6	0.00	****
Dopamine	1.21 ± 0.36	1.53 ± 1.04	0.29	NS
Tyrosine glucoside	305 ± 62.07	297.7 ± 45.77	0.26	NS
Ascorbic Acid	5087 ± 1370	950.9 ± 609.6	0.00	****
Dehydroascorbic Acid	853.6 ± 182.5	707.1 ± 204.5	0.11	NS

Peptidoglycan or water were added to paired replicate samples of M. sexta plasma diluted with anticoagulant solution containing no EDTA (n = 10). Asterisks indicate the level of significance at $P < 0.05$ (paired t-test).

The increased concentration of DOPA provides an explanation for the observation that total antioxidant concentration increased after treatment with peptidoglycan, even though the concentrations of the antioxidants ascorbate, cysteine, and GSH decreased. DOPA can act as an antioxidant (Rehmani et al., 2017). I measured the effect of DOPA concentration in the total antioxidant assay and found that DOPA concentrations in the physiological range of *M. sexta* plasma (80-800 µM) is detected by the total antioxidant assay and reaches a maximum signal at around 30 µM DOPA (Figure 8). The kit for measuring total antioxidant capacity uses copper, which has been shown to complex with DOPA at different pH levels (Kiss & Gergely, 1985). Additionally, the molar ratio between copper and DOPA can affect the solubility of the copper-DOPA complex (Kwik et al., 1974). Changes in solubility of the copper-DOPA complex may

also affect the solubility of free DOPA. It is also known that DOPA is a more efficient pro-oxidant than dopamine (Rehmani et al., 2017). Even though DOPA can act as an antioxidant, the change in DOPA solubility along with being a pro-oxidant may cause the redox potential to increase rapidly which could explain the plateaued total antioxidant capacity observed. DOPA produced by hydroxylation of tyrosine by PO, in addition to citrate in the buffer, unmeasured antioxidants, metabolites, and substrates may be responsible for the increased total antioxidant capacity detected after addition of peptidoglycan.

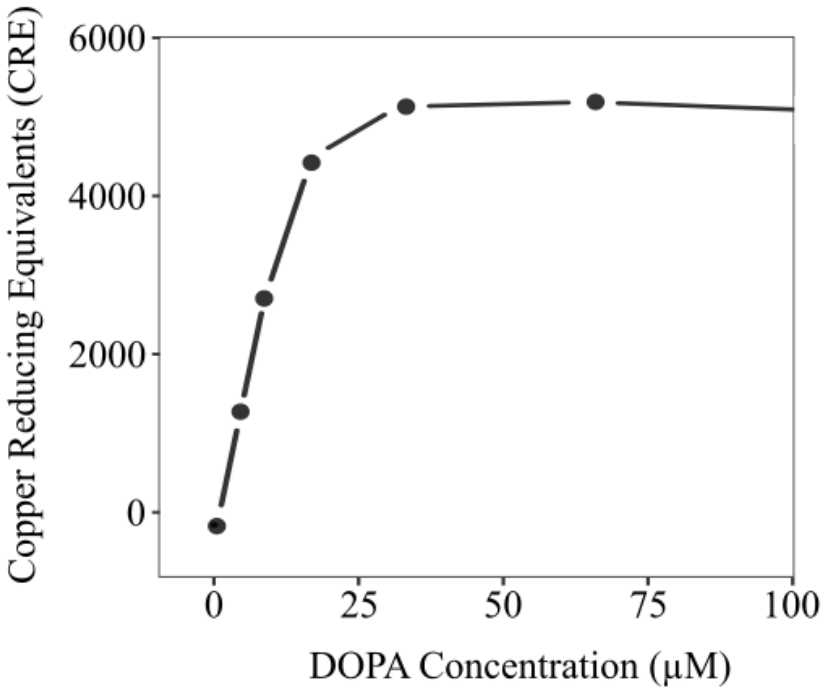

Figure 8. Total antioxidant capacity increases with increasing concentrations of DOPA. Standardized concentrations of DOPA were used to determine if DOPA affects the absorbance in the total antioxidant capacity assay.

