Review Article

MULTIFUNCTIONAL ROLE OF HEME PEROXIDASES IN HEMATOPHAGOUS INSECTS: A NOVEL TARGET TO ALTER MOSQUITO PHYSIOLOGY

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ABSTRACT

Heme-containing enzyme heme-peroxidases catalyzes various oxidative reactions, and has widespread existence throughout the kingdom of

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algae, bacteria, plants, insects, and animals. Like other animals, molecular and biochemical studies highlight that heme-peroxidases (HPXs) enzymes also play a diverse role in insect physiology and their lifecycle. Exploring their role, and targeting to manipulate the biology of blood-feeding mosquito vectors that transmits many life-threatening diseases such as dengue, chikungunya, malaria, is a fast-emerging field in the community of vector biologist. In this review, we aimed to update the available information on the mosquito HPXs, and briefly highlight the relevant functional importance for future exploration.

Keywords: insect, mosquito, heme peroxidase, cross-linking, feeding, immunity.

INTRODUCTION

Human pathogens carried by arthropod insects, especially those vectored by mosquitoes, are of global concern. For example, dengue, chikungunya viruses, and malaria parasites, which are transmitted by *Aedes* and *Anopheles* species, respectively, are life-threatening among several arthropod-borne diseases.[1-3] However, the rapid emergence of insecticide resistance in various diseases transmitting vector mosquitoes has prompted development of an alternative strategy to minimize vector-mediated disease transmission.[4-7] Gaining deep knowledge of the basic biology could provide alternative strategies not only to control the vector population but also to impede the pathogen development within the vector.[8,9] Blood feeding associated immune-stress physiology is a fast emerging topic of research, where peroxidases, especially heme-peroxidases which serve as potent antioxidant enzymes, are under intensive investigation to elucidate their potential role in multiple physiologies such as immunity, reproduction, and gut-homeostasis. This reviewexplains the diverse role heme-peroxidase (HPX) family members play in insect biology throughout its lifecycle, thus making it a suitable target to control vector population or to inhibit pathogen development within the vector.

Heme peroxidase, a heme-containing enzyme, catalyzes various oxidative reactions by utilizing hydrogen peroxide as the electron acceptor and is capable of oxidizing various substrates involved in many important biological processes such

as apoptosis, defense, and cell signalling. Peroxidase enzyme groups comprise a significant variety of heme-containing proteins.[10-12] and convey/sustain specific structural and biochemical properties, which empower them to work in extreme biological conditions.[13,14] HPX has widespread existence throughout the kingdom present in algae, bacteria, plants, and animals. Animal heme peroxidase belongs to the superfamily "Peroxidase-cyclooxygenase (PCOXS)", and comprises heme prosthetic group covalently bound to the protein peroxidase via two bonds. The prosthetic group of peroxidases is bound to the apoprotein, usually through a histidine residue that serves as a proximal ligand.[15,16] Thus the activity of these enzymes uses heme (as a prosthetic group) and hydrogen peroxide to catalyze one/ two-electron oxidation of the substrate H_2O_2, which is converted to water, and one-electron donor is oxidized to the corresponding radicals. Whereas in the case of a two-electron donor like halide is converted to hypohalous acid, as presented below:

(1) One-electron oxidation reaction: $H_2O_2 + 2AH_2 \rightarrow 2H_2O + 2 \cdot AH$

(2) Two-electron oxidation reaction: $H_2O_2 + H + X- \rightarrow H_2O + HOX$

In addition to the role of H_2O_2 as a pro-oxidant, it is important to activate the peroxidase enzyme, which then executes specific oxidation and reduction reactions *via* a free radical mechanism.[17] H_2O_2 is provided by the electron transport chain (ETC) of mitochondria, endoplasmic reticulum, and other metabolic reactions such as oxidation of xanthine, mono-amine, and NADPH oxidase.[18-20] Some members of the cyclooxygenase-peroxidase family depend explicitly on H_2O_2 provided by NADPH oxidase and in these particular cases, lack of the corresponding oxidase cannot be substituted by any other H_2O_2 sources.[20] The dependency of certain members of heme peroxidase on NADPH-oxidases or concerted action of oxidases highlights evolutionarily conserved cooperation among the oxidases and peroxidases.[21]

