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The *Drosophila melanogaster* Prophenoloxidase System Participates in Immunity against Zika Virus Infection

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Abstract

Drosophila melanogaster relies on an evolutionarily conserved innate immune system to protect itself from a wide range of pathogens, making it a convenient genetic model to study various human pathogenic viruses and host antiviral immunity. Here we explore for the first time the contribution of the *Drosophila* phenoloxidase (PO) system to host survival and defenses against Zika virus (ZIKV) infection by analyzing the role of mutations in the three prophenoloxidase (*PPO*) genes in female and male flies. We show that only *PPO1* and *PPO2* genes contribute to host survival and appear to be upregulated following ZIKV infection in *Drosophila*. Also, we present data suggesting that a complex regulatory system exists between *Drosophila PPO*s, potentially allowing for a sex-dependent compensation of *PPO*s by one another or other immune responses such as the Toll, Imd, and JAK/STAT pathways. Furthermore, we show that *PPO1* and *PPO2* are essential for melanization in the hemolymph and the wound site in flies upon ZIKV infection. Our results reveal an important role played by the melanization pathway in response to ZIKV infection, hence highlighting the importance of this pathway in insect host defense against viral pathogens and potential vector control strategies to alleviate ZIKV outbreaks.

Graphical Abstract

The prophenoloxidase cascade is an important antimicrobial response in *Drosophila*. Here, we have shown that the prophenoloxidase system is involved in the *Drosophila* immune signaling and function against Zika virus infection. This information is critical for understanding the dynamics of insect-flavivirus interactions.

Keywords

Prophenoloxidase; Zika virus; antiviral immunity; *Drosophila melanogaster*

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Authors' contributions

GTE and IE designed the study. GTE performed the experiments and analyzed the data. GTE wrote the manuscript and IE revised it. Both authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that they have no competing interests.

Background

Zika is a small arthropod-borne virus (arbovirus) with an enveloped positive-stranded RNA genome, in the family *Flaviviridae* (genus Flavivirus). Within the Flavivirus genus, Zika virus (ZIKV) is phylogenetically closely related to other mosquito-borne Flaviviruses of global public health significance, such as Dengue (DENV), West Nile (WNV), Yellow Fever (YFV), and Japanese Encephalitis (JEV) viruses [1]. ZIKV is primarily transmitted by multiple *Aedes* mosquito species including *A. aegypti* and *A. albopictus*, with a broad geographical distribution of human infection in sub-Saharan Africa, southeast Asia, and the Americas [2,3]. ZIKV has been connected to several unprecedented disease outbreaks around the world within the last decade, leading the World Health Organization (WHO) to declare an emergency of public health with international concern in 2016 [4, 5]. Though most infected individuals are asymptomatic or have a mild clinical disease with fever, headache, rash, arthralgia and/or conjunctivitis, ZIKV has been linked to a wide range of severe neuroimmunological disorders in newborns and adults [6]. Prenatal infection, for instance, leads to adverse pregnancy and birth outcomes, most notably microcephaly and other neurodevelopmental abnormalities; Guillain-Barre syndrome, stillbirth, and miscarriages [7–10]. Because of the increasing geographic expansion of both the virus and its mosquito vectors, ZIKV continues to pose a serious threat to public health around the globe [11]. However, no effective vaccine and/or antiviral drugs have been licensed or made available to prevent or treat ZIKV infection.

The driving factors for ZIKV emergence predominantly center on its mosquito vectors, with host-ZIKV dynamics being increasingly recognized as the key to controlling the explosive outbreaks caused by this virus. Despite more than a century of intensive efforts, development of effective strategies to prevent *Aedes*-transmitted viral diseases continues to lag behind the burden of the diseases themselves, in part because of large gaps in the knowledge of viral transmission, pathology, epidemiology, and immunology. Flaviviruses such as Zika carry out their life cycle by utilizing the machinery and functions of the host cells, making host-ZIKV interactions especially indispensable for understanding infection at the level of cellular and molecular mechanisms [12]. Many of these crucial interactions, however, remain elusive. Thus, the development of an in vivo model for identifying the number and types of molecular components that may directly or indirectly contribute to the host immune defenses against ZIKV is urgently needed. Such model would complement efforts in vaccine development and may represent the most effective and practical solution to circumvent ZIKV emergence and disease.

The use of the fruit fly *Drosophila melanogaster* has particularly led to major advances in the characterization of the molecular events leading to the activation of immune defenses against various infectious microorganisms, including viral pathogens [13]. In addition to being instrumental in the characterization of immune responses against viruses that naturally infect *Drosophila*, previous work shows that the fly is also an excellent model system for studying many human pathogenic viruses, such as DENV and ZIKV, and neurodegenerative disorders, such as Parkinson’s and Alzheimer’s diseases [14–17]. *Drosophila* has been advantageous in studies of host-pathogen interactions and infectious disease control due to several reasons, many of which are attributed to evolutionarily conserved features between

flies and vertebrates [18]. This includes innate immune cascades, signal transduction pathways, and transcriptional regulators involved, for instance, in the NF-κB (Nuclear Factor kappa B) and JNK (c-Jun N-terminal Kinase) immune signaling, as well as fundamental cellular processes such as phagocytosis and apoptosis [18–20]. In addition, the *Drosophila* model is amenable to powerful and sophisticated genetic manipulations, making it easier to screen for pathogen virulence factors and rapidly identify host effector molecules and pathways involved in antiviral immunity [21, 22].

Drosophila lacks an adaptive immune response and relies exclusively on innate immunity with both its humoral and cellular arms to fight off invading pathogens. Besides the well-documented production of antimicrobial peptides (AMPs) through the core signaling pathways Toll, Immune deficiency (Imd), and JAK/STAT, a major defense mechanism in *Drosophila* involves the melanization reaction [23–25]. This pathway acts as one of the most immediate responses to infection and is tightly regulated by several serpins to avoid a systemic activation [26]. It links the humoral and cellular immune responses and results in the localized and confined production of a black pigment called melanin at the site of infection or injury to contain the pathogen and facilitate wound healing in the host [27]. In *Drosophila*, a specific class of hemocytes (blood cells) known as crystal cells synthesizes phenoloxidase (PO), a key enzyme in melanin biosynthesis, as an inactive prophenoloxidase (PPO) precursor. The recognition of infectious microorganisms triggers a PPO-activating enzyme (PAE) proteolytic cascade culminating in the release of PO. Activated PO then catalyzes the oxidation of tyrosine-derived phenols to quinones, which subsequently polymerize into insoluble melanin [28, 29]. Reactive oxygen species (ROS) and other toxic compounds are also generated during this conversion, which are associated with the killing of microbes and pathogens while ensuring the protection of the host [30].

The *Drosophila* genome contains three PPO genes; *PPO1*, *PPO2*, and *PPO3*, all on the second chromosome [28]. *PPO1* and *PPO2* contribute to the bulk of melanization in the hemolymph and are activated differentially depending on the agent triggering the initial immune response. Crystal cells, which make up ˜5% of the *Drosophila* hemocyte population, rupture and release PPO1 into the circulating hemolymph to provide rapid melanization upon infection or injury whereas PPO2 is stored in these cells for a later phase of melanization [28, 31]. *PPO3*, on the other hand, is primarily expressed in lamellocytes, another specialized hemocyte lineage that is differentiated following parasite infection [25, 29]. Lamellocytes are larval hemocytes involved in the encapsulation of parasites such as parasitoid wasps [31]. PPO3 is likely produced in its active form because lamellocytes require an infection signal to begin the differentiation process, making further signaling to activate *PPO3* redundant. *PPO1* and *PPO2*, however, require a proteolytic cleavage to be activated [32]. The cleavage of PPO1 is characterized by a clip-domain serine protease (SP) called Hayan, which is stimulated through a stepwise process involving other SPs [33]. Most notably, studies suggest that the differences in spatial localization, immediate or late availability, and activation mode highlight the functional diversification of the three *Drosophila* PPOs, with each of them having non-redundant but overlapping functions [29].

Despite the significance ascribed to the melanization reaction in *Drosophila*, the precise contribution of PO activation to host survival during viral infections has not been

investigated, thus limiting our mechanistic understanding of the fundamental immune effectors involved in insect host defenses. We recently reported for the first time that infection with ZIKV in *Drosophila* increases the PO activity in the hemolymph and melanin deposition at the injection site, indicating a possible antiviral immune role that opposes ZIKV infection [34]. Furthermore, these findings are specific to ZIKV and not to the *Drosophila* natural pathogen DCV, which suggests that ZIKV can modulate the PO cascade in the flies. Here, we further investigate the molecular basis of the interaction between ZIKV and the PO/melanization cascade using single, double, and triple *PPO* mutants to determine if these mutations alter the *Drosophila* resistance to ZIKV, as well as sex-specific immune responses during infection. Results from this work will reveal original strategies to counter ZIKV infection and have the potential to provide novel tactics for restricting disease transmission through mosquito vector populations.