Discussion and Conclusion

Bacterial peptidoglycan stimulates PO activation in *M. sexta* plasma

Stimulation of PO activation by bacterial peptidoglycan was performed using various insects and peptidoglycans primarily from *E. coli, M. luteus, B. subtilis,* and *S. aureus* (An et al., 2009; Sumathipala & Jiang, 2010; Y. Wang et al., 2011, 2022). The bacterial peptidoglycan backbone is made of alternating β-1→4 linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. Stem peptides are covalently bound to each MurNAc residue via a lactyl bond. Bacterial peptidoglycans share a common stem peptide motif of alternating D and L enantiomer residue, ending with two L-alanine peptides bound together (Garde et al., 2021). The third position stem peptide residue is a dibasic acyl acceptor and forms a 3-4 cross linkage with neighboring position 4 acyl donor stem peptides(Vollmer et al., 2008). Gram-positive peptidoglycan usually have an L-lysine residue at position 3 while Gram-negative peptidoglycan usually have a meso-diaminopimelic acid (m-DAP) residue (Garde et al., 2021). *M. luteus* and *S. aureus* are lysine type peptidoglycans with cross links stem peptides using an interpeptide bridge. The interpeptide bridge in *M. luteus* contains the same motif as the stem peptides, whereas the interpeptide bridge in *S. aureus* consists of five glycine residues (Kim et al., 2015; Vollmer et al., 2008). *B. subtilis* is a unique type of Gram-positive peptidoglycan with m-DAP dibasic residue at position 3, which is common with Gram-negative peptidoglycan. *B. subtilis* and *E. coli* are DAP type peptidoglycans that have a direct 3-4 cross linkage between the m-DAP and L-alanine stem peptide residues (Lebar et al., 2013). A unique feature of *B. subtilis* peptidoglycan is the presence of a carboxamide group on the m-DAP residue instead of the common carboxyl group seen in *E. coli* (Figure 9) (Lebar et al., 2014).