According to the PeroxiBase database, in general, peroxidases can be divided into two main classes, heme, and non-heme peroxidases.[22] For the last two decades, diverse sequencing programs have expanded our knowledge about the orthologous and paralogs of heme peroxidase gene families (Table 1). Broadly heme peroxidases make up more than 80 percent of peroxidase, while non-heme peroxidase (alkyl hydro peroxidase, halo peroxidase, thiol peroxidase, and NADH peroxidase) is just a limited percentage of peroxidases (Fig. 1).

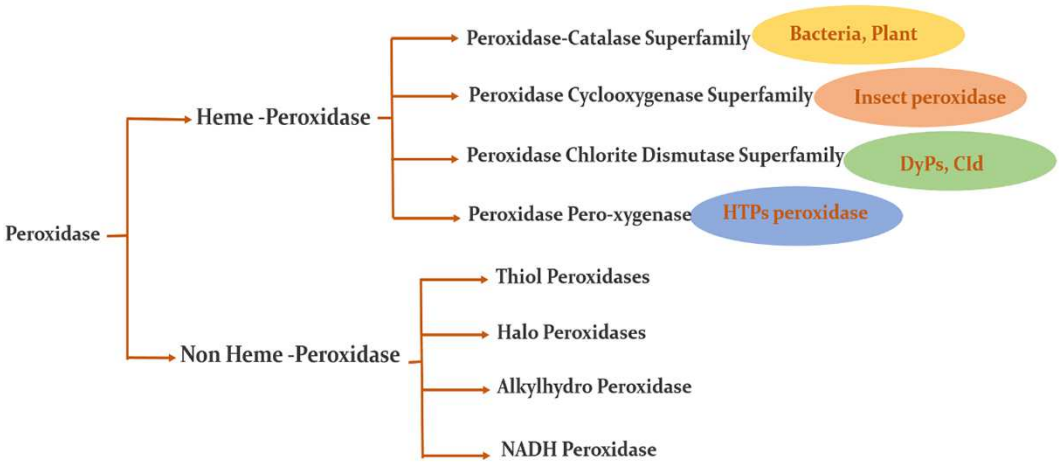

Fig. 1. General classification of Peroxidases; DyPs-dye decolorizing peroxidase, Cid-chloride dismutase, HTPs –heme thiol peroxygenase (*Source:* This work).

Heme peroxidase has been further classified based on the heme-binding site in four different superfamilies, namely the peroxidase-cyclooxygenase superfamily (PCOXS), peroxidase-catalase superfamily (PCATS), peroxidase chloride dismutase, and peroxidase pero-oxygenase. All families play an overall similar function to utilize free radicals and post-transcriptional modified heme binding to either histidine or cysteine residues. The description of these peroxidase superfamilies is presented in Table1.

Table1. Heme peroxidase superfamily classification: summary of features such as the domain, ligand binding, and their function

Features	**Peroxidase–catalase super family**	**Peroxidase–cyclooxygenase super family**	**Peroxidase–chlorite dismutase super family**	**Peroxidase peroxygenase superfamily**
Common name	Bacterial, fungal, plant heme – peroxidase	Animal heme peroxidase	Bacteria and fungi peroxidase	HTPs Heme peroxidase UPOs peroxygenases
Domain	Globular fold of this super family	Multi domain protein with one Heme	In this super family unique	Fold with ten alpha helices