Results

ZIKV infection activates the PO response in adult *Drosophila*

Previous research has demonstrated a potential involvement of the melanization reaction in the *Drosophila* antiviral immune defense during infection with ZIKV [34]. However, the exact role of the *PPO* genes during ZIKV infection has yet to be determined. To assess the contribution of each *PPO* gene to the *Drosophila* innate immune response against challenge with ZIKV, we first examined the expression of all three *PPO* genes in w^{1118} background control *Drosophila* adults at four time points following infection with ZIKV (Fig. 1). At 12 days post-injection (dpi), both female and male adults infected with ZIKV expressed significantly higher levels of *PPO1* and *PPO2* compared to other time points (Fig. 1A, B). In contrast, *PPO3* expression did not change in either sex at any time point (Fig. 1A, B). This suggests that both *PPO1* and *PPO2* may contribute to the *Drosophila* antiviral innate immune response, and likely play specialized roles during ZIKV infection. We next estimated ZIKV copy numbers in the infected flies using primer sequences against nonstructural protein 5 (NS5), the largest and most crucial protein in the zika viral replication complex [35]. NS5 contains a methyltransferase for RNA capping and a polymerase for viral RNA synthesis, and therefore forms an essential therapeutic target for interfering with viral RNA production [36]. We found that both infected w^{1118} females and males showed a significant increase in fold change at 12 dpi (6-fold increase compared to 4 dpi), which declined subsequently at 20 dpi (Fig. 1C, D). Collectively, these results show that ZIKV infection induces the transcriptional expression of *PPO1* and *PPO2* but not *PPO3* while replicating in wild-type flies.

Drosophila PPO1 and *PPO2* genes contribute to host survival against ZIKV infection

To further assess the role played by each *PPO* gene during ZIKV infection in *Drosophila*, we analyzed the survival of Δ*PPO* single, double, and triple loss-of-function-mutant adults, alongside that of the w^{1118} background controls. Fly survival ability was measured over the course of 20 days using ZIKV-infected or PBS control-treated flies. Infected Δ*PPO1* female and male flies showed no significant differences in survival compared to PBS and ZIKV w^{1118} control injections (Fig. 2A–D). However, the median survival of Δ*PPO1* male flies (Fig. 2D), but not Δ*PPO1* female flies (Fig. 2B), infected with ZIKV showed a significant

decrease at approximately 12 dpi compared to infected controls, indicating a potential sex-dependent role for *PPO1* in fly survival during ZIKV infection. Infected Δ*PPO2* females and males notably displayed increased sensitivity towards ZIKV infection compared to PBS- and ZIKV-injected w^{1118} controls (Fig. 2E–H), while Δ*PPO3* females and males exhibited a wild-type level of resistance upon challenge with ZIKV (Fig. 2I–L). Together, these results show that *PPO2* is required for resistance to ZIKV infection in both sexes whereas *PPO1* possibly enhances survivals in infected males only.

Consistent with the previous findings, survival rates of both infected female and male double *PPO* mutants (Δ*PPO1,2*) significantly decreased compared to PBS and ZIKV w^{1118} control injections (Fig. 3A–D). We also observed similar survival results in both female and male triple *PPO* mutants (Δ*PPO1-3*) infected with ZIKV (Fig. 3E–H). Notably, direct comparison of the survival rates of infected triple *PPO* mutants (Fig. 3G, H) (12 dpi) and those of the infected triple *PPO* mutants (Fig. 3E, F), shows a remarkable decrease in infected male survivals (Supplemental Fig. 1). These results confirm the involvement of *PPO1* and *PPO2* in the host immune response in a sex-specific manner, with adult male *Drosophila* exhibiting more susceptibility to ZIKV infection compared to female flies. To support our findings, we also estimated the ZIKV load in the infected single (Δ*PPO1*, Δ*PPO2*), double, and triple *PPO* mutants at 12 dpi using primer sequences against NS5, as previously described, and compared them to infected w^{1118} background controls. We found strongly elevated levels of *NS5* in all experimental groups except for the triple *PPO* mutants, both female (Fig. 4A) and males (Fig. 4B), compared to the controls, thus confirming that *PPO1* and *PPO2* loss-of-function mutations enhance ZIKV replication.

The *Drosophila* PO response participates in host immune signaling against ZIKV

To examine the correlation between the *Drosophila* PO response and innate immunity during ZIKV infection, we estimated the expression of genes regulated by immune signaling pathways in Δ*PPO* single, double, and triple mutants, in both female and male flies at 12 dpi. More specifically, we analyzed the activation of the RNAi pathway, a potent antiviral defense in *Drosophila* [18], the Toll and Imd pathways that regulate the activation of the NF-κB transcription factors DIF/Dorsal and Relish, respectively [13, 37], and the JAK/STAT pathway, a canonical mammalian antiviral pathway that is also induced in response to viral infections in the fly [38, 39]. We found that *Dicer-2*, which acts as a pattern recognition receptor in the *Drosophila* RNAi pathway [40], was significantly upregulated in infected Δ*PPO2*, Δ*PPO1,2*, and Δ*PPO1-3* female mutants compared to infected w^{1118} background controls (Fig. 5A). Expression levels of *Argonaute-2* (*Ago-2*), an essential component of the *Drosophila* RISC [41], were also strongly elevated in Δ*PPO2*, Δ*PPO1,2*, and Δ*PPO1-3* compared to infected controls (Fig. 5A). Notably, infected Δ*PPO1* female flies exhibited similar *Dicer-2* and *Ago-2* expression levels to their respective w^{1118} controls, indicating that only *PPO2* may interact with the *Drosophila* RNAi components (Fig. 5A). Both *Dicer-2* and *Ago-2* were strongly increased in infected Δ*PPO1-3* males compared to all other treatment groups except for Δ*PPO1* flies, which further confirms that *PPO1* does not correlate to the host RNAi pathway (Fig. 5B). Taken together, the significant upregulation of the RNAi components in flies that share a common mutation (*PPO2*) may suggest a

compensatory effect in the case of inactivation of the *PPO2* gene by the RNAi pathway in *Drosophila*.

In corroboration with the previous findings, we found a significant increase in the mRNA levels of the Toll-regulated AMP-encoding genes *Drosomycin* and *Metchnikowin* in *PPO* female mutants at 12 dpi (Fig. 5C). Compared to the expression levels in w^{1118} controls, *Drosomycin* was upregulated in *PPO2* and Δ*PPO1,2* female flies whereas *Metchnikowin* levels were only elevated in Δ*PPO1-3* mutants, therefore suggesting differential roles for the two Toll-pathway components that promote yet another compensatory effect in the case of inactivating *PPO* genes (Fig. 5C). *Drosomycin* levels were only upregulated in Δ*PPO1,2* male mutants, but *Metchnikowin* was significantly induced in both Δ*PPO2* and Δ*PPO1,2* female flies, which confirms a sex-specific ZIKV-pathology and a complex interplay between the *Drosophila PPO1* and *PPO2* genes (Fig. 5D).

We also observed elevated transcript levels of the Imd-regulated AMP-encoding genes *Diptericin* and *Cecropin-A1* in Δ*PPO* female flies at 12 dpi (Fig. 5E). *Diptericin* levels were markedly upregulated in Δ*PPO2*, Δ*PPO1,2*, and Δ*PPO1-3* compared to infected controls while expression of *Cecropin-A1* was only significantly increased in Δ*PPO2* female mutants, which highlights *PPO2* loss-of-function mutation as the key factor in these observations (Fig. 5E). Interestingly, we found no significant differences among any of the infected *PPO* male mutant groups compared to their w^{1118} controls, therefore indicating that ZIKV infection fails to activate Imd-mediated immunity in male *Drosophila* hosts (Fig. 5F).