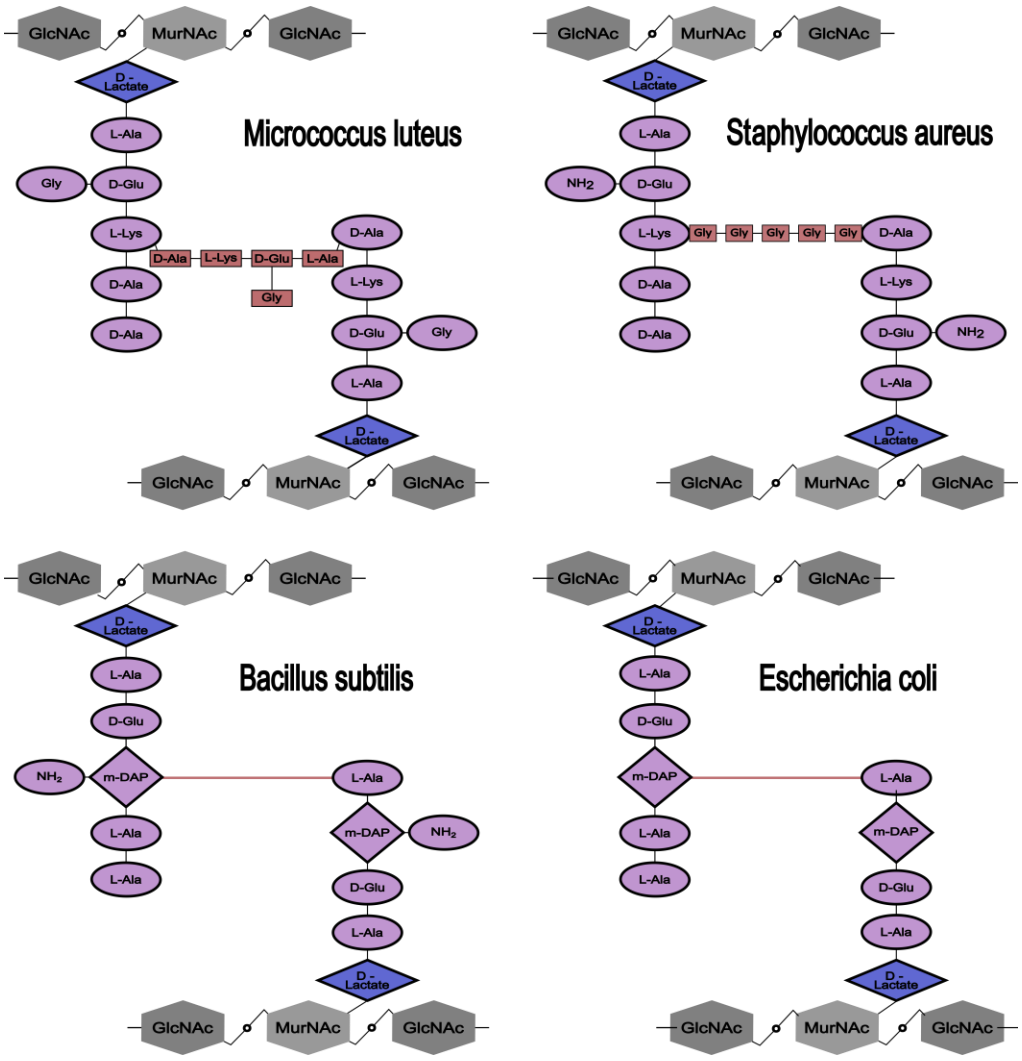

Figure 9. Bacterial peptidoglycan structure of the Gram-positive (M. luteus, S. aureus, B. subtilis) and the Gram-negative (E. coli). Commonly Gram-positive peptidoglycans are categorized as Lys type and Gram-negative as DAP type; B. subtilis is unique being a Gram-positive DAP type peptidoglycan. Alternating β-1→4 linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) form the glycan backbone. Peptide chains are connected to a MurNAc via a lactyl bond. The stem peptide consists of alternating D and L enantiomer residues, except for the endings which two D-Ala peptides can be bound together. The γ-carboxyl group of the D-Glu forms an isopeptide bond with the subsequent dibasic stem peptide (L-Lys or m-DAP). The four peptidoglycans contain a 3-4 cross linkage involving the acyl acceptor stem peptide (position 3) and the neighboring acyl donor stem peptide (position 4). M. luteus and S. aureus are Lys type peptidoglycan. M. luteus has a Gly bound to the D-Glu stem peptide while S. aureus instead has an amino group present. The stem peptides are cross linked by an interpeptide bridge bound to the ε-amino group of Lys and carboxyl end of the neighboring D-Ala (M. luteus [D-Ala-L-Lys-(Gly-D-Glu)-L-Ala] and S. aureus pentaglycine). B. subtilis and E. coli are DAP type peptidoglycans with a direct 3-4 cross linkage between the m-DAP ε-amino group and the C-terminus of L-Ala. B. subtilis m-DAP has a unique carboxamide group (NH_2) instead of the common carboxyl group.

I observed that *M. sexta* plasma incubated with different peptidoglycans at a relatively high concentration had similar increases in PO activity irrespective of peptidoglycan type. The similar stimulation of PO activation could be due to the multiple pattern recognition proteins having versatile peptidoglycan recognition (Kariyawasam et al., 2022; Y. Wang et al., 2011; Y. Wang & Jiang, 2010, 2017; Zhang et al., 2015). However, peptidoglycan concentration dependence experiments indicated that PO activity in *M. sexta* plasma is affected by the amount of peptidoglycan differently for different peptidoglycan types. PO was significantly stimulated with lower amounts, 1 ng to 100 ng of the three Gram-positive peptidoglycans tested than with the Gram-negative *E. coli* peptidoglycan. *B. subtilis* peptidoglycan stimulated PO at the lowest amount followed by *M. luteus* and *S. aureus*, with 10 ng and 100 ng respectively.