Features	**Peroxidase–catalase super family**	**Peroxidase–cyclooxygenase super family**	**Peroxidase–chlorite dismutase super family**	**Peroxidase peroxygenase superfamily**
	consists of twelve alpha-helices	peroxidase domain of predominantly alpha helical fold with a central Heme containing core of 5 alpha helices	feature a large dimeric a/b barrel structural	and five very short beta-sheets
Proximal heme ligand	His-Trp/Phe-Asp	His/Asp	Histidin	Cystein
Distal heme ligand	Arg-Trp/Phe-His	His /asparagine	Arginine	Aspartate
PFm id	PFOO141	PF03098	PF04261(Dyp) PF06778(Cld)	PFO1328
Function	Main function to degradation of lignin-containing soil debris	Major role in the innate immune system by the production of antimicrobial hypohalous acids	Important molecules in bioremediation as Clds catalyze the degradation of chlorite to chloride and molecular oxygen production	Oxidation of organic heteroatom's and inorganic halides, one-electron oxidations but also, epoxidations and dealkylations.
Examples	1) Family1 Mitochondrial cytochrome c peroxidases 2) Family II and III: Horseradish peroxidase(s) (HRP)	1) Myeloperoxidase (MPO) 2) Eosinophil peroxidase(EPO) 3) Lactoperoxidase (LPO) 4) Thyroid peroxidase (TPO)	1) Dye-decolorizing peroxidases (DyPs) 2) Chlorite dismutases (Cld)	Heme-thiolate peroxygenase
Reference	[10]	[16]	[15]	[10]

Insects comprise about ~1 million (900 thousand) species, and are able to adapt to diverse ecologies and environments. Current studies highlight that the evolution of insect peroxidase is featured by specific animal heme peroxidase domain. In insects, heme peroxidase is known to be involved in various physiological processes like innate immune response, wing maturation, strengthening of eggshell, and management of oxidative stress.[23,24] The blood-feeding behavioral evolution and its adaptation in the mosquitoes, makes them medically important disease transmitters, and therefore, exploring the role of the HPX family in the various physiological processes i.e. immunity, reproduction, and development is worth exploring. This review intends to highlight the important role of mosquito peroxidases that belong to the peroxidase-cyclooxygenase superfamily, and highlight key features of the missing links between functional variations among different heme peroxidases. In insects, a total of 889 heme peroxidases have been identified so far, and out of which 39 are unique to mosquitoes (Fig. 2). Heme peroxidases (HPXs) of Culicidae and other insects explain six highly conserved ancient HPX lineages, each of which is linked by gene duplication before the most recent common ancestor (MRCA) of Hemimetabola and Holometabola.[25]

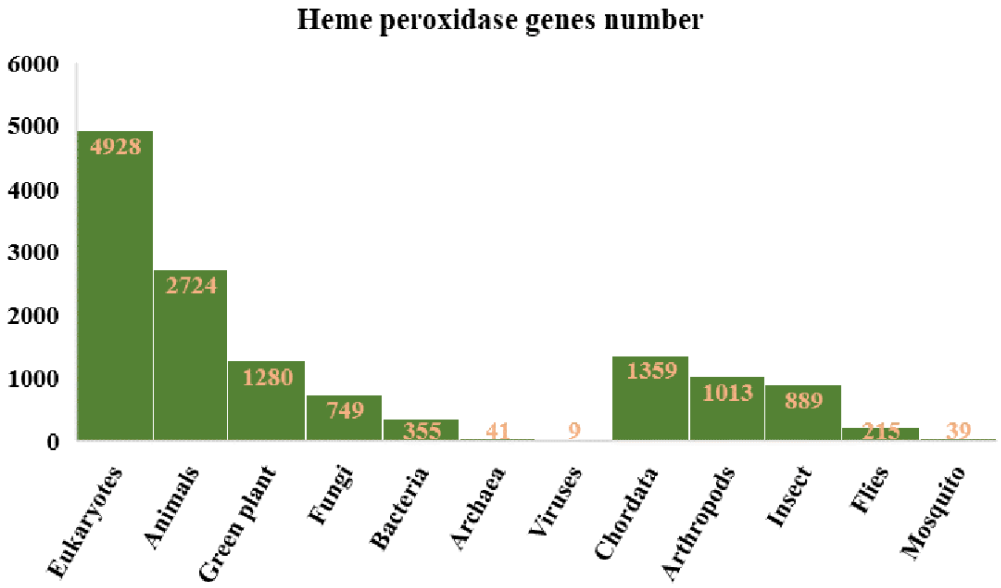

Fig. 2. Cataloguing of Heme peroxidase genes identified from different insect phyla (*Source* of data: NCBI).