Furthermore, we observed a strong upregulation of the JAK/STAT-regulated genes *TotA* and *TotM* in *PPO* female mutants at 12 dpi (Fig. 5G). *TotA* expression was specifically robust in Δ*PPO1* and Δ*PPO2* female mutants whereas *TotM* levels were higher in Δ*PPO1*, Δ*PPO2*, and Δ*PPO1,2* flies, which highlights an essential role for both genes that is compensated by JAK/STAT-mediated immunity (Fig. 5G). *TotA* was upregulated in Δ*PPO1* male flies but not in double or triple *PPO* mutants, which suggests an important role for *PPO1* that has not been observed in other immune pathways (Fig. 5H). *TotM* mRNA levels were enhanced in Δ*PPO1* and Δ*PPO1-3* male flies compared to the other infected groups (Fig. 5H). More notably, Δ*PPO1-3* male mutants had a significantly higher expression compared to the Δ*PPO1* flies, which indicates an important role for *PPO1* and a compensatory effect that is exacerbated in the case of inactivation of all three *PPO* genes (Fig. 5H). We found no differences in the expression levels of the STAT-regulated antiviral genes *Vago* and *Vir-1* at 12 dpi, (Fig. 5G, H). Together, these results suggest that Δ*PPO1* is a particularly debilitating mutation that triggers a highly potent JAK/STAT-mediated defense in *Drosophila*.

Drosophila PPO1 and *PPO2* are essential for hemolymph PO response and injury-mediated melanization following ZIKV infection

To further assess the effect of ZIKV infection on melanization, we measured the enzymatic PO activity with a previously established L-DOPA assay in adult hemolymph samples from wild-type flies and *PPO* mutants [42]. PO activity distinctly increased following ZIKV infection in female and male w^{1118} flies compared to their respective PBS-injected controls, which showed low levels of PO activity (Fig. 6A, B). No significant PO activity was detected in the hemolymph of unchallenged or infected Δ*PPO1* and Δ*PPO2* single,

double, or triple mutants. Consistent with these findings, we observed enhanced melanin formation at the injection spot in the thorax region of ZIKV-infected female and male w^{1118} flies compared to the PBS controls (Fig. 6C, D). In contrast, we found no melanization spot on the cuticle of PBS- or ZIKV-injected female and male *PPO* mutants, which collectively indicates that both *PPO1* and *PPO2* contribute to the melanization observed in the hemolymph and the wound site in flies upon ZIKV infection (Fig. 6C, D).

Discussion

Using *Drosophila* as an infection model for mosquitoes presents several limitations owing to significant physiological differences between the two insect species. Firstly, mosquitoes are hematophagous, feeding on vertebrate blood, while *Drosophila* primarily consume fruits. This dietary contrast is pivotal when investigating mosquito-borne infections like malaria, dengue fever, or Zika, as mosquitoes are direct vectors of these diseases [43]. Secondly, mosquitoes possess a more intricate and diversified immune system compared to *Drosophila*. Mosquitoes have evolved a specialized set of immune components, including distinct antimicrobial peptides, immune-related genes, and pathways, tailored to combat pathogens encountered during blood-feeding [44]. *Drosophila* lacks many of these specialized immune elements. Additionally, mosquitoes have unique functional organization of major body parts and gut regional specializations, which plays a crucial role in pathogen infection and dissemination—features absent in *Drosophila* [45]. Moreover, mosquito-borne pathogens have evolved specific strategies to evade the mosquito immune system, which may not be relevant in *Drosophila*.

Despite these physiological differences, *Drosophila* serves as a valuable model system for understanding the molecular functions of human pathogenic viruses, including flaviviruses, owing to its conserved developmental pathways shared with several vector mosquito species and the abundance of genetic resources available for studying gene function [46, 47]. Additionally, whole genome sequencing of mosquito vectors like *A. aegypti* and *Anopheles gambiae* has facilitated the characterization and comparison of antiviral immune genes, such as *Dicer-2* and *Ago-2* [48–50]. Studying fundamental immune processes in *Drosophila* provides critical insights into molecular mechanisms related to ZIKV infection. The current study's focus on *PPO* genes and their impact on viral infection, while possibly not directly translatable to mosquitoes, provides a foundational understanding of immune responses that could potentially have broader implications. By investigating the effects of depleting functional *PPO1* and *PPO2* in *Drosophila*, the present study enhances the applicability of its findings and offers valuable mechanistic insights. Furthermore, the use of an alternative model system like *Drosophila* allows for a controlled experimental setup and the ability to dissect specific pathways and mechanisms without the confounding factors often encountered in more complex organisms. This foundational knowledge can guide future research in mosquito systems and serve as a basis for hypothesis generation and experimental design. In the context of host pathology, forward genetic screens in *Drosophila* have identified genes regulating *Plasmodium* growth in *A. gambiae*, which suggests that *Drosophila* can be used effectively to identify relevant host factors in the mosquito [51]. Therefore, using *Drosophila* allows a thorough understanding of the host-virus interactions and the ZIKV-induced pathogenesis.

The MR766 ZIKV strain, which has a history of passage in mouse brain tissue, introduces the possibility of genetic and phenotypic alterations in the virus that may not represent the behavior and characteristics of other field strains of ZIKV [52]. Nonetheless, MR766 still holds value as a reference strain for certain aspects of ZIKV research. While passage in mouse brain could potentially introduce genetic and phenotypic alterations, it is important to recognize that such changes are not necessarily uniform or deterministic [53]. In some cases, passage may lead to adaptations that enhance viral replication but do not fundamentally alter the virus's core characteristics. Comparing the MR766 strain with more recent field strains can allow researchers to identify both shared and distinct features, shedding light on the evolution and progression of the virus over time.

The melanization reaction is a major immune response in arthropods that involves the rapid synthesis of melanin at the site of infection and injury. Flies carrying loss-of-function mutations in one or more *PPO* genes have already been generated without compromising larval viability [28, 29]. These models have particularly expanded our knowledge on the specific contribution of each *PPO* to host immune reactions. Two *Drosophila PPO* genes, *PPO1* and *PPO2*, have been ascribed critical roles in wound healing and microbe encapsulation while a third, *PPO3*, has been implicated in the anti-parasite melanization response [28, 29, 53]. Nonetheless, the melanization reaction remains one of the less characterized facets of *Drosophila* innate immune responses, especially during viral infections. Our work here takes advantage of the *PPO* mutant models and demonstrates for the first time that *Drosophila PPO1* is essential for male survival following ZIKV infection while *PPO2* is crucial for both female and male flies (Fig. 7A, B). Moreover, infected female and male wild-type flies upregulate *PPO1* and *PPO2* but not *PPO3* in response to the infection, thus inferring an important role for these two genes during viral infection. The lack of change in *PPO3* expression suggests that it is either 1) dispensable in host protection against ZIKV infection, 2) suppressed by zika viral components, or 3) largely restricted to lamellocytes and contributes to the encapsulation of a wasp egg, as established in previous studies [29]. Consistent with these findings is the elevated levels of the ZIKV crucial molecule NS5 in *PPO1* and *PPO2* single and double mutants. Interestingly, infected *PPO* triple-mutants had similar *NS5* levels to ZIKV-injected wild-type flies, which implicates other host signaling functions that prevent viral replication.

When a particular host defense component or pathway is inactivated, infection susceptibility will depend on whether the defect/mutation can be compensated for by the remaining pathways [54]. Therefore, combining the various mutations allowed us to show the different *PPO* functions to optimize melanization and immune responses in case of a viral infection. *PPO1* single mutation does not induce significant changes in the RNAi, Toll, or Imd signaling, but appears to interact with the JAK/STAT pathway in both females and males during infection. *PPO2* single mutation, on the other hand, induces higher RNAi, Toll, Imd, and JAK/STAT immune signaling in female flies. The same mutation in males, however, does not affect the induction of any of these pathways, thus indicating sex-specific immune responses and underlining the significance of host physiology during ZIKV infection (Fig. 7A, B). *PPO1* and *PPO2* double mutation parallels the results from *PPO2* single mutation in infected female flies, which suggests a complex regulatory immune system allows for the compensation of *PPO2* loss-of-function mutation by other immune pathways. *PPO1* and

PPO2 double mutation in male flies leads to the upregulation of only the Toll signaling and not the other pathways, which suggests *PPO1* and *PPO2* together interfere with the Toll pathway in male *Drosophila* hosts (Figure 7B). Remarkably, triple *PPO* mutations result in enhanced RNAi and Toll signaling but not Imd or JAK/STAT levels in female flies while male triple mutant males exhibited only higher RNAi and JAK/STAT signaling activity. These observations complement biochemical and genetic studies citing the *PPO* cascade as a mechanism that cooperates with other immune pathways in the integrated *Drosophila* defense against microbial infections [55, 56]. For instance, Toll activation has been linked to melanization regulation, with many SPs and serpins involved in both pathways at transcriptional levels [55, 56]. More specifically, research implicates a role for the combined action of the SP Hayan and the SP processing enzyme Persephone (Psh) in propagating Toll signaling downstream of pattern recognition receptors (PRRs) activating either Toll signaling or the melanization response [57]. The increased mortality in *PPO1* and *PPO2* single and double mutants could result from the less-efficient compensation by overactivated host immune pathways. We show that ZIKV cannot kill wild-type flies, which means that the immune pathway activation was already sufficient enough to fight off the infection in these flies. The various *PPO* mutations result in excessive immune activation of these pathways and may carry a cost for host fitness and health, as evidenced from the survival data we present here [58]. Because the melanization reaction is an immediate response, *Drosophila PPO*s may act as early sensors that rapidly detect ZIKV infection and activate other protective effector responses such as the RNAi, Toll, Imd, and JAK/STAT pathways in a sex-dependent manner. The specific function of each *PPO* gene and its involvement in these signaling pathways remains unclear and requires further molecular characterization. In addition, studies identifying the mechanisms of *PPO* activation in *Drosophila* during viral infections would be particularly useful.