Additionally, *M. luteus* had the highest capacity to stimulate PO activity. This could be due to the interpeptide bridge having the same motif as the stem peptides, possibly enhancing peptidoglycan recognition from PGRP, MBP, or HP14. PGRP1 is the most abundant PGRP in the *M. sexta* hemolymph. Tyr90 and Asn89 are the binding residues in PGRP1 and the binding site is stabilized by either 17 residues or 21 residues depending on if DAP or Lys type peptidoglycan are present (Hu et al., 2019). Lys type peptidoglycans have lower docking energy and a higher dissociation constant compared to DAP peptidoglycans (Hu et al., 2019). Additionally, HP14 has highly specific binding for Lys type peptidoglycans in the LDL_{1-5}, Sushi, and Wonton domains (Y. Wang & Jiang, 2010). The specificity of HP14 and low docking energy of PGRP1 might contribute to the sensitivity of Gram-positive peptidoglycan recognition and stimulation capacity of *M. luteus*. Another possible explanation for the sensitivity of Gram-positive peptidoglycan needed to stimulate PO could be due to the PO cascade sharing proteins

that also activate the Toll pathway, HP1, HP6, and HP8, ensuring that PO is activated under immune pressure (An et al., 2009; He, Wang, Yang, et al., 2017; Y. Wang et al., 2020; Y. Wang & Jiang, 2008). With multiple shared proteins being activated, less peptidoglycan may be needed to stimulate PO due to the activation of multiple immune pathways.

Recognition proteins involved in the activation of PO, such as MBP, PGRPs, and proHP14 bind Gram-positive peptidoglycan (Hu et al., 2019). Some PGRPs and recognition proteins have improved binding and recognition to specific peptidoglycan types, and differentiation in PO (Shen et al., 2021; Sumathipala & Jiang, 2010). The capacity for specific peptidoglycans to stimulate PO and melanization may be determined by the length, solubility, or linkages of the peptidoglycans (Hu et al., 2019; Sumathipala & Jiang, 2010; Q. Wang et al., 2019; Y. Wang et al., 2022; Y. Wang & Jiang, 2010). Higher amounts of *E. coli* peptidoglycan were needed to stimulate PO activation and increasing amounts of *E. coli* peptidoglycan further increased PO activity. This was not observed for the Gram-positive peptidoglycans, which reached a maximum PO activity with 1000 ng of peptidoglycan. Interestingly, plasma with peptidoglycan exceeding 1000 ng had decreased PO activity. The need for increasing amounts of *E. coli* might be caused by preferential binding of PGRP1 to DAP peptidoglycans and the need for proHP14 to form a complex for activation by DAP peptidoglycans (Y. Wang & Jiang, 2017). Low amounts of *E. coli* peptidoglycan might lead to weak activation conditions due to competitive DAP peptidoglycan binding by MBP, PGRP1, and proHP14 LDLa and sushi domains (Y. Wang et al., 2022). MBP concentrations are low in plasma so insufficient amounts of peptidoglycan could lead to improper complex association (Y. Wang et al., 2011). Amounts of Gram-positive peptidoglycan exceeding 1000 ng led to lower PO activity. This observation might be explained by unproductive and inefficient activation of HP14 and possibly interfering

with positive feedback regulation, which was observed for excess MBP (Y. Wang & Jiang, 2017).

PO activation alters metabolite concentrations.

Analysis of whole hemolymph and plasma fractions using mass spectrometry revealed that cysteine and glutathione are primarily located in the plasma, rather that intracellular in hemocytes. Increases in PO activity affect the redox environment in plasma, and additionally the redox environment may affect PO activity. A role for glutathione in reducing oxidative damage and insect defense has been suggested (Barbehenn et al., 2013; Jeschke et al., 2016; Sadowska-Bartosz et al., 2017). Another vital antioxidant, ascorbate, has been measured in *Galleria mellonella*, under immune response, *Heliothis virescens*, and *M. sexta* using electron paramagnetic resonance (EPR), colorimetric methods, and HPLC (Grizanova et al., 2018; Johnson & Felton, 2001; K. J. Kramer et al., 1981). Ascorbate concentrations in *G. mellonella* was the lowest at about 40 µM and was the highest in *H. virescens* midgut at about 1.03 M. Previously reported ascorbate concentration in *M. sexta* hemolymph using HPLC was comparable to the colorimetric results (Figure 6) at about 2 mM.