To comprehend the functional relationship, we updated and evaluated the functional correlation of putative HPX proteins identified from the mosquito genome, so far. The nomenclature of individual proteins and their putative orthologues was based on names provided by Vector Base (http://www.vectorbase.org) for *A. gambiae*, and other heme peroxidases retrieved by NCBI database.

PHYLOGENETIC RELATIONSHIP

Genome sequence and availability of quality annotation for the mosquito *Aedes aegypti*, *Anopheles gambiae,* and *Culex quinquefasciatus*, serve as the base to establish a phylogenetic relationship, and update the current knowledge on the HPX research status (Fig. 3). All the amino acid sequences belonging to three important mosquito vector species were downloaded from NCBI and Vector Base databases. All the retrieved sequences were aligned by ClustalW algorithm and the phylogenetic tree was constructed in the MEGA 6 program by using the maximum likelihood (ML) method. Criteria selected for the phylogenetic analysis are the following: 'WAG' option was selected as the model for amino acid substitution as this model best fits our data and to define gaps and missing data, we used the 'all sites' option. Branching pattern reliability was tested for ML tree by 1000 bootstrap replicates. The resulting phylogenetic tree was analyzed based on clusters and nodes formed. Based on *An. gambiae* peroxidase representative sequences, the clades for animal heme peroxidases are further divided into subclades (Nomenclature was adopted from Vector Base database) in the cluster of the particular subclades.