In *Drosophila*, there is a growing body of evidence suggesting that immune pathways exhibit a degree of compensation, wherein the activation of one pathway can lead to the modulation or reinforcement of others. This phenomenon highlights the intricate and interconnected nature of the immune response in the fly. Several primary studies lend support to this notion. One notable example can be found in the research conducted by Tzou et al., where the authors demonstrated that the Toll and Imd pathways can functionally compensate for each other in certain contexts [59]. They observed that the activation of one pathway could enhance the expression of antimicrobial peptides mediated by the other pathway, indicating a degree of crosstalk and compensation between these immune pathways. Another study revealed that the Toll pathway can compensate for the loss of the Imd pathway in the response to certain Gram-negative bacterial infections [60]. The authors found that Toll pathway activation could partially rescue the susceptibility of Imd pathway-deficient flies to these infections, underscoring the potential compensatory interactions between these pathways. In another investigation, Buchon et al. explored the interplay between the JAK-STAT and Imd pathways in the context of gut immunity [61]. Their findings demonstrated that the JAK-STAT pathway could compensate for the loss of the Imd pathway in the regulation of antimicrobial peptide expression and host defense against oral infections. A more recent study suggests a potential compensatory mechanism, where the immune system may prioritize the expression of other PPO genes (PPO1 and PPO2)

to compensate for the lack of response from PPO3. The study indicates that compensation within the *Drosophila* immune response system might involve intricate interactions between various immune pathways. Different PPO mutations lead to varying effects on immune signaling pathways, such as RNAi, Toll, Imd, and JAK/STAT pathways, revealing that the immune system can adapt and compensate for disruptions in specific pathways by activating others [42]. Collectively, these primary studies provide compelling evidence for the existence of compensatory mechanisms among immune pathways in *Drosophila*. Such inter-pathway interactions contribute to the robustness and flexibility of the immune response in *Drosophila*, ensuring effective defense against a diverse array of pathogens.

Our study further confirms that both *Drosophila PPO1* and *PPO2* are crucial for the PO activity in the hemolymph and melanin formation at the injection site (Figure 7A, B). No PO activity was observed in any of the single, double, or triple mutants. In addition, the effect of ZIKV infection on melanization in *Drosophila* is also evident by the enhanced size and intensity of melanin spot at the wound site on the thorax of ZIKV-injected wild-type flies compared to flies injected with PBS. These findings indicate an interaction between ZIKV infection and the activity of the PO/melanization cascade in *Drosophila* adults. Future work will include identification and characterization of the ZIKV molecular components that regulate PO activity in *Drosophila* by examining the efficacy of these responses in transgenic flies overexpressing ZIKV core and non-structural proteins ubiquitously or in a tissue-specific manner. Results from this work are expected to reveal original strategies to counter ZIKV infection that may lead to novel tactics for restricting disease transmission through mosquito populations.

Methods

Fly stocks

w^{1118} flies were used as wild-type controls. Δ*PPO1*, Δ*PPO2*, Δ*PPO3*, Δ*PPO1,2*, and Δ*PPO1-3* flies were described previously [28, 29]. Δ*PPO1*, Δ*PPO2*, Δ*PPO3* flies were obtained from the Bloomington Stock Center. Δ*PPO1,2*, and Δ*PPO1-3* were obtained from the Lemaitre lab (EPFL, Switzerland). All fly stocks were reared on Bloomington *Drosophila* Stock Center cornmeal food (LabExpress), supplemented with yeast (Carolina Biological Supply), and maintained at 25°C with a 12:12-h light:dark photoperiodic cycle. Both sexes were selected from the same generation and randomly assigned to experimental groups.

Zika Virus stocks

Stocks of ZIKV strain MR766 were prepared as previously described [39].

Fly infection

Injections were performed by anesthetizing flies of the stated genotypes with carbon dioxide. For each experiment, 2–5-day-old adult female and male flies were injected with ZIKV suspensions in PBS (pH 7.5) using a nanoinjector (Nanoject II for immunostaining experiments and Nanoject III for all other experiments; Drummond Scientific). ZIKV stocks were prepared in PBS (pH 7.5). Live ZIKV solution (11,000 PFU/fly) (100 nl) were injected

into the thorax of flies, and control flies were injected with the same volume of PBS. Following infection, flies were maintained at 25°C and transferred to fresh vials every three days for the duration of the experiment. Fly deaths occurring within one day of injection were attributed to injury and were not included in the results.

Fly survival estimation

For each fly strain, three groups of 20 adult male and female flies were injected with ZIKV, and control groups were injected with PBS. Following injection, flies were maintained at a constant temperature of 25°C with a 12-hour light/dark cycle, and mortality was recorded daily.

Quantitative reverse-transcription PCR

Flies were collected at 12 days post injection and directly processed for RNA analysis. For each experiment, total RNA was extracted from 10 male or female flies, using TRIzol (Invitrogen) according to manufacturer's protocol. Total RNA (500 ng–1 μg) was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) experiments were performed with two technical replicates and gene-specific primers (Table 1) using a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories). Cycle conditions were as follows: 95°C for 2 min, 40 repetitions of 95°C for 15 s followed by 61°C for 30 s, and then one round of 95°C for 15 s, 65°C for 5 s, and finally 95°C for 5 s.

Hemolymph extraction and PO activity

One hour post injection, 20 female or male flies from each treatment group were collected into a Pierce® Spin Column (10 μM, ThermoFisher) and incubated for 10 minutes on ice. Protease inhibitor (20 μl, 2.5×) and five 4 mm glass beads were added to the spin column and centrifuged at 13,000 ×*g* for 20 min at 4°C. The supernatants were transferred to a new microcentrifuge tube containing 10 μl of 2.5× protease inhibitor on ice. The protein concentration of extracted hemolymph was adjusted with a Pierce BCA Protein Assay Kit (Thermo Fisher). A sample volume of 40 μl, containing a mixture of 15 μg of protein, 5 mM $CaCl_2$, and 2.5× protease inhibitor, was added to 160 μl L-DOPA solution (20 mM in phosphate buffer, pH 6.6) in a clear, 96-well flat bottom plate (Greiner Bio-One). A blank of L-DOPA solution was used as a negative control. The samples were incubated at 29°C in the dark in a Synergy HTX Mutli-mode Reader (BioTek) for 30 minutes. During the incubation, the optical density (OD) was measured at 492 nm at a frequency of 2-min intervals. The experiment was repeated three times with biological duplicates and technical triplicates. Two tailed t-tests were performed using the GraphPad Prism 9 software.

Melanization response

Adult flies (2–5-day-old) were separated into experimental groups containing 10 male or 10 female adults with two biological replicates and injected with either ZIKV or PBS as negative control, as described above. Three hours post treatment, photographs of flies were taken on a Keyence VHX-5000 digital microscope at 400X fitted with a VH-Z00T lens.

Quantification and statistical analysis

All analyses were conducted with data from three independent experiments with biological duplicates. For survival curves, pairwise comparisons of each experimental group with its control were carried out using a log-rank (Mantel–Cox) test. Data from quantitative reverse-transcription PCR was analyzed with gene-specific primers in duplicates, with at least three independent experiments for both test and control treatments. Fold changes were calculated with the $2^{-\Delta\Delta C}{}_{T}$ method using *Ribosomal protein L32* (*RpL32*), also known as *rp49*, as a housekeeping gene [62, 63]. All error bars represent standard error of mean. GraphPad Prism software 9 was used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Prof. Bruno Lemaitre (EPFL, Switzerland) for sharing the *PPO* mutants. We thank colleagues in the Department of Biological Sciences at GW for comments on the manuscript. This work was supported by a supplement to grant R01AI110675 to IE from the National Institute of Allergy and Infectious Diseases, and a grant to IE from the Columbian College of Arts and Sciences at George Washington University. GTE was funded through a Wilbur V. Harlan summer research fellowship from the George Washington University Department of Biological Sciences.