It has been observed that the addition of antioxidants, such as ascorbate, directly into hemolymph can decrease ROS concentrations in *G. mellonella* or inhibit melanization and virucidal activity in *G. mellonella* and *H. virescens* (Shelby & Popham, 2006; Tonogawa et al., 2021). Increased ROS production caused by active PO or ingested phototoxic plant constituents has been shown to decrease concentrations of ascorbate. *T. ni* and *D. pastinacella* fed xanthotoxin and *Antheraea mylitta* infected with microsporidia exhibited decreased ascorbate concentrations in hemolymph when ROS production was increased during an immune response

or exposure to UVB radiation respectably (Jena et al., 2016; Timmermann et al., 1999). *H. zea* eating plants containing quinones show high ascorbate free radical reductase enzymatic antioxidant activity in the midgut at 168 nmoles/min/mg protein, providing evidence that ascorbate is used to reduce ROS produced from dietary quinone detoxification (Felton & Duffey, 1992). The significant decrease in ascorbate concentration in plasma with active PO that I observed suggests that antioxidants, such as ascorbate, are affected by increases in PO activity by reducing quinones, ROS, or other self-damage (Felton & Duffey, 1992; Jena et al., 2016; Timmermann et al., 1999; Tonogawa et al., 2021). I expected that the oxidative compounds produced by PO activation might oxidize ascorbate to dehydroascorbate. However, even though ascorbate concentration decreased, the concentration of dehydroascorbate did not significantly change. The fate of the ascorbate that was lost is not clear. Plasma with increased PO activity was shown to have increased total antioxidant capacity. This observation is contrary to expectations and observations of decrease levels of non-enzymatic antioxidants like ascorbate and GSH (Clark et al., 2010), and may be due to increased concentrations of DOPA, which has antioxidant activity (Rehmani et al., 2017).

Effect of PO Activation on non-Enzymatic Antioxidant Environment in Plasma

The concentration of ascorbate measured using mass spectroscopy, 5.1 ± 1.4 mM, in the control plasma is comparable with previous HPLC measurements of ascorbate in *M. sexta* hemolymph, 2.72 ± 2.27 mM (K. J. Kramer et al., 1981). Ascorbate concentrations differed between the Oxiselect™ kit and mass spectroscopy by about 3-fold. However, the decrease in ascorbate concentration after PO activation was consistent with both measurement methods. We

expect that the mass spectroscopy method provided more accurate measurements compared with the somewhat indirect colorimetric assay.

The high concentrations of reduced thiols and ascorbate, 367.6 μM and 5087 μM respectively, in *M. sexta* plasma suggests a highly reducing environment, which further increased when tyrosine was converted to DOPA. PO activation caused a decrease in the concentration of cysteine, glutathione, and particularly ascorbate; However, the decrease in concentration was not accompanied by an increase in total oxidized thiols or dehydroascorbic acid, which is difficult to explain. It was clear that activation of PO alters the redox environment in *M. sexta* plasma and decreases concentration of the reducing agents glutathione, cysteine, and ascorbate.

Previous studies and my experiments showed that concentration of tyrosine in plasma decreased when PO is activated, consistent with a conclusion that tyrosine is being oxidized to DOPA (Clark, 2015; Clark & Strand, 2013). The concentration of DOPA was about 70-fold higher than dopamine in control plasma and increased nearly 500-fold after PO activation (Table 7). The PO substrate dopamine did not significantly change with stimulated PO activity, suggesting that DOPA may be the metabolite primarily oxidized to form quinones for melanization. Tyrosine glucoside has been detected in hemolymph of a majority of surveyed Lepidoptera (K. J. Kramer et al., 1980). The presence of tyrosine glucoside is dependent on larval growth stages. Tyrosine glucoside is not detectable in fourth instar larvae but significantly increased in the fifth instar into late wandering stage (K. J. Kramer et al., 1980). Concentrations in mature fifth instar *Plodia interpunctella* and *Ephestia cautella* were approximately 3.2 mM and 6.1 mM respectively. Concentrations found in *M. sexta* were higher at 15 mM in day four fifth instar and 30 mM for wandering larvae (Ahmed et al., 1983). Previously measured tyrosine glucoside concentrations in day two fifth instar larvae was approximately 873 μM (Ahmed et al.,