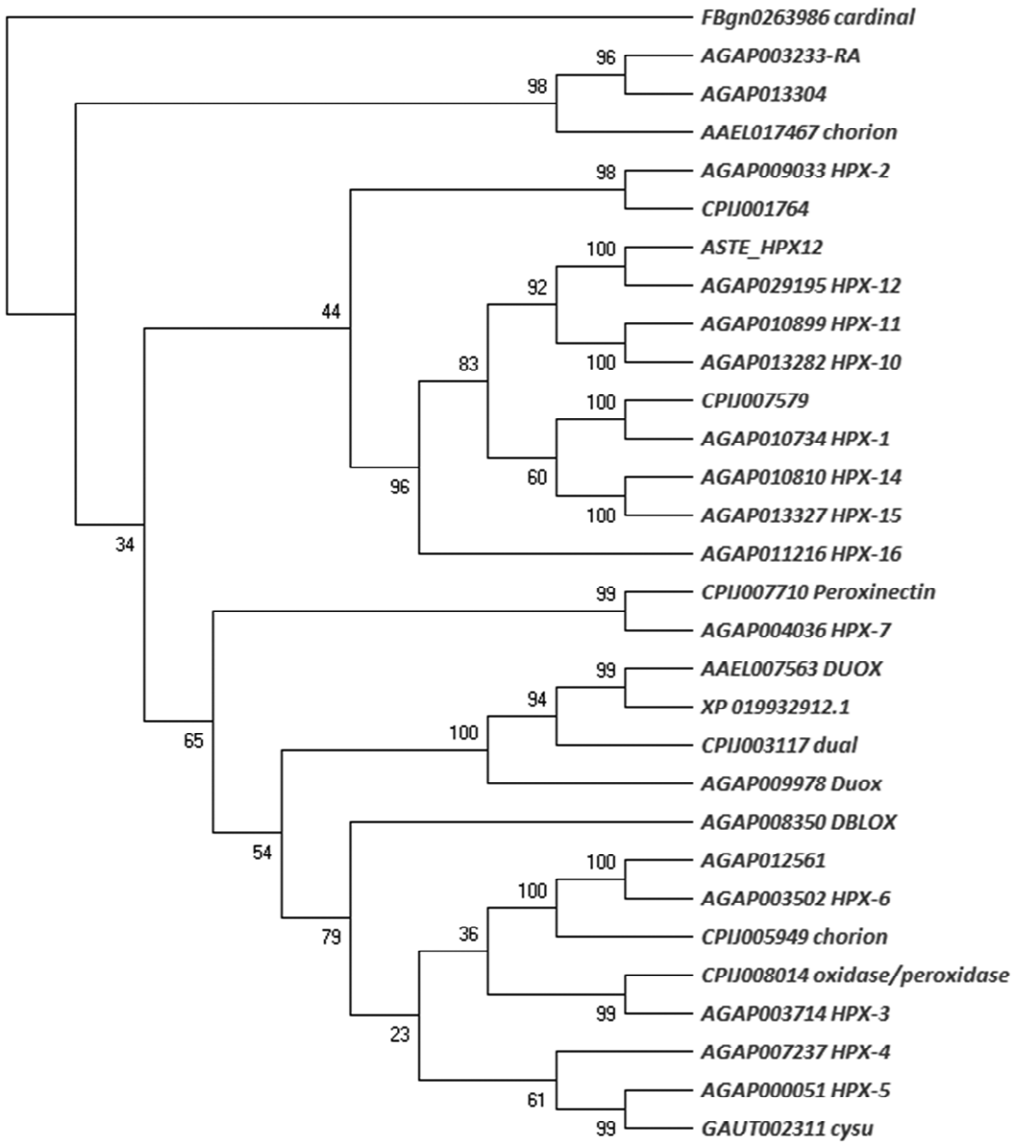

Fig. 3. Insect heme peroxidase and their phylogenetic relationship: *An. gambiae* shows a close link to different mosquito species, but a distant relationship to other non-mosquito species for example *Drosophila.* The gene labels signify as *ASTE* (*An. stephensi*), *AGAP* (*An. gambiae)*, *AAEL(Ae. aegypti),* and *CPIJ* (*Culex quinquefasciatus*).

FUNCTIONS OF HEME PEROXIDASE

Numerous physiological processes in living beings can produce reactive oxygen species including hydrogen peroxide. One important possible source of hydrogen peroxide is NADPH oxidases which belong to Nox/Duox family. Generally, the Nox/Duox protein family is considered to be the source of two major reactive oxygen species which are superoxides and hydrogen peroxides.[26] Duox proteins also can be included under heme peroxidases, even though it lacks enzymatic activity of their N-terminal peroxidase domain.[21] However, dual oxidase (Duox) proteins also require either of the two major maturation factors DuoxA1 or DuoxA2 for proper folding and membrane targeting,[27,28] which acts as a catalytic unit dedicated to producing hydrogen peroxide. It has also been observed that certain members of the cyclooxygenase-peroxidase family have a specific demand for hydrogen peroxide generated by an NADPH-oxidase. Such peroxidase family strictly needs only H_2O_2, which cannot be supplemented by any other source. This feature highlights that the mode of action between heme peroxidases and NADPH-oxidases is very well conserved which can be explored in other insects.

Current studies in insects highlight the various functions which enzyme heme peroxidase performs including extracellular matrix stabilization, detoxification, immunity development etc., and these peroxidases may likely have similar physiological functions in the mosquitoes. Given below is a representation of the various functions which heme peroxidase performs in insects/mosquitoes:

(1) Maturation of wings: In insects, wing development is a very complex process that requires the expression of a subset of genes. Duox and Cysu function together during the final step of the wing maturation process. Cysu is a heme peroxidase, also known as a curly suppressor, that participates in the *Drosophila* wing maturation. The cuticle is stabilized during the cuticular sclerotization process with the aid of the inclusion of phenolic compounds; hydrogen peroxide generated by Duox is used by Cysuheme peroxidase to crosslink the protein *via* tyrosine residues. Mutation in either Duox or Cysu results in less tyrosine cross-linking which finally affects the sclerotization and melanization of adult wings.[24] Specific studies have shown that in the insect cuticular sclerotization process tyrosine can crosslink the protein and thus stabilize the wings after egg hatching (Fig. 4).