Data availability statement

The datasets supporting the conclusions of this article are included within the article.

Abbreviations

ZIKV	Zika virus
DENV	Dengue virus
WNV	West Nile virus
YFV	Yellow Fever virus
JEV	Japanese Encephalitis virus
WHO	World Health Organization
NF-κB	Nuclear Factor kappa B
JNK	c-Jun N-terminal Kinase
AMPs	antimicrobial peptides
Imd	immune deficiency
PO	phenoloxidase
PPO	prophenoloxidase

PAE	PPO-activating enzyme
ROS	reactive oxygen species
SP	serine protease
Psh	Persephone
PRR	pattern recognition receptor

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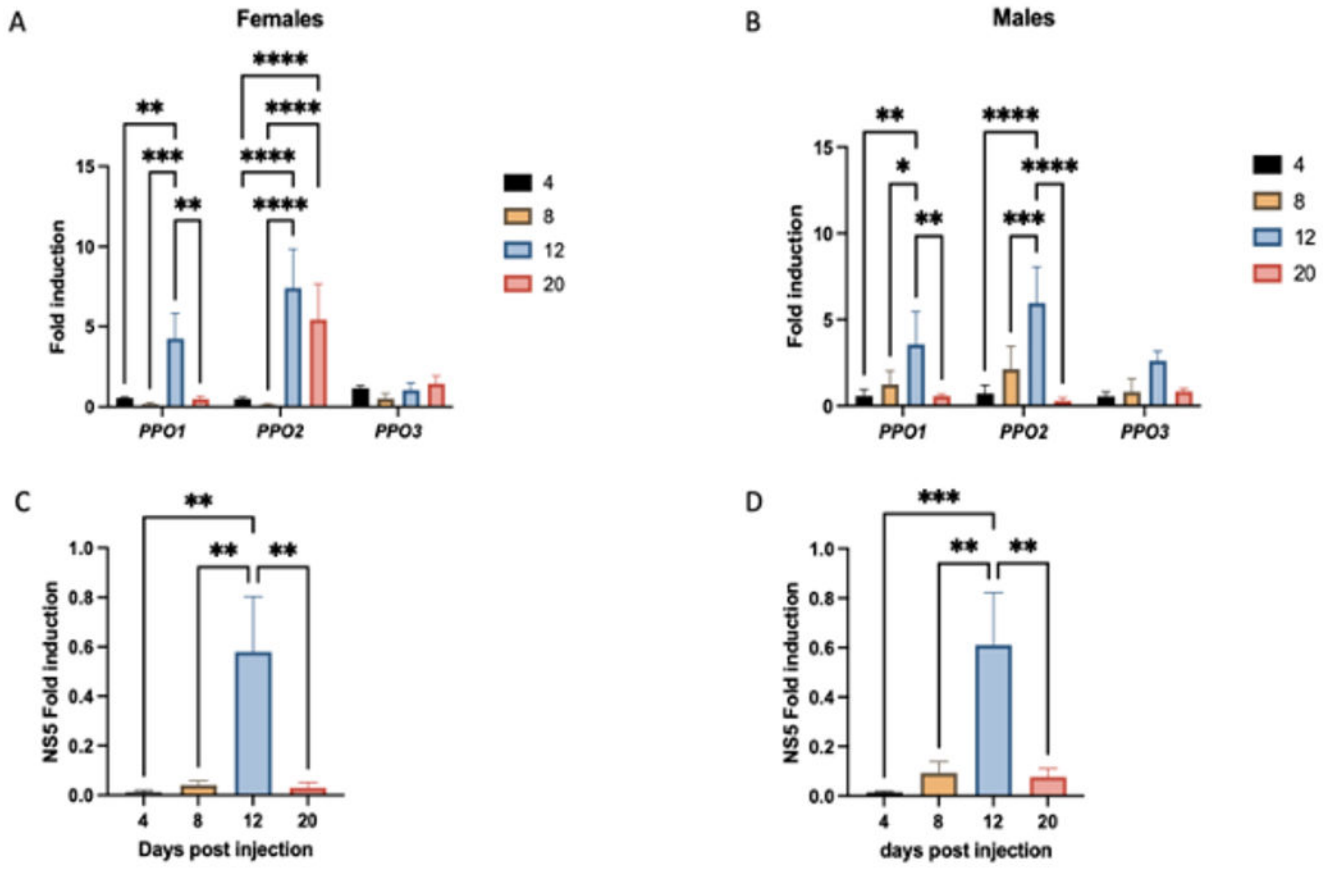

Fig. 1.

Relative fold change of *PPO* genes and viral load in w^{1118} *Drosophila melanogaster* adults following zika virus infection. *PPO* gene expression levels were determined by qRT-PCR in *Drosophila* (**A**) female and (**B**) male w^{1118} flies several days-post-injection (dpi), indicated by color codes, with Zika virus (African strain MR766; 11,000 PFU/fly). Zika virus load was also measured using qRT-PCR analysis and *NS5* gene-specific primers in (**C**) female and (**D**) male w^{1118} flies several days-post-injection. (One-way ANOVA, **p < 0.001, ***p = 0.0001, ****p < 0.0001).

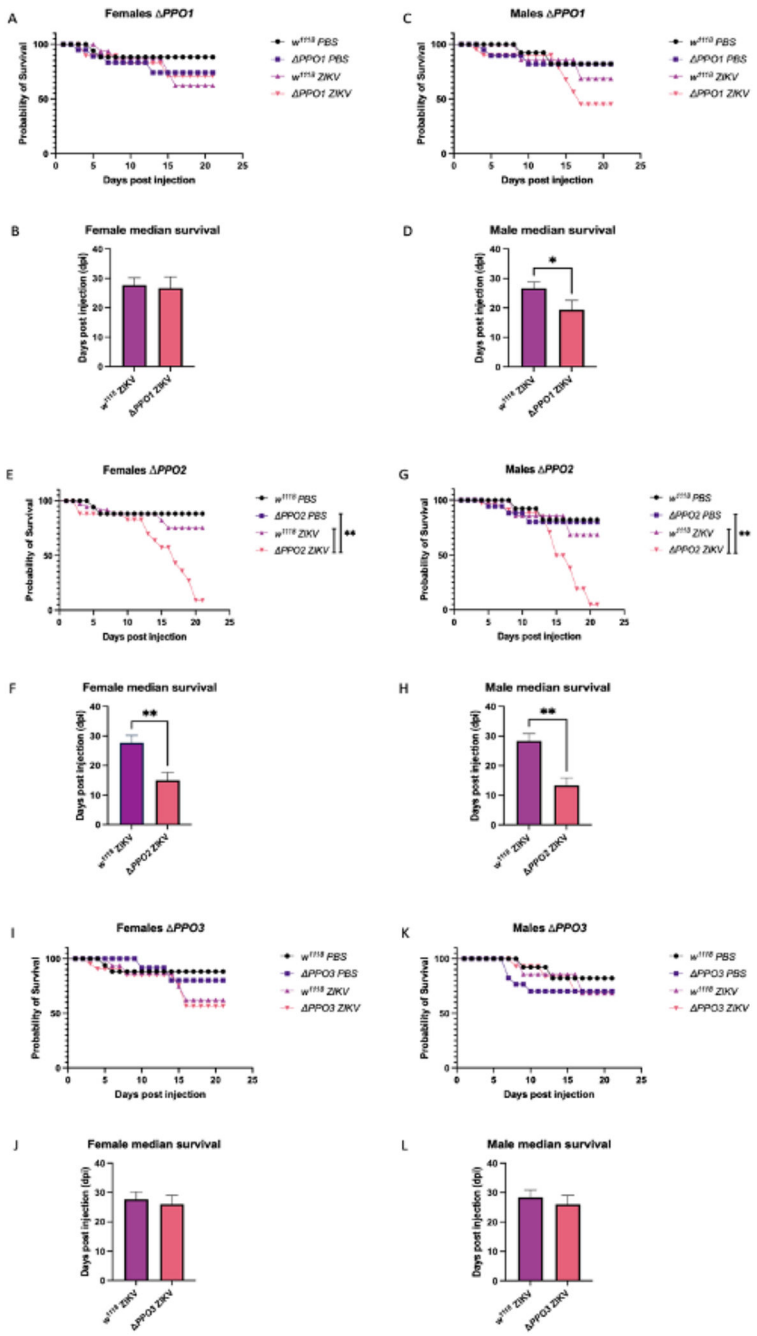

Fig. 2.