1983), compared to the 305 μM measured in this study using mass spectrometry Tyrosine glucoside concentrations did not significantly change with increased PO activity, suggesting that tyrosine stored as tyrosine glucoside and used in the pupal ecdysis and pupal-pharate adult period (Lu et al., 1982) is not a source of tyrosine for immune melanization.

The concentration of total GSH measured in my *M. sexta* plasma samples, 124.5 μM, is higher than the average concentration of GSH observed in *P. includens*, 55 μM (Clark et al., 2010). When compared to previously reported GSH concentrations in *M. sexta*, the concentration of total GSH, 124.5, is comparable to fifth instar day 1 larvae, approximately 130 μM, but lower than what was observed in other fifth instar day 2 larvae, approximately 370 μM (Adamo et al., 2016; McMillan et al., 2018). After addition of bacteria or peptidoglycan to plasma, there is a lag period before the dopachrome product of DOPA oxidation begins to accumulate (Clark et al., 2010), which corresponds with decreased concentration of GSH in *P. includens* (Clark et al., 2010). In my PO assays with *M. sexta* plasma, this lag period was typically 2 to 8 minutes (data not shown). After 15 minutes of incubation with peptidoglycan, GSH concentrations decreased approximately 53%. It could be inferred that melanization could be correlated to a decrease in GSH of 37%-53% with sample variance relating to additional factors, such as fluctuations in various antioxidant concentrations or intrinsic variability of enzymatic activity, contributing to increased PO activity and melanization. These observations are consistent with an interpretation that as PO begins to oxidize DOPA to quinones, they may be reduced again by the antioxidants in plasma, and when the antioxidants are sufficiently consumed, the dopachrome and its product indole quinone accumulate and lead to polymerization to form melanin.

The composition of GSSG was about 5% of the total glutathione, which is close to the expected range of 1-3%. GSSG is derived from the oxidation and disulfide linkage of 2 GSH

molecules, meaning there is double the amount of GSH present than GSSG. Additionally, GSH is an important factor in defenses in oxidative damage, so GSSG is readily converted to GSH to maintain the highly reducing environment homeostasis, thus keeping concentrations of GSSG low in hemolymph (Barbehenn et al., 2013). Increased PO activity caused a greater decrease of free cysteine concentration than reduced GSH. The concentrations of cysteine related metabolites also significantly changed, possibly indicating cysteine bound metabolites may be a more sensitive indicator of oxidative damage (Fu et al., 2019). Depending on the type of tissue or sample used, a certain fraction of total cysteine is used to produce GSH. The fraction of total cysteine used to produce total GSH measured was 24%, similar to observations in another generalist insect, *Lymantria dispar*, which was between 21-23% (Barbehenn et al., 2013). Cysteine can form disulfides with various thiols and with free cysteine in proteins. Cysteine can be used to reduce ROS, but its concentration may also decrease due to various disulfide formation, such as Cys-ss-Cys, CysGly-ss-Cys, GSH-ss-Cys, and protein bound cysteine (protein-ss-Cys) (Fu et al., 2019). Cysteine may act as a storage molecule to maintain availability of various antioxidants and retain metabolites that would otherwise be excreted (Fu et al., 2019). Additionally, disulfides formed during oxidative stress could play a role in influencing the balance of total cysteine and exposed proteins in the plasma (Fu et al., 2019). GSH and ascorbate may be generalized reductants that are rapid and efficient metabolites for ROS reduction, while cysteine and its disulfide derivatives provide a more intricate method in supporting oxidative damage and direct influence of redox potential.