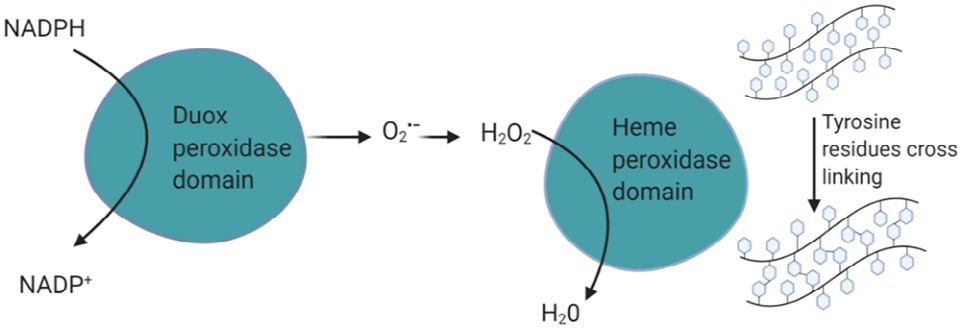

Fig. 4. Role of heme peroxidase in wing maturation: the peroxidase domain of Cysu exploit Duox as an H_2O_2 generator to oxidize catechol compounds, and utilizes tyrosine moiety as a mediator of cross-linking between the protein chains, a sclerotization process of wing maturation (*Source:* This work, Created in BioRender.com).

(2) HPX role in sperm storage and reproductive physiology: Preserving sperm viability during storage is essential to the reproductive success of the mosquitoes. *Drosophila* studies have shown that the spermatheca defends and nourishes sperm to preserve sperm viability, but the molecular and functional mechanism is yet unexplored. In the mosquito *An. gambiae*, heme peroxidase-15 (HPX-15) is one of the factors that has been found to maintain sperm functionality for several weeks, which is induced in spermatheca after mating. As mating takes place only once in the mosquitoes lifetime, therefore, it is necessary that the sperms are stored in a specialized organ (spermatheca) that preserves the viability of sperm before it is used for fertilization. The induction of HPX-15 is regulated by sexually transferred steroid hormone, ecdysone that is produced in the male accessory glands and transferred to female mosquitoes during copulation. Increased reactive oxygen species (ROS) rates in stored sperm can induce insect infertility. HPX-15 induction seems to play a role in protecting the stored sperm from the oxidative challenge.

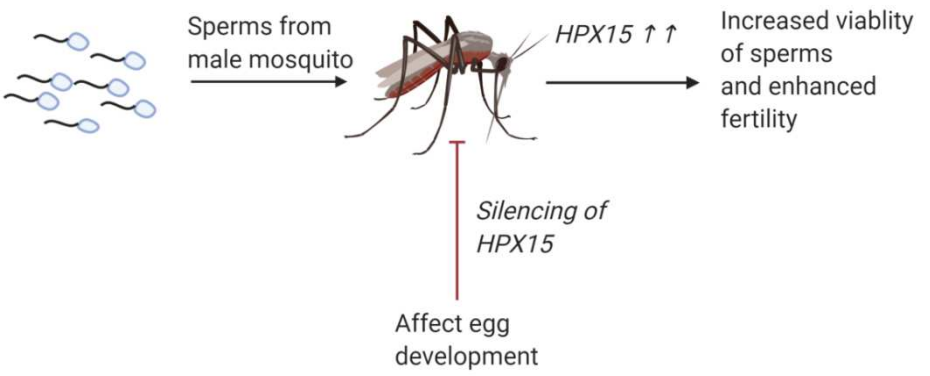

Fig. 5. Heme peroxidase role in the mosquito reproductive physiology: during mating, the sperm in the spermatheca are labile to reactive oxygen species, which can affect sperm viability. The presence of HPX15 in spermatheca not only quenches these reactive oxygen species but also help maintain the optimal physiological homeostasis for long term storage of healthy, active, and viable sperms (*Source:* This work, Created in BioRender.com).

Besides defending against harmful ROS, HPX15 can also work *via* a cross-linked matrix to shield sperm from the female immune system. HPX-15 gene silencing impairs the development of the egg which further indicates its importance in female reproductive success.[29] Additionally, HPX-12 in *An. stephensi* also seems to contribute functionally to the male reproductive system as the mRNA depletion of HPX-12 in testes impairs the oxidant-antioxidant system, which in turn reduces the male fertility, and affects the egg development in the mated female mosquito.[30] Figure 5 represents a schematic overview of the HPX's role in reproductive physiology.