Survival response of *Drosophila melanogaster* single Δ*PPO* adults to Zika virus infection. The survival curve and median survival of: Δ*PPO1* (**A**, **B**) female and (**C**, **D**) male mutants; Δ*PPO2* (**E**, **F**) female and (**G**, **H**) male; and Δ*PPO3* (**I**, **J**) female and (**K**, **L**) male, as well as their background line controls w^{1118}, were assessed after intrathoracic injection with Zika virus at 24-hour intervals for 20 days. Injections with PBS served as negative controls. Significant differences between survival curves were calculated using

Kaplan-Meier analyses and median survivals were compared using One-way ANOVA. (*P < 0.01, **p < 0.001).

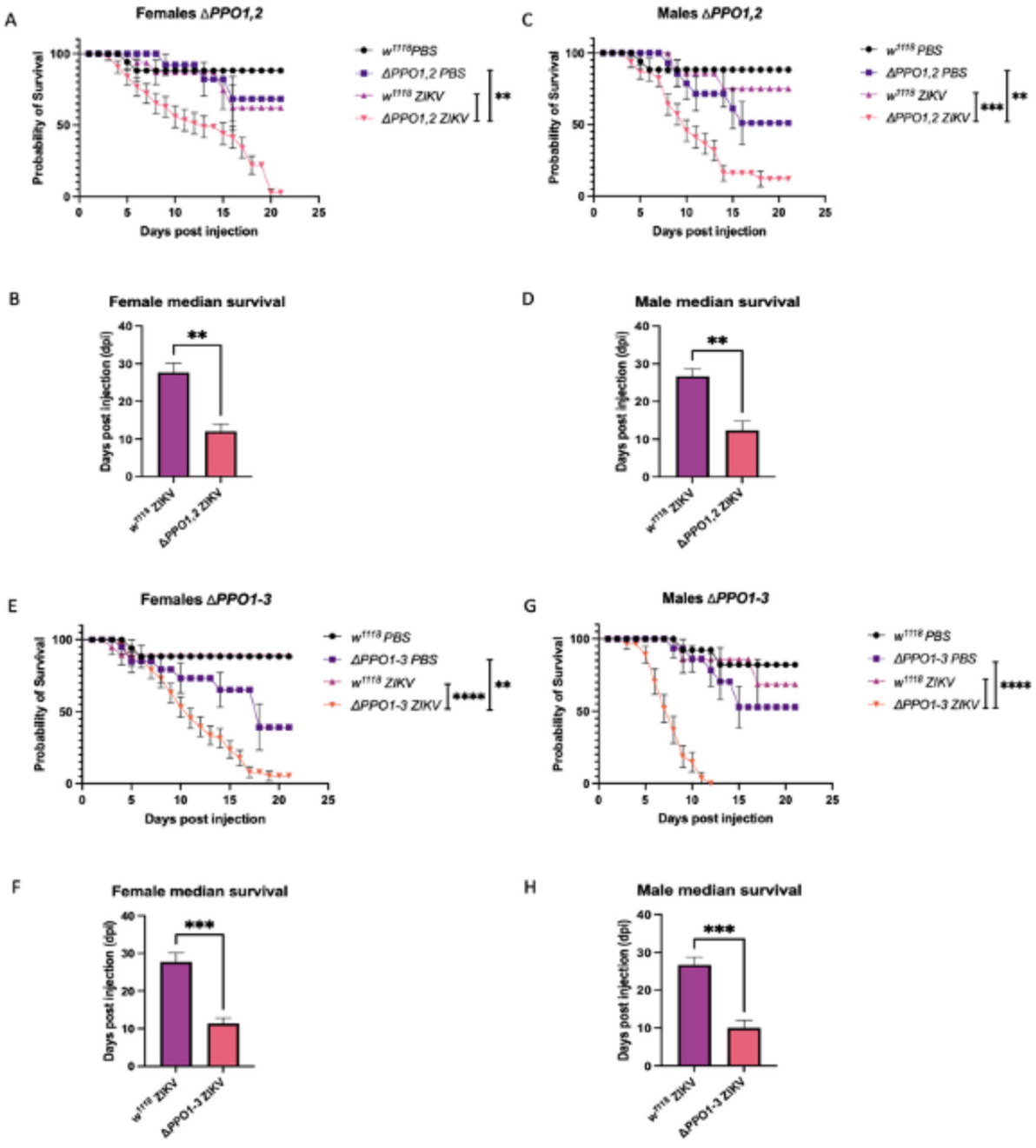

Fig. 3.

Survival response of *Drosophila melanogaster* Δ*PPO2* double and triple mutants to Zika virus infection. The survival curve and median survival of: Δ*PPO1,2* (**A**, **B**) female and (**C**, **D**) male mutants; Δ*PPO1-3* (**E**, **F**) female and (**G**, **H**) male mutants, as well as their background line controls w^{1118}, were assessed after intrathoracic injection with Zika virus at 24-hour intervals for 20 days. Injections with PBS served as negative controls. Significant differences between survival curves were calculated using Kaplan-Meier analyses and

median survivals were compared using One-way ANOVA. (**p < 0.001, ***p = 0.0001, ****p < 0.0001).

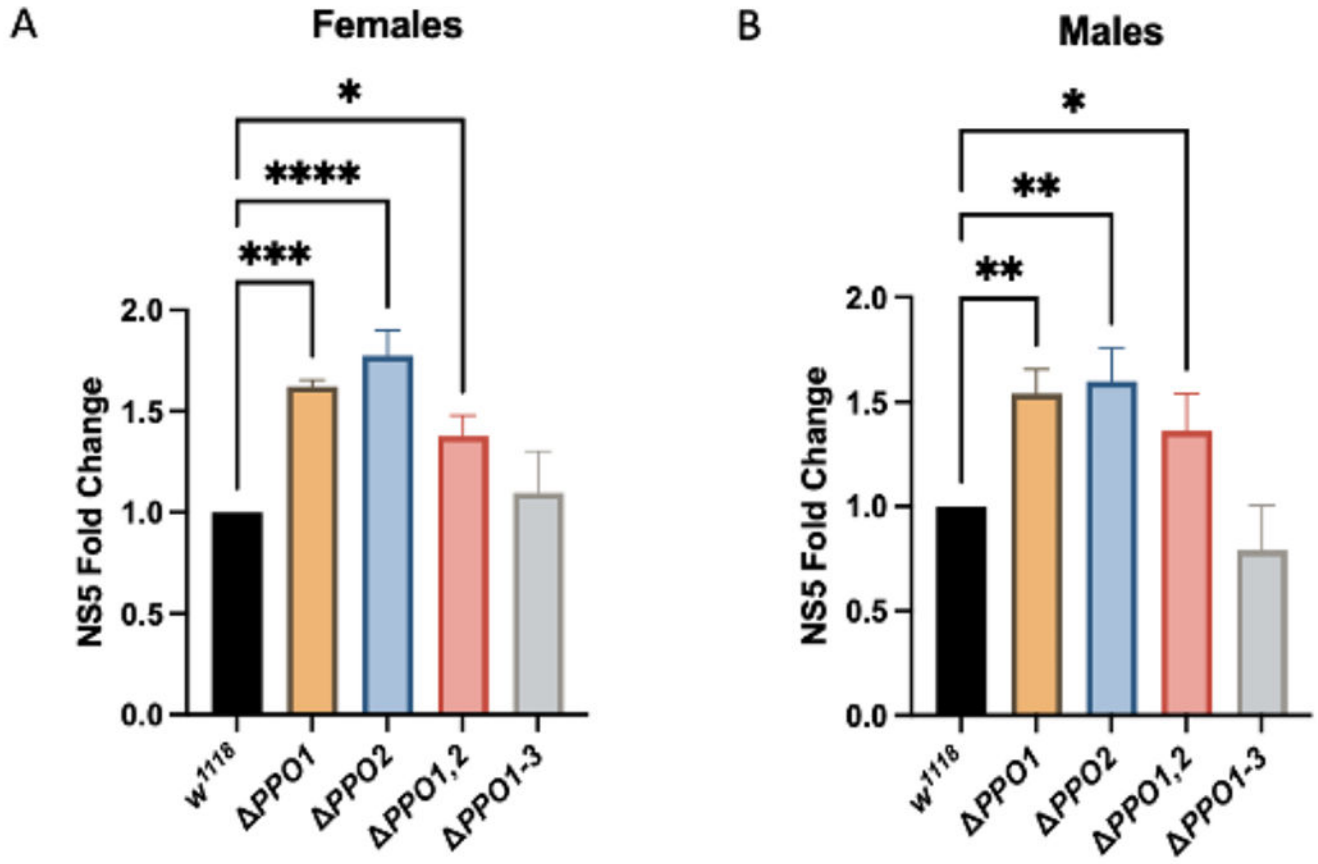

Fig. 4.