The *M. sexta* plasma environment has high concentrations of non-enzymatic thiol antioxidants, ascorbate, and the substrate tyrosine. Activation of PO and melanization produces ROS that can aid in eliminating foreign material and infections but may also be damaging to the

host organism (Nappi & Christensen, 2005). A highly reducing environment would allow for regulation and prevention of host damage (Kanost & Gorman, 2008). The *M. sexta* plasma environment contains high concentrations of reductants that decrease when PO activity is increased by peptidoglycan recognition (Table 4, Table 7, Figure 6). Decreases in reducing potential can modulate the concentrations of reducing and oxidizing agents in the plasma (Sadowska-Bartosz et al., 2017). The non-enzymatic antioxidants in plasma may act as the preliminary mechanism for mitigating host damage resulting from ROS during immune responses to infections (Tonogawa et al., 2021).

During the encapsulation response, intracellular enzymatic antioxidant activity of superoxide dismutase (SOD), catalase, and GST has been shown to significantly decrease after initial infection, eventually increasing post PO activation. This pattern may indicate that non-enzymatic antioxidants might be the primarily extracellular while enzymatic antioxidants may be the intracellular response that aids in regulating the balance of non-enzymatic antioxidants during an immune response (Dubovskii et al., 2010; Lozinskaya et al., 2004). Since PO substrate and antioxidants are primarily in plasma, non-enzymatic antioxidants play a significant role in the immediate immune response. Management of initial immune reaction allows for proper execution of nodulation, AMP production, and hemocyte activity (Charles & Killian, 2015; Miller et al., 1994; Xu et al., 2012). Nylon implants were used to activate melanization in *G. mellonella* hemocytes and showed that the enzymatic antioxidants SOD, catalase, and GST exhibited decreased activity for the first 4 hours after implant incorporation. The activity of these enzymatic antioxidants increased to normal activity after 24 hours (Dubovskii et al., 2010). However, nylon implants used with *G. mellonella* plasma to activate melanization showed GST activity in plasma increased after 30 minutes to 4 hours and decreased to normal activity after 24

hours (Grizanova et al., 2018). These results demonstrate the relationship between hemocyte and plasma enzymatic antioxidants. Increased plasma GST activity can help maintain the redox environment when exposed to prolonged melanization. Hemocyte GST activity decreasing and then returning to normal activity after 24 hours could indicate that hemocytes are responsible for returning the plasma redox environment to homeostasis.

The environment in insect plasma is highly reduced and may be regulated by the concentrations of plasma antioxidants. There is a strong relationship between non-enzymatic antioxidants and neutralization of ROS generated during the melanization and PO response. Antioxidants such as thiols and ascorbate appear to be the primary mechanism for prevention of host damage during an immune response. Enzymatic antioxidants such as SOD, catalase, and GST might serve different functions depending on if they are intracellular or extracellular but share the same purpose of regenerating and maintaining the redox environment in *M. sexta*, either through rapid regeneration of non-enzymatic antioxidants or regulation of redox homeostasis after an immune response. Investigation of the change in metabolite concentrations using different concentrations of peptidoglycan to activate varying levels of PO would be beneficial in further understanding the importance of specific non-enzymatic antioxidants and reduction function of these metabolites to prevent host damage and elimination of infection.

Maintenance of the redox environment in *M. sexta* plasma seems to be regulated by specific mechanisms, signals, enzymes, and stimulus. The direct and indirect interactions between hemolymph and plasma molecules during melanization and homeostasis are intricate. Further analysis of additional substrates and metabolites in plasma with increased PO activity from bacterial peptidoglycan would be beneficial for understanding the redox environment and insect defense against pathogens and host damage. The effects of active PO in plasma on

substrates and metabolites was investigated. Analysis of these same substrates and metabolites in hemolymph containing hemocytes would be beneficial in confirming if intracellular production and regulation has a significant impact on the plasma redox environment during melanization by offsetting the significant decreases in antioxidants. It has been observed that tyrosine hydroxylase is absent in *M. sexta* plasma and is thought to be more effective at hydroxylating tyrosine than PO (Gorman, An, et al., 2007). Increased concentrations of DOPA during an immune response by tyrosine hydroxylase may promote increased oxidation of DOPA by PO which may further alter concentrations of substrates and metabolites or be unaltered due to increased involvement of hemocytes.

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