(3) **Role in blood-feeding:** Although sugar meal is sufficient for the survival of both male and female mosquitoes, however, blood-feeding is an indispensable requirement for a female mosquito to develop eggs. The blood-feeding is accomplished with the help of saliva, secreted by the salivary glands. The saliva comprise a cocktail of molecules like anti-homeostatic components, anti-regulatory factors, and anti-inflammatory components that counteract the host defense against blood loss and facilitates successful blood feeding. In female *An. albimanus* mosquitoes, salivary enzyme heme peroxidase located in the posterior lobes have been reported to act during

blood-feeding by destroying hemostatically active vasodilators amine serotonin released by the host in response to tissue injury.[31] Further observation of a decrease in peroxidase activity after blood-feeding in the mosquito *An. albimanus*[19] and enhanced probing propensity after silencing in the mosquito *An. stephensi,*[32] advocates its role in blood-feeding associated behavioral properties.

(4) Eggshell hardening and waterproofing: The insect eggshell or chorion is a multilayer structure that confers both biological and physical protection to the developing embryos. In *Aedes aegypti* the hardening of chorion occurs after oviposition, however, in the case of *Drosophila melanogaster* and *Rhodnius prolixus* (vectors of Chagas disease) it takes place while the eggs are still in the ovary. This hardening of eggshell is attributed to the cross-linking of chorion proteins *via* tyrosine residues mediated by peroxidase. In *Drosophila*, the oxidase located at the apical surface of follicle cells likely serves as a source of H_2O_2 which is then utilized by the peroxidase to cross-link the chorion protein. In *Rh. prolixus* the ovarian Dual oxidase (Duox), an NADPH oxidase, generates H_2O_2, which activates the peroxidase and chorion protein cross-linking during eggshell hardening.[33]

(5) Agonist and antagonist activity of HPX against microbial pathogens: The midgut of the mosquito houses a large number of commensal bacteria. Midgut epithelial cells defend the host from pathogenic bacteria, without increasing immune responses to these commensal bacteria. However, after a blood meal, the rapid proliferation of the gut microbiota may presumably raise the risk of an enhanced immune response.

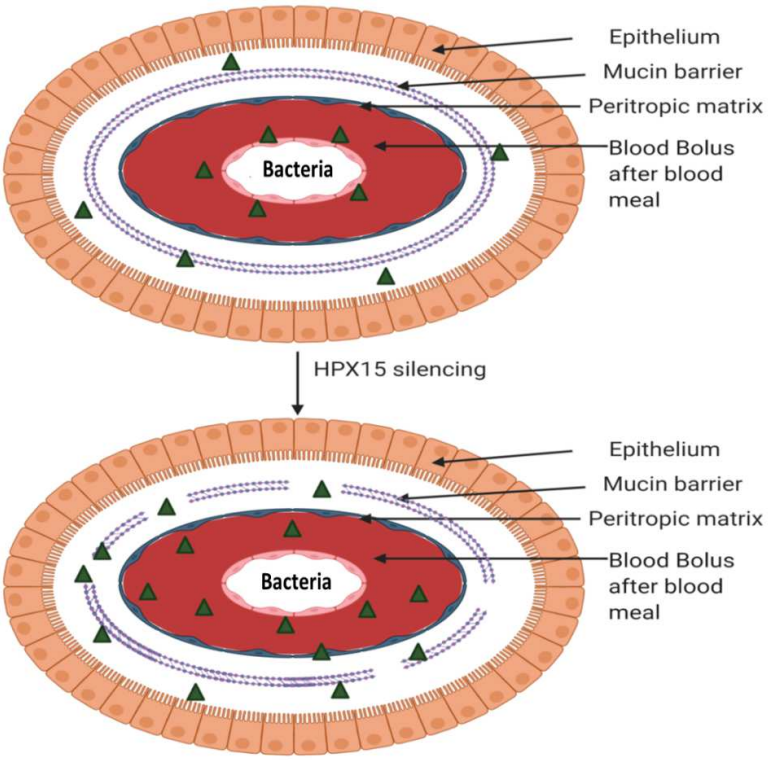

Fig. 6. Model of mosquito midgut immunity and heme peroxidase relationship: heme peroxidase 15 catalyzes the formation of mucin layer that inhibits direct interaction of mosquito immunity elicitors such as microbes with midgut epithelium. When silenced the absence of mucin layer favours direct interaction of the pathogen with bacteria and activates the mosquito immune system (*Source:* This work, Created in BioRender.com).