Zika virus load estimates in *Drosophila melanogaster PPO* single-, double-, and triple-mutants. Data from (**A**) female and (**B**) male adult mutants were collected using gene-specific primers designed against *NS5* and normalized to the housekeeping gene *RpL32* shown relative to infected w^{1118} control flies at 12 days-post-injection (dpi). (One-way ANOVA; *p < 0.01, **p < 0.001, ***p = 0.0005, ****p < 0.0001).

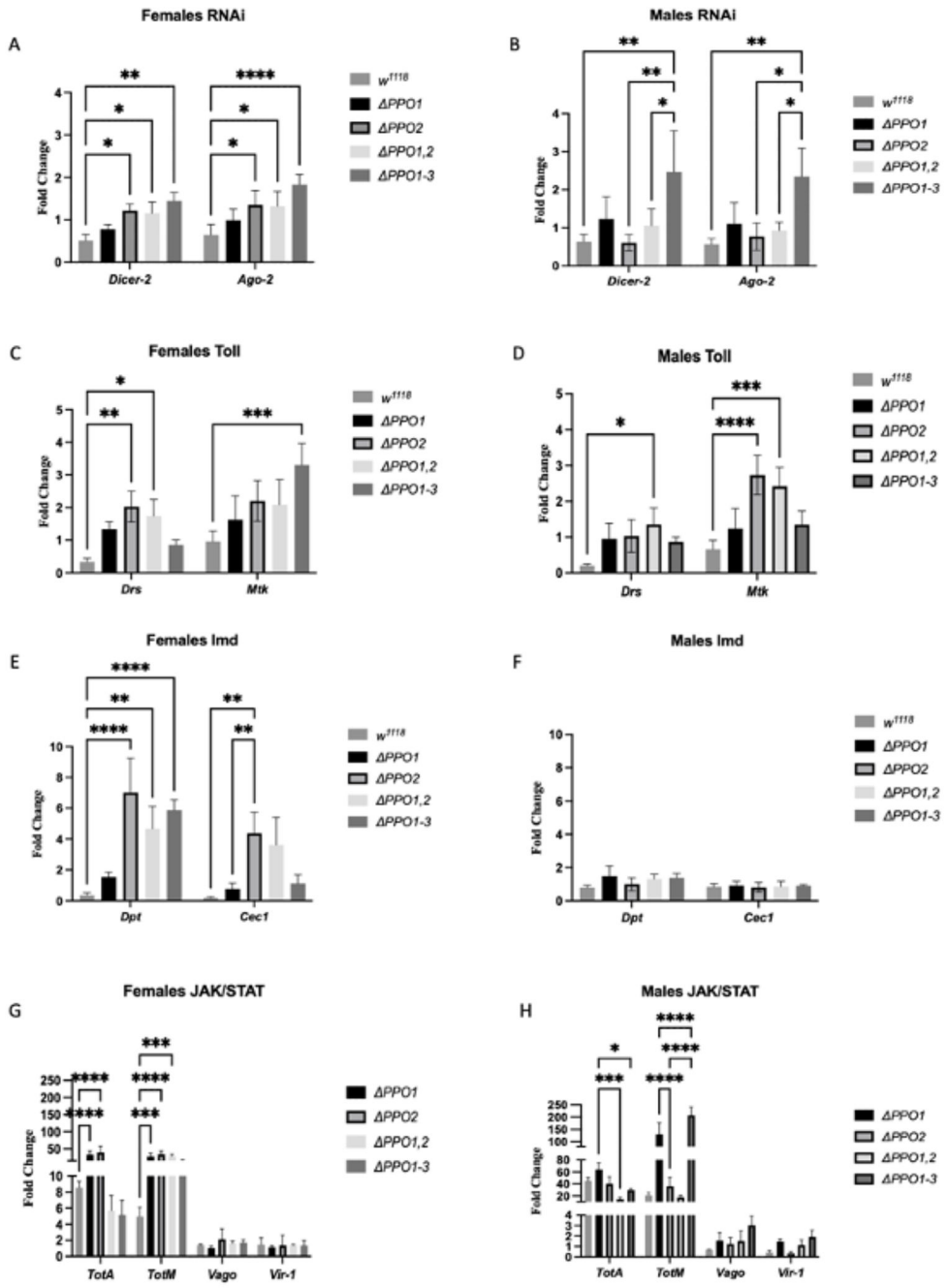

Fig. 5.

Δ*PPO Drosophila melanogaster* immune responses to Zika virus infection. Transcript levels of RNAi signaling in *Drosophila melanogaster PPO* single-, double-, and triple-mutants. (**A**) Female and (**B**) male ZIKV-infected flies were processed for RNA analysis, and gene expression levels of the RNAi machinery, *Ago-2* and *Dicer-2* were determined by qRT-PCR at 12 days-post-injection (dpi). Gene expression levels of the Toll signaling gene readouts *Drosomycin* (*Drs*) and *Metchnikowin* (*Mtk*) were determined by qRT-PCR at 12 days-post-injection (dpi) in Δ*PPO* (**C**) Female and (**D**) male mutants infected with Zika

virus. Transcript levels of the Imd signaling in (**E**) female and (**F**) male flies were also analyzed using gene readouts *Diptericin* (*Dpt*) and *Cecropin* (*Cec*) at 12 dpi. (**G**) Female and (**H**) male ZIKV-infected flies were processed for RNA analysis, and transcript levels of JAK/STAT gene targets, including *TotA* and *TotM*, and the antiviral STAT-regulated target genes *Vago* and *Vir-1*, were quantified via qRT-PCR. Results were normalized to the housekeeping gene *RpL32* and shown relative to PBS controls. (Two-way ANOVA; *p < 0.01, **p < 0.001, ***p = 0.0001, ****p < 0.0001).

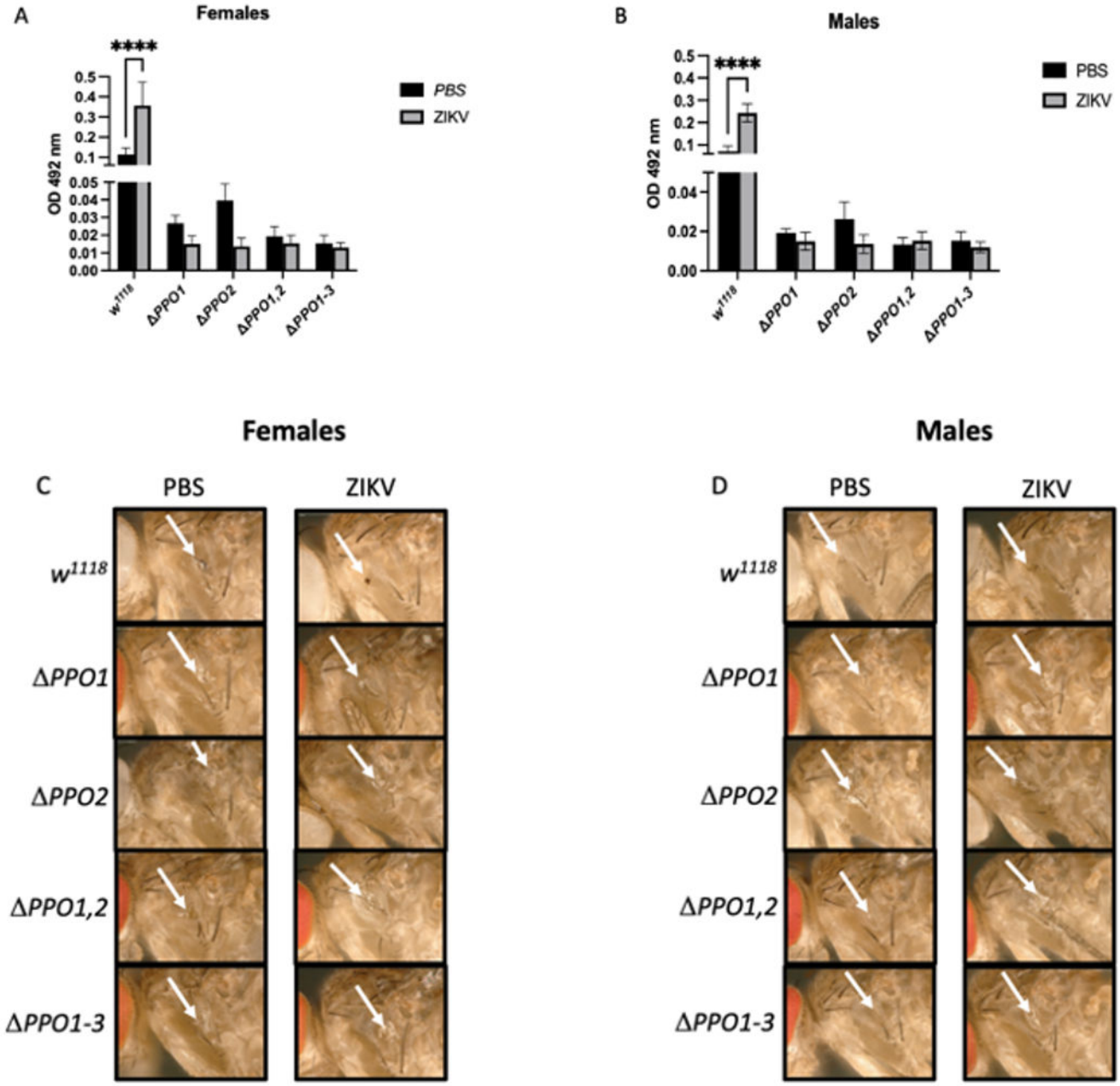

Fig. 6.

Hemolymph phenoloxidase activity and melanization formation in *Drosophila melanogaster PPO* single-, double-, and triple-mutants. Phenoloxidase (PO) activity was measured via the L-DOPA assay in hemolymph samples extracted from (**A**) female and (**B**) male ZIKV-infected flies and compared to PBS controls. Melanin formation at the injection spot in the thorax region of Zika virus infected *Drosophila melanogaster* mutants. The formation of melanization in (**C**) female and male (**D**) adults was observed following infection with zika virus. *Arrows* indicate the injection site. (One-way ANOVA, ****$p < 0.0001$).

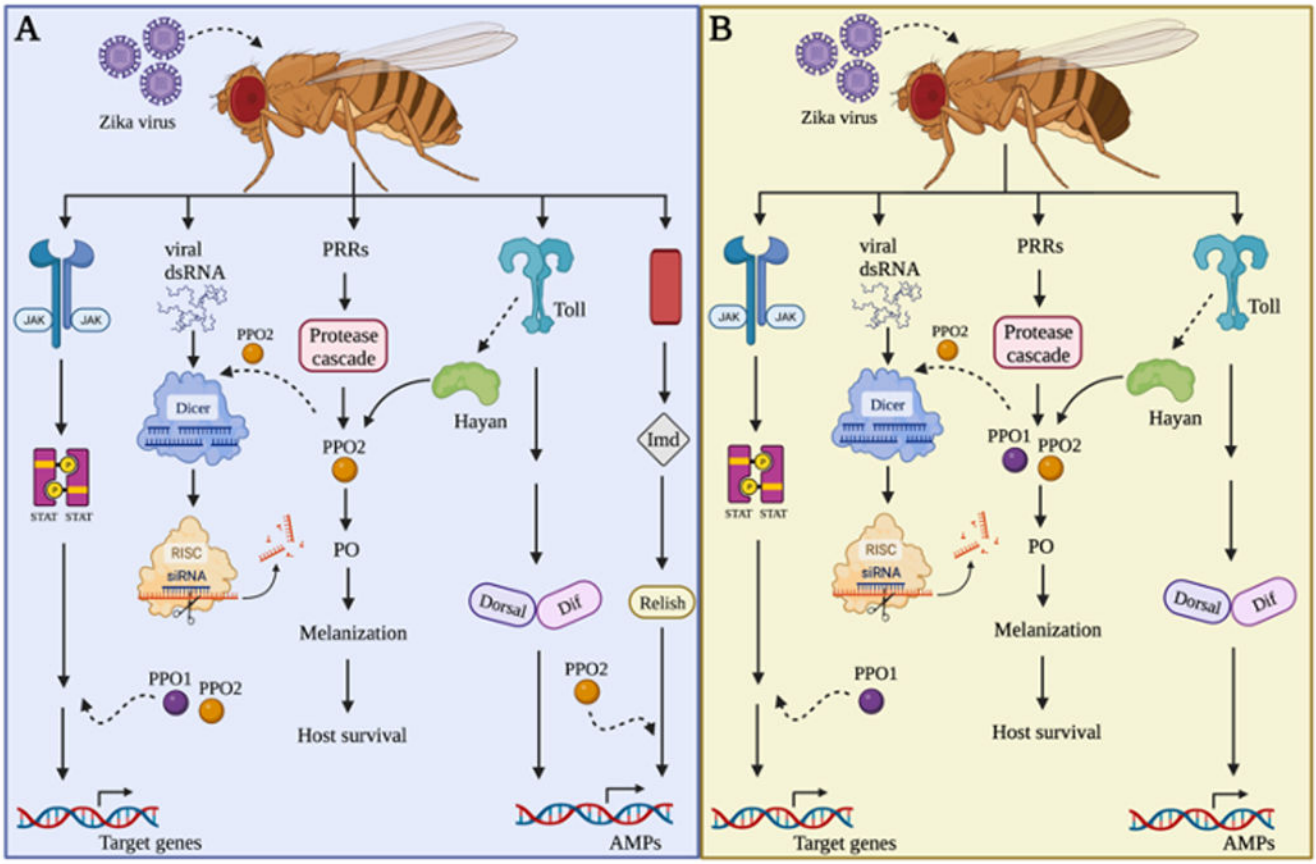

Fig. 7.

A model of the complex, sex-dependent regulatory system between *Drosophila melanogaster PPO*s and other host immune responses against Zika virus infection. **(A)** *Drosophila PPO2* interacts with the RNAi, Toll, and Imd, immune signaling in female flies to promote survival following ZIKV infection. Both *PPO1* and *PPO2* appear to regulate the JAK/STAT pathway, induce the PO activity in the hemolymph, and trigger melanin formation at the injection site in infected females. **(B)** *PPO1* and *PPO2* promote survival and interfere with the Toll signaling in infected male flies. Unlike female hosts, male *PPO*s do not interact with the Imd pathway. *PPO1* plays a role in the JAK/STAT signaling during infection and cooperates with *PPO2* to induce the PO activity in the hemolymph and melanin formation at the injection site in infected males. Arrows indicate activation of downstream components or steps. Dashed arrows indicate steps that have not been experimentally characterized in *Drosophila*.

Table 1.

List of the gene-specific primers used in quantitative qPCR experiments

Gene	Forward	Reverse
Rpl32	5′-GATGACCATCCGCCCAGCA-3′	5′-CGGACCGACAGCTGCTTGGC-3′
NS5	5′-CCTTGGATTCTTGAACGAGGA-3′	5′-AGAGCTTCATTCTCCAGATCAA-3′
Dicer-2	5′-GTATGGCGATAGTGTGACTGCGAC-3′	5′-GCAGCTTGTTCCGCAGCAATATAGC-3′
Argonaute-2	5′-CCGGAAGTGACTGTGACAGATCG-3′	5′-CCTCCACGCACTGCATTGCTCG-3′
Diptericin	5′-GCTGCGCAATCGCTTCTACT-3′	5′-TGGTGGAGTTGGGCTTCATG-3′
Cecropin A1	5′-TCTTCGTTTTCGTCGCTCTC-3′	5′-CTTGTTGAGCGATTCCCAGT-3′
Drosomycin	5′-GACTTGTTCGCCCTCTTCG-3′	5′-CTTGCACACACGACGACAG-3′
Metchnikowin	5′-TCTTGGAGCGATTTTTCTGG-3′	5′-AATAAATTGGACCCGGTCTTG-3′
TotA	5′-GAAGATCGTGAGGCTGACAAC-3′	5′-GTCCTGGGCGTTTTTGATAA-3′
TotM	5′-GCTGGGAAAGGTAAATGCTG-3′	5′-AGGCGCTGTTTTTCTGTGAC-3′
Vago	5′-TGCAACTCTGGGAGGATAGC-3′	5′-AATTGCCCTGCGTCAGTTT-3′
Vir-1	5′-GATCCCAATTTTCCCATCAA-3′	5′-GATTACAGCTGGGTGCACAA-3′