To protect the gut microbiota a mucin layer is secreted between the midgut epithelium and peritrophic matrix. Along with Duox, HPX15 (IMPer) cross-links the tyrosine residue in the mucin layer, which decreases the permeability of immune elicitors and suppresses the innate immune responses against gut microbiota (Fig. 6). The malaria-causing *Plasmodium* parasite takes an advantage of this low immunity area for its development in the mosquito host.[27] Silencing of midgut HPX15 in *An. stephensi* reduces the *P. berghei* oocyst load by inducing manifold expression of anti-*Plasmodium* gene encoding nitric oxide synthase (NOS) enzyme.[34]

Table 2. Some of the important heme peroxidases in mosquitoes

Name of heme peroxidase	Function	References
HPX-15	Suppresses Midgut immunity to support *Plasmodium* development in *An. stephensi*	[34]
HPX-2	Critical mediators of midgut epithelial nitration and anti-plasmodial immunity that enhance nitric oxide toxicity in *An. gambiae*.	[35]
HPX-15	Role in preserve the functionality of stored sperm and long-term fertility in *An. gambiae*.	[29]
Salivary catechol oxidase/ peroxidase	Salivary peroxidase displaying an oxidates reaction towards catecholamines in *An. albimanus*	[31]
HPX-8	Antibacterial Peroxidase in *An. stephensi*	[23]
HPX-8C	Antiviral response against DENV in *Ae. aegypti*	[36]
As-DBLOX	Have role in pupal stage of development	[27]
Chorion peroxidase	Involved in the formation of a rigid and insoluble egg chorion by catalyzing chorion protein cross-linking through dityrosine formation and phenol oxidase-catalyzed chorion melanization in *D. melanogaster*	[33]
Cysu	Play role during the wing maturation process in *D.melanogaster*	[24]
HPX-12	Play a role to help blood meal acquisition in female mosquito *An. stephensi*	[32]
HPX-12	Play a role to maintain healthy sperm in male mosquito *An. stephensi*	[30]

After entering the midgut lumen, *Plasmodium* gametocytes fuse to form a zygote that turns into the ookinete stage. Ookinetes trespass the midgut epithelium and try to dodge the mosquito immune response so that it is not recognized by the complement system.[34] In *An. gambiae* the heme peroxidase (HPX2) and NADPH oxidase 5 (NOX5) are induced in ookinete- invaded midgut cells.[35] Along with NOS, both HPX2 and NOX5 mediate protein nitration, which enables the *Plasmodium* ookinete to be recognized by the mosquito complement system and results in the lysis of ookinete through activation of thioester protein (TEP1).[35,36] Though HPX's role against salivary sporozoites remains poorly understood, an

ongoing study shows altered expression of HPX12 in response to *P. vivax* thus highlighting its possible role against sporozoites.[32]

Summarily, we update the current knowledge over HPX family proteins (Table-2) in hematophagous insects and highlight their potential role for future exploration in mosquito physiology. Further elucidation of molecular, biochemical, and cellular mechanisms that demonstrate how HPXs enzymes regulate complex responses may help identification of novel targets to disrupt immune-physiology of blood-feeding mosquitoes and other insect vectors. Establishing a functional correlation of HPX15 in the anti-Plasmodium immunity, as well as in the reproductive physiology of adult female mosquitoes may serve as a unique target. Likewise, a comprehensive understanding of how HPX12 regulates sex-specific behavioural physiology of host-seeking in the adult female mosquitoes, and mating behavior of the male mosquitoes, could be crucial in disrupting mosquito-human interaction inthe control of vector populations in disease-endemic areas.

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Conflict of Interest: Authors declare no competing interest**.**

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